Biochemical Pharmacology, 1964, Vol. 13, pp. 1291–1303. Pergamon Press Ltd., Printed in Great Britain.

CARCINOGENIC AZO DYES, DYE-BINDING AND LIVER GLUTATHIONE

W. J. P. NEISH, HILDA M. DAVIES and P. M. REEVE

Cancer Research Unit and Department of Statistics, The University, Western Bank, Sheffield, 10

(*Received 27 February* 1964; *accepted* 4 *May* 1964)

Abstract—Good correlations have been found between the relative carcinogenic activities of azo dyes and the ability of these compounds to increase the liver glutathione (GSH) content and to form bound dye (B.D.) in the livers of male rats 24 hr after intraperitoneal (i.p.) injection of equimolar amounts of the dyes. The size of the product B.D. × GSH seems to be a useful measure of the carcinogenic potency of an azo dye. It is suggested that the simultaneous occurrence of dye-binding and GSH increase is essential for azo dye hepatocarcinogenesis.

THE glutathione (GSH) content of male rat liver was found to be increased markedly 24 hr after a single i.p. injection of a convenient arbitrary dose (16·5 mg/100 g rat body wt. of the powerful hepatocarcinogen 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB).[1, 2] Under similar conditions, the weaker carcinogenic isomer 4′-methyl-4-dimethylaminoazobenzene (4′-MeDAB) caused some increase in liver GSH while the non-carcinogenic compound 2-methyl-4-dimethylaminoazobenzene (2-MeDAB) produced a decrease in liver GSH.

GSH-inducing capacities as well as the extent of dye-binding[3] of some azo dyes have now been compared with their relative carcinogenic activities. In the present work it was found that liver GSH increase is maximal 24 hr after injection of 8·25 mg 3′-MeDAB/100 g body wt. Therefore, for comparative studies, azo dyes have been injected in amounts corresponding on a mole for mole basis with this dose of 3′-MeDAB. It must be noted however that these doses are not necessarily optimal for the the induction of liver GSH by the azo dyes in question.

Good correlations were observed especially between the GSH-inducing capacities of azo dyes and their relative carcinogenic activities and also between the extent of dye-binding and carcinogenicity. A strong correlation between the degree of dye-binding and the GSH content of livers of dye-injected rats suggested that changes in these two factors could be interdependent to some extent. However the non-carcinogenic dye 2-MeDAB gave a moderate degree of dye-binding without causing an increase in liver GSH. Conversely, the non-carcinogenic 2-hydroxy-4-dimethylaminoazobenzene (2-HODAB) caused some increase in liver GSH but gave rise to practically no bound dye.

As a working hypothesis, it is suggested that an azo dye will act as a complete carcinogen only if it provokes both bound dye (B.D.) formation and an increase in liver GSH. The size of the product B.D. × GSH appears to be a useful index of the

hepatocarcinogenic potency of an azo dye and might serve as a guide to the possible carcinogenic activity of otherwise unscreened azo dyes.

Azo dyes which provoked formation of B.D. or an increase in liver GSH but not both may prove to be incomplete carcinogens. If the simultaneous occurrence of both factors is essential for hepatocarcinogenesis we could expect that a mixture of the non-carcinogenic dyes 2-MeDAB and 2-HODAB would be carcinogenic for rat liver, the former contributing B.D. and the latter increasing the liver GSH content. Prior to an investigation of this possibility by feeding experiments we have found that injections of a mixture of these dyes into male rats resulted in a simultaneous increase in both liver B.D. and GSH. The size of the product B.D. $\times$ GSH suggested that the carcinogenic potency of the combined dyes would be of the order of that of 4-dimethylaminobenzene (DAB).

Dye-binding and GSH increase may prove to be linked with the phenomena of nuclear injury and cell proliferation respectively which have been regarded by Maini and Stich[4] as essential conditions for hepatocarcinogenesis.

EXPERIMENTAL

Pairs of stock male albino rats ($\sim$250 g body wt.) were injected i.p. with various doses of 3′-MeDAB (1–33 mg) in arachis oil (0·6 ml) per 100 g body wt. or with other dyes in equimolar amounts based on the standard injection of 8·25 mg 3′-MeDAB 100 g body wt. Control rats received injections of arachis oil. The animals had access to Diet No. 86 and water. Rats (in batches of 12–18) were killed with ether during 10.30 a.m. and 12 noon approximately 24 hr after injection. The livers, perfused with ice-cold normal saline, were collected and frozen at once in solid carbon dioxide.

Glutathione estimation

GSH was determined on pooled 10% trichloracetic acid (TCA) extracts of 5-g samples of frozen livers from pairs of identically-treated rats by the cuprous mercaptide method already described.[1, 2] In most cases, GSH was also determined by a more rapid colorimetric method due to Saville[5] which depends on the ability of thiol compounds (RSH) to form S-nitroso derivatives (RSNO). Under the influence of mercuric ions, RSNO decomposes with the production of an equivalent amount of nitrous acid which is used to diazotise sulphanilamide. The resulting diazonium salt on coupling with N-1-naphthylethylenediamine produced a stable red dye (absorption maximum at 540 mμ). This method which probably permits nearly 100% recovery of GSH confirmed that recoveries of only about 60–70% were being obtained with the cuprous mercaptide procedure.

For Saville's method, 1-ml aliquots were removed from each pair of TCA extracts of 5-g portions of liver and pooled. One ml of this mixture was then mixed with 9 ml N sulphuric acid and a 1-ml aliquot of this dilution was used for the estimation. Reaction time in nitrous acid was 4 min, and 2 min was allowed for breakdown of excess nitrous acid by ammonium sulphamate. For each series of estimations, calibration curves were prepared by diluting appropriate aliquots of a fresh stock GSH solution (10 mg GSH in 5 ml 10% TCA) to 10 ml with N sulphuric acid and using 1-ml aliquots of these dilutions for colour development.

Although Saville's method is not specific for GSH, interference from other thiol compounds is probably negligible in the present experiments. In each experimental

series, when GSH values obtained by the mercaptide method and by the colorimetric method were plotted the points usually lay on or near a single straight line.

Bound dye (B.D.)

One-g portions of each frozen liver from a pair of rats were pooled and homogenised in de-ionised water (8 ml). Molar acetate buffer (pH 5, 10 ml) was added to the homogenates and the mixtures heated for 3 min in boiling water. The protein coagulum was collected and bound dye (optical density at 520 mμ, 1-cm cells; Unicam S.P. 500 Spectrophotometer) was determined on extracts of alkaline hydrolysates of the protein as described by Miller and Miller.[3] It must be noted that not all bound dyes exhibit an absorption maximum at 520 mμ. Some give maxima at 490 mμ. However for the present experiments it was decided to use the optical density reading at 520 mμ in every case.

Azo dyes

DAB and orthoaminoazotoluene (oAAT) were commercial samples. Other dyes were prepared by diazotisation and coupling procedures outlined by Miller and Miller,[6] Miller, Sapp and Miller[7] and Miller, Miller and Finger.[8]

An attempt was made to prepare 2-HODAB by coupling diazotised aniline with an oil which resulted from the distillation of the methiodide of meta-dimethylaminophenol under reduced pressure as described by Stedman.[9] The product, recrystallised from ethanol gave the expected microanalytical values for C, H and N: Calc. for $C_{14}H_{15}N_3O$: C = 69·71; H = 6·27; N = 17·42. Found: C = 69·53; H = 6·41; N = 17·06, but the substance after several recrystallisations from ethanol had a sharp melting point of 105–106° not in agreement with the value of 163–165° quoted by Miller, Sapp and Miller[7] for authentic 2-HODAB.

When benzene diazonium chloride was coupled with meta-dimethylaminophenol (m.p. 84°; recrystallised commercial product), the resulting azo dye which gave the following analysis: C = 69·88; H = 6·03; N = 17·32%, had m.p. 162–163°.

Injection of either the low or high melting materials resulted in an increase in rat liver GSH the low melting product being somewhat more active than the high melting substance. It is interesting to note that Miller and Miller[10] have described two compounds 2-methoxy-4-dimethylaminoazobenzene (m.p. 104–105°) and 2-methoxy-4-monomethylaminoazobenzene (m.p. 114–115°). Pending further investigation of the nature of our low melting product which might bear some relationship to these 2-methoxyl derivatives it is referred to as 2-HODAB (low m.p.) in the present paper.

N-dimethylamino oxides of DAB and 3′-MeDAB were prepared by the method of Pentimalli.[11]

RESULTS AND DISCUSSION

Effect of dose of 3′-MeDAB *on liver* GSH *content and B.D. level*

The mean results of several separate experiments performed in April–May 1963 are given in Table 1.

The GSH-inducing ability of 3′-MeDAB was maximal at a dose of 8·25 mg dye/100 g body wt. Thereafter, with increasing dose, the liver GSH content at first declined but began to rise again with doses of 3′-MeDAB greater than 21 mg/100 g body wt. There was initially a linear relation between the extent of dye-binding and GSH

content up to the dose of 8·25 mg. At higher doses, however, this simple relationship was lost. Dye-binding was maximal 24 hr after an injection of 21 mg 3′-MeDAB/100 g body wt. but declined at higher dose levels.

TABLE 1. EFFECT OF DOSE OF 3′-MeDAB ON RAT LIVER B.D. LEVEL AND GSH CONTENT 24 hr AFTER INJECTION

Dose of 3′-MeDAB (mg/100 g body wt.)	Total no. of rats used	B.D. (E at 520 mμ)	GSH* (mg %)	B.D. × GSH
0	6	0·045	95·8	4·3
1	2	0·051	122.3	6·2
2	2	0·070	142·4	10·0
4·1	4	0·105	195·5	20·5
8·25	6	0·150	238·8	35·8
12·4	4	0·170	231·8	39·4
16·5	6	0·195	208·7	40·7
20·6	2	0·208	185·0	38·5
26·0	2	0·175	195·0	34·1
33·0	2	0·148	219·0	32·4

*Cuprous mercaptide method.

If the size of the product B.D. × GSH is important for hepatocarcinogenesis, the results suggest that there could be an optimal dose of 3′-MeDAB for tumour production. It would be interesting to determine whether feeding 3′-MeDAB at doses higher than those ordinarily employed (0·06%) would result in some inhibition of tumourigenesis. Rudali and Royer[12] were unable to obtain tumours in rats and mice treated with higher-than-usual doses of the carcinogen 4-acetylaminobiphenyl.

Correlations between the relative carcinogenic activities of various azo-dyes and the GSH *content and B.D. level of livers of dye-injected rats.*

The results of 8 experiments are given in Table 2 for the GSH-inducing capacity and extent of dye-binding of 7 azo dyes with relative carcinogenic activities ranging from 0 to 10–12 according to Miller, Miller and Finger.[8]

In these experiments which were carried out between 13 Sept. 1963 (Expt. No. 1) and 4 Dec. 1963 (Expt. No. 8) there is some indication of a decline in GSH content for dye-treated and control animals which might be due to seasonal variation in rat liver GSH level. At the time of these experiments the animals were housed at 15–20°. British Summer Time ended 28 Oct. 1963 between Expt. No. 4 and Expt. No. 5 so that Expt. Nos. 5, 6, 7 and 8 were begun and terminated about 1 hr earlier than Expt. Nos. 1, 2, 3 and 4. Analyses of variance in Table 2 appear to show a greater day-to-day variability in GSH than in B.D. There was a fairly consistent decrease in GSH content over the experimental period (Sept.–Dec.).

The correlation coefficient between the forty pairs of B.D. and GSH observed in Table 2 was 0·885 ($P < 0·001$). For the same observations the correlation coefficient between the relative carcinogenic activity and GSH was 0·891 ($P < 0·001$) which is significantly greater than that of 0·824 ($P < 0·001$) between relative carcinogenic activity and B.D. ($P < 0·01$). The correlation coefficient between B.D. + GSH and relative carcinogenic activity was 0·830 ($P < 0·001$).

TABLE 2. BOUND DYE (B.D.) AND GSH* CONTENT OF RAT LIVER 24 hr AFTER INJECTION OF VARIOUS AZO DYES

Dye† injected	Relative‡ carcinogenic activity	Experiment No. 1		2		3		4		5		6		7		8		Mean		B.D × GSH
		B.D.	GSH	B.D.	GSH	B.D.	GSH	B.D.	GSH	B.D.	GSH	B.D.	GSH	B.D.	GSH	B.D.	GSH	B.D.	GSH	
4′-EtDAB	10	0·129	244·6	0·130	248·7	0·121	234·5	0·141	243·1		226·0							0·130	239·4	31·1
4′-FDAB	11 (10–12)	0·084	212·8	0·073	207·4	0·062	170·3	0·085	193·5	0·075	168·8							0·076	190·6	14·5
3′-MeDAB	11 (10–12)	0·155	236·9	0·134	253·1	0·122	210·6	0·142	212·8	0·106	171·1						185·0	0·132	211·6	27·9
DAB	6			0·067	193·5	0·097	185·8	0·074	158·7	0·070	163·3	0·093	161·7	0·066	147·1‖	0·051	157·1	0·074	166·7	12·3
2′-MeDAB	2·5 (2–3)			0·049	141·7	0·045	175·7	0·042	145·5	0·034	117·7							0·043‖	145·2	6·2
4′-MeDAB	1 (<1)					0·074	144·7	0·063	117·7	0·058	127·0							0·065	129·8	8·4
2-MeDAB	0					0·063	125·4	0·049	97·5	0·040	104·5					0·046	70·4	0·050	99·5	4·8
nil		0·032	107·6	0·026	124·7	0·025	108·3	0·018	99·1	0·028	95·2	0·030	101·4	0·024	85·9	0·018	103·0	0·025	103·2	2·6

* Cuprous mercaptide method. Recovery experiments (11) with 10-mg samples of GSH; range 56·8–69·5; Mean recovery = 63·23% ± 1·34 (S.E.)
Recovery experiments (11) with 15-mg samples of GSH; range 54·4–73·4; Mean recovery = 65·90% ± 2·15 (S.E.)

† Derivatives of 4-dimethylaminoazobenzene (DAB) with ethyl (Et), fluoro (F) or methyl (Me) substituents at the positions indicated.

‡ Values in brackets show range quoted by Miller, Miller and Finger[8]. Values outside brackets were those employed for statistical calculations.

Effect of miscellaneous azo dyes on rat liver GSH *and B.D. levels*

As shown in Table 3 the N-dimethylamino oxides of DAB and 3′-MeDAB (DABO and 3′-MeDABO respectively) were almost as active as the parent dyes in increasing the GSH content of rat liver but were less active in producing B.D. In contrast to the parent azo compounds, little or no dye remained in the peritoneal cavity 24 hr after injection of fine suspensions of the oxides in arachis oil. The water-soluble oxides are

TABLE 3. EFFECT OF VARIOUS AZO DYES ON RAT LIVER GSH AND B.D. LEVELS 24 hr AFTER INJECTION

Dye injected	Relative carcinogenic activity‡	No. of rats used	Mean			B.D. × GSH (i)
			B.D. (F at 520 mμ)	GSH* (mg %) (i)	(ii)	
DAB	6	6	0·070	155·3	234·7	10·9
DABO		2	0·036	148·6	231·0	5·3
3′-MeDAB	10–12	4	0·125	181·1	271·2	22·6
3′-MeDABO		4	0·089	168·4	273·6	15·0
AB	<1	4	0·046	145·6	210·1	6·7
MAB	6	2	0·125	157·9	227·1	19·7
EAB	0	2	0·048	95·2	165·0	4·6
MEAB	6	4	0·087	108·8	160·0	9·5
4′-MeODAB	3	2	0·103	169·5	228·4	7·9
4′-EtODAB		2	0·066	119·2	164·5	4·0
oAAT (7·8 mg)	2†	6	0·030	148·6	228·6	4·6
mAAT (7·8 mg)		6	0·082	124·6	200·0	10·2
oAAT (15·5 mg)		2	0·074	233·7	338·0	17·3
mAAT (15·5 mg)		2	0·157	225·3	314·4	35·4
2-HODAB (low m.p.)		4	0·028	144·4	203·0	4·0
2-HODAB (authentic)	0	2	0·019	137·8	207·3	2·6
2′-HODAB	0	4	0·017	118·5	193·2	2·0
3′-HODAB	0	4	0·025	157·2	226·4	3·9
2-HODEAB		2	0·007	127·0	200·1	0·9
azobenzene		2	0·024	136·2	221·9	3·3
3′-MeODAB	10–12	2	0·105	226·0	305·0	23·7
3′-nitroDAB	5	2	0·034	209·8	269·5	7·1
3′-EtODAB	<1	2	0·058	125·4	186·4	7·3
nil		8	0·025	99·5	174·0	2·5

* (i) Cuprous mercaptide method.
(ii) Saville's method.
† See ref. 4.
‡ See refs. 6, 7 and 8.

probably metabolized more rapidly than the parent dyes. On our hypothesis both oxides, especially 3′-MeDABO, might have some carcinogenic activity. Terayama[13] regards DABO as a likely intermediate in the metabolism of the DAB in the rat.

Among the N-substituted 4-aminoazobenzene derivatives which have been studied, 4-monoethylaminoazobenzene (EAB; zero carcinogenic activity) gave some B.D. but no GSH increase. Thus it resembled 2-MeDAB (see Table 2) and in our opinion EAB might be capable of acting as an incomplete carcinogen. A considerable degree

of B.D. but only a slight GSH increase was produced by 4-methylethylaminoazobenzene (MEAB; activity 6). 4-Monomethylaminoazobenzene (MAB: activity 6) gave as high a level of B.D. as did 3′-MeDAB and was quite active in increasing the liver GSH content. Some dye-binding and GSH increase were produced in the livers of our rats by the feebly active carcinogen, 4-aminoazobenzene (AB).

The compound 4′-methoxyDAB (4′-MeODAB; activity 3) gave strong dye binding and a moderate increase in liver GSH. On the other hand, 4′ethoxyDAB (4′-EtODAB) hardly affected the liver GSH content though it was fairly active in producing B.D. Thus this substance might be expected to act as an incomplete carcinogen but would have little or no carcinogenic activity by itself. Indeed Arcos and Simon[14] obtained 4 liver tumours at 11 mos. in 14 rats fed 4′-methoxyDAB but no tumours at this time in 18 rats fed 4′-ethoxyDAB.

Orthoaminoazotoluene (oAAT; 2′-methyl-3-methyl-4-aminoazobenzene) which is considered to have some carcinogenic activity (see Crabtree [15]) produced very little B.D. in rat liver but was fairly active in increasing the GSH content. On the other hand its isomer, 3′-methyl-2-methyl-4-aminoazobenzene (known as mAAT in Table 3) which is said to be non-carcinogenic[15] for rat liver gave a high level of B.D. but only a slight increase in GSH. It must be noted, however, that when the dose of oAAT or mAAT was increased to 15·5 mg/100 g body wt. (i.e. twice the dose which is equimolar with 8·25 mg 3′-MeDAB/100 g body wt.) the levels of B.D. and GSH were increased appreciably by each substance so that the product B.D. $\times$ GSH for the supposedly non-carcinogenic mAAT exceeded that for the standard injection of 3′-MeDAB. If the product B.D. $\times$ GSH is significant for hepatocarcinogenesis, it is expected that feeding experiments with mAAT at doses higher than that (0·06%) employed by Crabtree[15] might reveal carcinogenic activity for this substance.

Among the mono-hydroxy substituted DAB derivatives which have been examined, none was capable of producing more than traces of B.D. in rat liver. However injections of 2-HODAB (authentic or low m.p. compound) and 3′-HODAB resulted in moderate increases in rat liver GSH. 2′-HODAB was only slightly active in this respect. The compound 2-hydroxy-4-diethylaminoazobenzene (2-HODEAB) produced no bound dye but caused some increase in liver GSH.

Injections of azobenzene resulted in no B.D. formation but caused a slight increase in rat liver GSH.

B.D. and GSH levels were determined in the livers of rats injected with 3′-methoxy DAB (3′-MeODAB; activity 10–12) 3′nitroDAB (activity 5) and 3′-ethoxyDAB (3′-EtODAB; activity $<$ 1). The increase in liver GSH was closely related to the relative carcinogenic activities of the dyes, but the weak carcinogen 3′-ethoxyDAB was a little more active in producing B.D. than was the stronger carcinogen 3′-nitroDAB.

Unfortunately, at the dose selected for comparative studies, the non-carcinogenic dyes 4-diethylaminoazobenzene and 4′-hydroxy-4-dimethylaminoazobenzene were highly toxic. At post-mortem of animals which died some hours after injection of these substances the lung cavity contained a large volume of nearly colourless fluid and sometimes ascites was present in the peritoneal cavity. The livers appeared shrunken. So far, only non-carcinogenic dyes have shown these toxic effects. Similar toxic reactions have been encountered in other work with the non-carcinogenic dye 2-MeDAB at 16·5 mg/100 g body wt. and an occasional death has occurred with this

dye at the 8·25-mg dose. Recently Hansen *et al.*[16] described hydrothorax and ascites in rats fed the azo dyes Yellow AB (non-carcinogenic) and Yellow OB (mild carcinogen).

Effect of mixtures of azo dyes on the GSH content and B.D. level in rat liver

If the simultaneous occurrence of B.D. and liver GSH increase is necessary and sufficient for hepatocarcinogenesis by azo dyes, it is conceivable that liver tumours could be produced in rats by feeding them pairs of non-carcinogenic or weakly active dyes provided that one of the dyes has the capacity to bind to liver protein and that the other can induce an increase in liver GSH. A suitable combination for study would be 2-MeDAB and 2-HODAB.

We have examined B.D. and GSH increase in livers of male rats which have received injections of mixtures of 2-MeDAB with 2-HODAB (low m.p.) or with authentic 2-HODAB.

The results are given in Table 4.

TABLE 4. EFFECT OF 2-MeDAB AND 2-HODAB SINGLY AND COMBINED ON LEVEL OF B.D. AND GSH IN RAT LIVER 24 hr AFTER INJECTION

Series	Dye injected*	B.D. (E at 520 mμ)	GSH† (mg %)		B.D × GSH
			(i)	(ii)	
A	2-MeDAB	0·053	—	126·4	—
	2-MeDAB + 2-HODAB (low m.p.)	0·087	—	267·1	—
	2-HODAB (low m.p.)	0·027	—	232·6	—
	nil	0·022	—	171·5	—
B	2-MeDAB	0·044	83·6	164·9	3·7
	2-MeDAB + 2-HODAB (low m.p.)	0·086	164·9	246·4	14·2
	2-MeDAB + 2-HODAB	0·051	154·0	222·3	7·9
	2-HODAB low m.p.)	0·026	178·8	250·6	4·6
	2-HODAB	0·019	137·8	207·3	2·6
	nil	0·024	109·1	194·8	2·6

* 8·25 mg 2-MeDAB and/or 8·3 mg 2-HODAB (authentic or low m.p.)/100 g body wt.
† (i) Cuprous mercaptide method.
(ii) Saville's method.

Series A experiments give mean results for pooled liver samples from three pairs of male rats. These experiments were carried out with 2-HODAB (low m.p.) which produced an appreciable increase in GSH but gave only a small amount of B.D. Injections of this dye together with 2-MeDAB more than offset the decline in liver GSH due to 2-MeDAB alone and led to an increase in B.D. over that due to 2-MeDAB alone. It is not known that the alleged 2-HODAB is non-carcinogenic but from its behaviour in our experiments it may be presumed to be without hepatocarcinogenic activity.

For Series B experiments which were carried out when authentic 2-HODAB (m.p. 162°, non-carcinogenic) became available, only 1 pair of rats was used per injection. It is seen that authentic 2-HODAB was not quite as active as the unknown azo compound in promoting an increase in rat liver GSH. However, it appears that

a mixture of 2-MeDAB with either alleged or authentic 2-HODAB could have carcinogenic activity for rat liver probably of the order of potency of DAB (see Table 3).

Some examples of synergism in relation to the carcinogenic action of mixtures of azo dyes are recorded in the literature. McDonald *et al.*[17] found that a mixture of 0·032% 4′-MeDAB and 0·03% DAB fed to rats was a more effective hepatocarcinogenic stimulus than either 0·03% DAB or 0·064% 4′-MeDAB alone, although the mixture was less effective than 0·06% DAB. Perhaps this synergism could be accounted for by increased levels of B.D. and GSH due to the mixture as compared with the contributions due to each dye separately.

Similarly an enhancement of the carcinogenic action of 0·02% 3′-MeDAB by 0·04% 2-MeDAB was noted by Arcos and Griffiths.[18] In 3 separate experiments (3 pairs of rats per dye or dye mixture injection) with a mixture of 3′-MeDAB and 2-MeDAB or each dye separately we found the mean values for B.D. and GSH shown

TABLE 5. EFFECT OF 3′-MeDAB AND 2-MeDAB SINGLY AND COMBINED ON LEVEL OF B.D. AND GSH IN RAT LIVER 24hr AFTER INJECTION

Dye injected*	B.D. (E at 520 mμ)	GSH† (mg %)
3′-MeDAB	0·126	267·4
3′-MeDAB + 2-MeDAB	0·152	248·5
2-MeDAB	0·053	126·4
nil	0·022	171·5

* 8·25 mg of each dye per 100 g body wt.
† Saville's method.

in Table 5. Evidently the mixture of dyes gave a higher B.D. × GSH value than either dye separately. It must be noted that our dye ratio of 1 : 1 (3′-MeDAB : 2-MeDAB) differs from the ratio of the fed dyes (1 : 2) which produced enhancement according to Arcos and Griffiths.[18] On our hypothesis however it is difficult to explain the loss of synergism noted by Arcos and Griffiths[18] when the concentration of 3′-MeDAB in their mixed diet was increased to 0·035%.

CONCLUSION

Maini and Stich[4] found for a series of hepatocarcinogens including azo dyes that the percentage of mitotic irregularities (P.M.I.) produced by the carcinogens correlated well with their carcinogenic activities. The exception in the series was the non-carcinogenic dye 2-MeDAB which gave almost the same P.M.I. as the highly active carcinogen, 3′-MeDAB. Maini and Stich[4] showed however that whereas 3′-MeDAB could also stimulate liver cell proliferation, 2-MeDAB failed to do so.

Thus we have been led to equate dye-binding ability with P.M.I. or nuclear damage and GSH increase with liver cell proliferation. In Table 6 we have compared some of our values for B.D. and B.D. × GSH with P.M.I. values quoted by Maini and Stich[4] (see their Table 1) after about 4 weeks of azo dye feeding.

The correlation coefficient between P.M.I. and B.D. was found to be 0·967 (significant at 1% level) and between P.M.I. and B.D. × GSH 0·973 which was almost significant at the 0·1% level. We have not included the values for 2-MeDAB in these correlations. If the dye-binding phenomenon is closely associated with P.M.I., there

TABLE 6. CORRELATION BETWEEN PERCENTAGE MITOTIC IRREGULARITIES (P.M.I.) DUE TO AZO DYES AND THEIR B.D. AND B.D. × GSH VALUES

Azo dye	Relative carcinogenic activity	P.M.I.*	B.D.	B.D. × GSH
3′-MeDAB	10–12	22·1	0·132	27·9
MAB	6	19·1	0·125	19·7
oAAT	2	7·7	0·030	4·6
4′-MeDAB	1	8·7	0·065	8·4
AB	0	4·8	0·046	6·7
2-MeDAB	0	19·9	0·050	4·8
nil		3·2	0·025	2·6

* data of Maini and Stich.[4]

should be no discrepancy with 2-MeDAB It is known from work of Miller and Miller[19] that dye-binding occurs more slowly with 2-MeDAB than with 3′-MeDAB when these dyes are fed to rats. Eventually however the extent of binding due to 2-MeDAB exceeds that due to 3′-MeDAB.

In a recent experiment we have compared the extent of dye-binding and change in rat liver GSH content due to 3′-MeDAB and 2-MeDAB at 48 hr as well as at 24 hr after injection. The results are shown in Table 7.

TABLE 7. COMPARISON OF THE EFFECT OF 3′-MeDAB AND 2-MeDAB ON RAT LIVER B.D. LEVEL AND GSH CONTENT 24 AND 48 hr AFTER INJECTION

Dye injected	B.D. (E at 520 mμ)	GSH* (mg %)		B.D. × GSH (i)
		(i)	(ii)	
3′-MeDAB (24 hr)	0·134	211·3	297·5	28·3
3′-MeDAB (48 hr)	0·109	178·0	254·5	19·4
2-MeDAB (24 hr)	0·058	72·8	139·4	4·2
2-MeDAB (48 hr)	0·081	170·3	216·0	13·8
nil—(24 hr)	0·022	120·8	185·4	2·7

* (i) Cuprous mercaptide method.
(ii) Saville's method

With 3′-MeDAB the amount of B.D. and GSH were less at 48 than at 24 hr. On the other hand, the B.D. level after 2-MeDAB injection was higher at 48 hr than at 24 hr. Moreover the liver GSH content was greater than normal at 48 hr so that the value for B.D. × GSH was now in the 'carcinogenic' range. Evidently a considerable increase in liver GSH content can occur in the recovery period following a single injection of 2-MeDAB. If the size of B.D. × GSH is important for hepatocarcinogenesis it would seem that conditions might be found under which 2-MeDAB could

exhibit carcinogenic properties. That 2-MeDAB does not ordinarily act as a carcinogen in feeding experiments[25] (0·064% in diet) may be related to the prevention of GSH increased by continual application of the dye. It has been stated[26] that the hydrochloride of 2-MeDAB (0·4–2·5% in diet) is hepatocarcinogenic for albino rats. Probably because of lower toxicity, higher solubility and more rapid excretion of the hydrochloride the effective dose of 2-MeDAB is lower than with the parent dye. This lower dose might permit the attainment of levels of B.D. and GSH sufficiently high for hepatocarcinogenesis.

Some results of Price *et al.*[20] point to a correlation between the carcinogenicity of an azo dye and the liver cellularity of rats 4 weeks from the start of dye feeding. It was of interest to compare their values for the number of nuclei per gram of fresh liver tissue with liver GSH content as determined in the present work. These values are shown in Table 8.

TABLE 8. CORRELATION BETWEEN THE NUMBER OF NUCLEI PER GRAM OF RAT LIVER AND THE GSH CONTENT FOLLOWING THE APPLICATION OF VARIOUS AZO DYES

Azo dye	Relative carcinogenic activity	No. of nuclei* per gram ($\times 10^6$)	GSH† (mg %)
3′-MeDAB	10–12	336	211·6
4′-FDAB	10–12	229	190·6
2′-MeDAB	2–3	160	145·2
2-MeDAB	0	126	99·5
nil		137	103·2

* Data of Price *et al.*[20]
† Cuprous mercaptide method.

Here it is seen that there is a close parallel between liver cellularity and GSH content. The correlation coefficient between the number of nuclei per gram and GSH content was 0·934 ($P = 0·025$) which suggests that the liver GSH content is closely related to the cellularity of the tissue.

Although the extent of dye-binding appears to be closely associated with percentage mitotic irregularities it need not necessarily be responsible for these irregularities. Some other less easily detectable parallel reaction may be involved. In 1958, Neish[21] found a correlation between the carcinogenic activity of some azo dyes and their ability to suppress rat serum copper and caeruloplasmin (a copper-containing enzyme) levels. Later studies (Neish, unpublished results) showed that the non-carcinogen 2-MeDAB had also some caeruloplasmin-suppressing action. These suppressive effects on serum copper due to carcinogenic dyes (e.g. 4′-EtDAB) and to the non-carcinogen, 2-MeDAB, may be related to disturbances in liver copper metabolism which might also be responsible for mitotic irregularities and nuclear damage in the liver.

Other hepatocarcinogens such as ethionine and tannic acid have been found to suppress rat serum caeruloplasmin.[22] According to Maini and Stich[4] ethionine produced 12% of mitotic irregularities in rat liver and this same hepatocarcinogen has been found by Calcutt[23] to increase the level of thiol in rat liver. Evidently ethionine could operate as a hepatocarcinogen in much the same way as azo dyes.

Our results are in accord with a statement by Calcutt *et al.*[24] that "an elevation of tissue —SH levels is an essential prerequisite for tumour formation." It would appear however that compounds which produce only an elevation of liver GSH are not necessarily carcinogenic although they may turn out to be incomplete carcinogens or cocarcinogens. At least one other factor appears to be necessary for hepatocarcinogenesis—a factor probably concerned with the nuclear damaging action described by Maini and Stich.[4] It is suggested here that this factor may involve disturbances of copper metabolism in cell nuclei possibly as a result of the chelating potentialities of carcinogens or their metabolites. The increase in liver GSH (cause or consequence of liver cell proliferation?) may be invoked for detoxication of carcinogen metabolites or it might arise as the result of derangements in cellular copper metabolism due to carcinogens.

Note added in proof

It has now been found that the compound known as 2-HODAB (low m.p.) is in fact 2-methoxy-4-dimethyl-aminoazobenzene (2-MeODAB). It gave no melting point depression when mixed with an authentic sample of 2-MeODAB (m.p. 104°) which has been described by Miller and Miller.[10] Both compounds gave identical visible absorption spectra (in ethanolic HCl). Moreover, authentic 2-MeODAB gave the same low degree of dye binding and the same high degree of GSH increase in rat liver as found for the unknown compound.

M. A. McKinnon, the microanalyst, found that under normal combustion conditions (sample burnt as approximately 750°), authentic 2-MeODAB tended to give low carbon values which were nearer to those expected for 2-HODAB. Using a higher combustion temperature of 800–850° samples of authentic 2-MeODAB and of the unknown azo compound gave C, H and N values in agreement with 2-MeODAB. It seems likely that under normal combustion conditions there is a tendency for a methyl group to form methane. This would account for the low C and H values and high N value quoted for the unknown azo compound.

In our original attempt to prepare N, N-dimethyl-m-aminophenol methiodide it would appear that excessive methylation led to the formation of N, N-dimethyl-m-anisidine. On coupling the product with benzene diazonium chloride 2-MeODAB rather than the expected 2-HODAB was obtained.

According to Miller and Miller (1961) 2-MeODAB is not carcinogenic for rat liver. Therefore it will be suitable to use in conjunction with 2-MeDAB in rat feeding experiments to test the hypothesis outlined in this paper.

Acknowledgements—Thanks are due to Mr. D. A. Taylor, computing assistant in the Dept. of Statistics. Microanalyses were carried out by Miss M. McKinnon, (Chemistry Dept., University of Sheffield) to whom our special thanks are due for her skilful and patient co-operation.

REFERENCES

1. W. J. P. NEISH and A. RYLETT, *Biochem. Pharmacol.* **12**, 893 (1963).
2. W. J. P. NEISH and A. RYLETT, *Biochem. Pharmacol.* **12**, 1147 (1963).
3. E. C. MILLER and J. A. MILLER, *Cancer Res.* **7**, 468 (1947).
4. M. M. MAINI and H. F. STICH, *J. Nat. Cancer Inst.* **26**, 1413 (1961).
5. B. SAVILLE, *Analyst*, **83**, 670 (1958).
6. J. A. MILLER and E. C. MILLER, *J. exp. Med.* **87**, 139 (1948).

7. J. A. MILLER, R. W. SAPP and E. C. MILLER, *Cancer Res.* **9**, 652 (1949).
8. J. A. MILLER, E. C. MILLER and G. C. FINGER, *Cancer Res.* **17**, 387 (1957).
9. E. STEDMAN, *Biochem. J.* **20**, 719 (1926).
10. J. A. MILLER and E. C. MILLER, *Cancer Res.* **21**, 1068 (1961).
11. L. PENTIMALLI, *Tetrahedron* **5**, 27 (1959).
12. G. RUDALI and R. ROYER, *Compt. rend. soc. biol.* **146**, 1531 (1952).
13. H. TERAYAMA, *Gann.* **55**, 195 (1963).
14. J. C. ARCOS and J. SIMON, *Arzneimittel-Forschung* **12**, 270 (1962).
15. H. G. CRABTREE, *Brit. J. Cancer* **3**, 387 (1949).
16. W. H. HANSEN, A. A. NELSON and O. G. FITZHUGH, *Toxicol. appl. Pharmacol.* **5**, 16 (1963).
17. J. C. MCDONALD, E. C. MILLER, J. A. MILLER and H. P. RUSCH, *Cancer Res.* **12**, 50 (1952).
18. J. C. ARCOS and G. W. GRIFFITHS, *Brit. J. Cancer* **15**, 291 (1961).
19. E. C. MILLER and J. A. MILLER, *Cancer Res.* **12**, 547 (1952).
20. J. M. PRICE, E. C. MILLER, J. A. MILLER and G. M. WEBER, *Cancer Res.* **10**, 18 (1950).
21. W. J. P. NEISH, *Experientia* **14**, 287 (1958).
22. W. J. P. NEISH, *Experientia* **15**, 20 (1959).
23. G. CALCUTT, *Brit. J. Cancer* **15**, 683 (1961).
24. G. CALCUTT, D. DOXEY and J. COATES, *Brit. J. Cancer* **15**, 149 (1961).
25. J. A. MILLER and C. A. BAUMANN, *Cancer Res.* **5**, 227 (1945).
26. N. NAGAO, *Gann.* **35**, 280 (1941) cited by J. L. HARTWELL in *Survey of compounds which have been tested for carcinogenic activity*, p. 370 U.S. Public Health Service Publ. No. 149, 2nd Ed., 1951.