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AGENTS AFFECTING BOUND DYE AND LIVER GLUTATHIONE OF RATS INJECTED WITH 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE

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Abstract—Sodium salicylate and aluminon strongly inhibited dye-binding and liver glutathione increase due to injections of 3′-methyl-4-dimethylaminoazobenzene into male rats.

L-thyroxine decreased the glutathione content but had no effect on dye-binding. 3,5-diiodotyrosine failed to affect dye-binding or liver glutathione content. Copper sulphate injections reduced the amount of dye-binding and the glutathione level.

THE LIVER carcinogen, 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB) caused a marked rise in glutathione (GSH) content and produced an appreciable amount of bound dye (B.D.) in male rat liver.[1] With other aminoazo dyes of varying degrees of hepatocarcinogenicity it was found that the more potent ones gave a greater degree of B.D. and a greater increase in liver GSH than did the weaker ones. On the other hand some non-carcinogenic azo dyes such as 2-methyl-4-dimethylaminoazobenzene produced bound dye but did not increase the liver GSH content whereas others such as 2-methoxy-4-dimethylaminoazobenzene failed to bind but caused an increase in liver GSH.

An attempt has now been made to examine a possible relationship between the degree of dye-binding and GSH-inducing capacity of 3′-MeDAB through the application of various substances including sodium salicylate, thyroxine and cupric sulphate which are themselves capable of influencing the GSH content of rat liver.

EXPERIMENTAL

Pairs of stock male albino rats [~250–350 g bodyweight (b.w.)] were injected intraperitoneally with 8·25 mg of 3′-MeDAB in 0·6 ml of arachis oil per 100 g bodyweight. Control animals received arachis oil only. Other compounds such as L-thyroxine were dissolved or suspended in arachis oil with or without 3′-MeDAB. Water-soluble materials such as sodium salicylate were injected intraperitoneally as aqueous solutions immediately after the appropriate oil injection. Control animals received comparable volumes (usually 0·33 ml/100 g b.w.) of de-ionised water intraperitoneally. The rats, which had access to Diet No. 86 and water *ad libitum*, were killed with ether 24 hr after injection and the livers, perfused with ice-cold normal saline, were frozen at once in solid carbon dioxide.

Bound dye estimations

One g samples of liver from each of a pair of identically treated rats were pooled and processed according to the method of Miller and Miller.[2] Alkaline hydrolysis

was carried out on 50 mg samples of dried liver powder and the B.D. content determined as previously described.[1]

GSH estimation

One g samples of liver from each of a pair of identically treated rats were pooled and homogenised with 2 ml of 10% trichloroacetic acid (TCA) in an all-glass homogeniser. The homogenate was washed into a centrifuge tube with 1 ml of 10% TCA and supernatant was collected after centrifugation for 5 min at 2,500 rev/min. The precipitate was resuspended in 1 ml of 10% TCA, centrifuged as before, and the supernatant added to the first supernatant. The washing process was then repeated with another 1 ml of 10% TCA. A 1 ml aliquot of the combined supernatant (total volume 4·5–5·0 ml) was made up to 10 ml with 1 N sulphuric acid and 1 ml of this solution was used for GSH determination by the method of Saville as already described.[1]

RESULTS

Effect of sodium salicylate (sodium *ortho*-hydroxybenzoate: OHB) and related compounds on B.D. and GSH contents of livers of 3′-MeDAB-injected and control rats.

TABLE 1. EFFECT OF SALICYLATES AND RELATED COMPOUNDS ON THE B.D. AND GSH CONTENTS OF LIVERS OF MALE RATS INJECTED WITH 3′-MEDAB IN ARACHIS OIL OR WITH ARACHIS OIL ONLY

No. of pairs of rats used	Supplementary injection		Main injection			
			3′-MeDAB in arachis oil		Arachis oil only	
	Substance	Dose mg/ 100 g b.w.	B.D. E at 520 mμ	GSH mg %	B.D. E at 520 mμ	GSH mg %
2	Water		0·114	317·5	0·021	199·2
1	OHB	25	0·063	277·5	0·013	205·8
2	OHB	50	0·039	222·1	0·024	210·7
1	MHB	50	0·102	338·4	0·026	213·6
1	PHB	50	0·105	274·4	0·021	205·8
3	Water		0·116	281·0	0·023	174·3
1	Aluminon	50	0·043	204·7	0·012	218·6
1	PAS	65·9	0·121	301·4	0·036	174·8
1	BAS*	8·35	0·096	315·0	0·013	127·5
1	OAB	45·3	0·090	247·5	0·032	172·8
1	PAB	45·3	0·092	232·1	0·034	165·6
1	HOQ*	10	0·102	268·8	0·028	153·0

* Incorporated in arachis oil. BAS dose equivalent to standard dose of 3′-MeDAB. All other substances in aqueous solution. Aluminon dose arbitrary. Other doses are molar equivalents of sodium salt corresponding to 50 mg sodium salicylate (OHB) per 100 g b.w.

From Table 1 it is seen that injections of OHB (50 mg sodium salt/100 g b.w. applied as 15% aqueous solution) almost completely prevented binding of 3′-MeDAB to rat liver protein, and markedly suppressed the increase in liver GSH. Injections of OHB at 25 mg/100 g b.w. also prevented dye-binding and GSH increase though to a lesser extent than did the 50 mg dose. OHB alone (25 or 50 mg dose) caused a slight increase in the liver GSH content of control rats injected with arachis oil.

The isomers sodium *meta*- or *para*-hydroxybenzoates (MHB or PHB) failed to

influence dye-binding and only in the case of PHB was the liver GSH content somewhat reduced. In control rat livers, both MHB and PHB, like OHB, caused some increase in liver GSH.

Aluminon (ammonium salt of aurintricarboxylic acid) a triphenylmethane dye composed of 3 salicylate residues each linked through position 5 to the methane carbon atom behaved like OHB in that it suppressed the dye-binding and GSH increase due to 3′-MeDAB and increased the GSH content of control rat livers. Salicylic acid derivatives with an amino group at position 4 (sodium *para*-aminosalicylate : PAS) or a benzeneazo group at position 5 (benzeneazosalicylic acid; BAS) failed to suppress B.D. and GSH formation due to 3′-MeDAB. BAS by itself gave no bound dye. It decreased the GSH level in control rat liver.

It is interesting that powders prepared from livers of aluminon-injected rats for B.D. studies, had a faint pink colour quite distinct from the usual grey colour of powders from 3′-MeDAB-treated or control rat livers. During alkaline hydrolysis the powders became bright crimson and dissolved gradually to give solutions which had about the same colour intensity whether the liver had been treated with 3′-MeDAB or not. After several hours of hydrolysis at 80° the crimson colour disappeared. Evidently aluminon or a derivative—free or bound—is associated with the liver powders and is released and degraded slowly by alkaline hydrolysis.

The ability of OHB and aluminon to suppress dye-binding and GSH increase due to 3′-MeDAB might be associated with the metal-chelating potentiality of the ortho substituents. It was of interest to study the effect of replacing the hydroxy group by an amino group as in sodium anthranilate (sodium *ortho*-aminobenzoate: OAB). This compound failed to inhibit dye binding though it prevented to some extent the rise in GSH. Its isomer sodium para-aminobenzoate (PAB) had little or no effect on B.D. but caused some decrease in liver GSH. The well-known chelating agent 8-hydroxyquinoline (HOQ) had no effect on dye-binding but it brought about some reduction in the GSH content of 3′-MeDAB-injected and control rat livers.

Effect of L-*thyroxine and other compounds on the B.D. and GSH content of livers of 3′-MeDAB-injected and control rats*

It has been stated[3] that thyroidectomy, thyroxine injections or the application of thyroid drugs such as thiouracil give rise to increased levels of GSH in various tissues. However, Charkey and Hougham[4] noted that iodocasein decreased liver GSH in cockerels. It seemed possible that the increased levels of GSH in rat liver following injections of hepatocarcinogens might result from disturbances in thyroid gland metabolism.

L-thyroxine (monosodium salt) or DL-3,5-diiodotyrosine at various doses were injected intraperitoneally as suspensions in arachis oil or in arachis oil containing 3′-MeDAB. The results of these experiments are given in Table 2.

With increasing dose of L-thyroxine, the level of GSH in both normal and 3′-MeDAB livers declined markedly but the extent of dye-binding of 3′-MeDAB was hardly affected. Diiodotyrosine caused no marked reduction in the GSH content of 3′-MeDAB-treated or normal livers and it had little or no effect on dye-binding.

Since thyroxine and OHB are both phenolic substances with ortho substituents it was of interest to compare the effects of the closely related substances tyrosine (1 mg/100 g b.w.) and dihydroxyphenylalanine (DOPA; 2 mg/100 g b.w.) on B.D. and

TABLE 2. EFFECT OF L-THYROXINE AND OTHER SUBSTANCES ON THE B.D. AND G.S.H. CONTENT OF LIVERS OF MALE RATS INJECTED WITH 3′-MEDAB IN ARACHIS OIL OR WITH ARACHIS OIL ONLY

No. of pairs of rats used	Supplementary injection		Main injection			
			3′-MeDAB in arachis oil		Arachis oil only	
	Substance*	Dose mg/100 g b.w.	B.D. E at 520 mμ	GSH mg %	B.D. E at 520 mμ	GSH mg %
4	nil		0·112	294·7	0·023	191·8
1	L-thyroxine	2	0·109	262·7	0·010	177·1
2		4	0·102	251·5	0·027	145·7
1		8	0·121	221·7	0·036	139·1
2	Diiodotyrosine	1	0·127	316·3	0·025	194·4
3		2	0·114	308·9	0·030	169·0
2		4	0·125	287·4	0·028	180·2
1	tyrosine	1	0·112	237·7	0·024	175·0
1	DOPA	2	0·107	292·6	0·038	201·5
1	Water		0·104	306·3	0·013	187·5
1	Copper sulphate	1	0·053	150·4	0·008	181·7

* With the exception of copper sulphate, materials were incorporated as fine suspensions in the arachis oil injections.

GSH increase due to 3′-MeDAB. Neither compound affected dye-binding but tyrosine suppressed the GSH level in both 3′-MeDAB-treated and control rat livers.

Copper salts are known to exert a protective action against liver carcinogenesis by aminoazo dyes.[5] In the present study, rats received the standard injection of 3′-MeDAB or of arachis oil followed by injection of an aqueous solution of copper sulphate (3 mg of copper sulphate pentahydrate/ml) at the dose of 1 mg of copper sulphate/100 g b.w. As shown in Table 2, dye-binding was reduced appreciably as was the GSH content of the livers of 3′-Me-DAB-treated rats injected with copper sulphate.

DISCUSSION

Sodium salicylate and aluminon strongly suppressed dye-binding and liver GSH increase due to injections of 3′-MeDAB into rats. If these phenomena are important for hepatocarcinogenesis as has been suggested,[1] salicylate and aluminon should protect rat liver against azo dye carcinogenesis.

The cause of the inhibitory effects of salicylates on dye binding remains to be found. Meanwhile it is interesting to note that aluminon and salicylic acid are the most effective of a series of related substances in protecting mice against beryllium poisoning.[6] Among substances which were ineffective were *meta*- and *para*-hydroxybenzoic acids, anthranilic acid, DOPA and 8-hydroxyquinoline. These same substances had no effect on the degree of dye-binding of 3′-MeDAB to rat liver. Lindenbaum *et al.*[6] found that not all salicylic acid derivatives were active in preventing beryllium poisoning. For instance several dyes closely related to benzeneazosalicylic acid (BAS) were inactive and in the present work it was found that BAS did not inhibit dye-binding. However *para*-aminosalicylic acid failed to inhibit dye-binding though this compound

was shown to be quite active in increasing the survival percentage of beryllium-treated mice.[6]

In general there is a close parallel between the inhibitory effects of certain salicylate derivatives on 3′-MeDAB dye-binding in rat liver and their ability to prevent beryllium poisoning in mice. This latter effect has been attributed to chelation of beryllium with the active compounds. A possible explanation for the inhibition of dye-binding by salicylates might lie in the ability of these substances to chelate some element which is involved directly or indirectly with the linkage of azo dye or its metabolite to rat liver protein. Aluminon has a strong affinity for proteins and is known to be retained in rat liver for several months.[7] It might inhibit dye-binding simply by blocking some reactive site in rat liver protein.

There appears to be no direct interrelation between dye-binding and GSH increase. Often marked inhibition of dye-binding is accompanied by a large decrease in liver GSH. However some substances are able to cause a moderate decline in liver GSH without affecting 3′-MeDAB binding.

L-thyroxine caused a marked decrease in liver GSH in both control and 3′-MeDAB-injected rats but it failed to influence dye-binding. In this case, the mechanism of GSH suppression is probably different from that operating in the cases of salicylate or aluminon which are able to increase the level of control rat liver GSH. The decrease in liver GSH due to thyroxine might result from conversion of GSH to the oxidised form GSSG which would not be estimated by Saville's method. Similarly catalytic oxidation of GSH by copper could account for the lower levels of GSH encountered in rat liver after copper sulphate injection.

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