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AZO DYES AND RAT LIVER GLUTATHIONE

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Abstract—The level of male rat liver glutathione (GSH) rapidly increased following intraperitoneal injection of the strong heptocarcinogen, 3′-methyl-4-dimethylamino-azobenzene (3′-MeDAB). A more gradual, less-pronounced increase resulted from injection of the weaker hepatocarcinogen, 4′-methyl-4-dimethylaminoazobenzene (4′-MeDAB). On the contrary, 24 hr after injection of the non-carcinogenic azo dye, 2-methyl-4-dimethylaminoazobenzene, the level of rat liver GSH dropped below normal.

Paper chromatography of trichloracetic acid extracts of 3′-MeDAB-treated liver showed that besides the increase in GSH, other changes occurred including an increase in phosphoethanolamine (PE). The pattern of changes was markedly similar to that observed by other workers in extracts of regenerating rat liver. The only marked change with the weak carcinogen 4′-MeDAB was the increase in GSH.

A survey is given of substances capable of increasing liver GSH levels. These include the carcinogen 3,4:5,6-dibenzcarbazole, the anti-tumour agent, nitromin and two anti-inflammatory agents salicylic acid and antipyrine which are not known to be carcinogenic. Attention is directed to the metal-chelating agent, 8-hydroxy-quinoline which has both anti-inflammatory properties and carcinogenic activity. It is suggested that anti-inflammatory substances should be carefully screened for carcinogenic and/or cocarcinogenic activity.

IN A previous investigation[1] we noted that the level of glutathione (GSH) in the livers of male rats injected with the strong hepatocarcinogen, 3′-methyl-4-dimethylamino-azobenzene (3′-MeDAB) in arachis oil was greater than in the livers of control rats which received injections of arachis oil only. Thus 72 hr after injection, the GSH levels were 167 mg per cent and 122 mg per cent respectively as determined by the cadmium–cuprous mercaptide method of Waelsch and Rittenberg.[2] These authors claimed that although their method permits only 55–70 per cent recoveries of rat liver GSH, the cuprous mercaptide samples were analytically pure, containing no thiol other than GSH. We found that when cuprous mercaptide samples from normal or 3′-MeDAB-treated rat livers were suspended in water, decomposed with hydrogen sulphide and the resulting copper-free supernatants examined by paper-electrophoresis, only spots due to GSH could be detected. Thus the mercaptide procedure appeared to be quite specific for liver GSH and it was felt that it could be usefully applied (in spite of low recoveries) in a comparative investigation of the effects of various azo dyes on the level of rat liver GSH.

We have now determined the GSH content of 3′-MeDAB-treated rat livers and of control rat livers at 24, 48, 72 and 96 hr after injection of the dye in arachis oil or of arachis oil only, and a similar experiment has been carried out in which the weak carcinogen 4′-MeDAB was injected instead of 3′-MeDAB.

The free ninhydrin-positive components of TCA extracts of livers of dye-treated and control rats have been examined by two-dimensional chromatography[1] and it has been found that injection of the strong carcinogen 3′-MeDAB leads not only to elevation of liver GSH levels but to other changes similar to those observed by Sano and Roberts[3] and by Ferrari and Harkness[4] in extracts of regenerating rat liver following partial hepatectomy.

EXPERIMENTAL

Glutathione determination

Eight stock adult male albino rats were each given a single intraperitoneal injection of a solution/suspension of finely-divided 3′-MeDAB or 4′-MeDAB in arachis oil (16·5 mg azo dye in 0·6 ml arachis oil per 100 g body weight). Eight control rats received intraperitoneal injections of arachis oil only (0·6 ml per 100 g body weight). The animals had access to water and Diet No. 86 *ad libitum*.

Pairs of dye-injected rats and of control rats were killed with ether at 24, 48, 72 and 96 hr after injection and the livers perfused (for several minutes) with ice-cold normal saline. The injection times were arranged so that all 16 livers could be collected in as short a time as possible (11 a.m.–1 p.m. on the same day.) Livers were stored at −15° for approximately 2 hr before GSH determination, which was carried out as follows.

A 5 g portion of each frozen liver was homogenized in an all-glass homogenizer with 5 ml ice-cold 10% TCA solution and the homogenate transferred to a centrifuge tube. The homogenizing tube and pestle were washed with 2·5 ml 10% TCA and the washings added to the centrifuge tube. After centrifugation for 5 min at 3000 rev/min, the clear supernatant was collected and the protein precipitates were washed twice on the centrifuge (5 min at 3000 rev/min)with 2·5 ml portions of 10% TCA. The supernatants from these washings were combined with the first supernatant. At this stage a 1 ml sample of the TCA supernatant was removed and reserved for chromatographic examination as described later. The remaining TCA extracts from pairs of identical livers were then pooled and the cadmium–cuprous mercaptide procedure[2] was applied to the pooled extracts.

According to Waelsch and Rittenberg,[2] the cuprous mercaptide of GSH is obtained as a hydrate unless it is dried for at least 2 hr *in vacuo* over P_2O_5 at 78°. C and H analyses on the mercaptide prepared by us from authentic GSH and stored overnight at room temperature *in vacuo* over P_2O_5 (conditions employed in handling the liver cuprous mercaptides) indicated that it contains 1·5 moles water per mole GSCu.

For $C_{10}H_{16}N_3O_6SCu.\ 1{\cdot}5\ H_2O$

Calcd.	C = 30·26%	Found	C = 30·30%
	H = 4·82%		H = 4·71%

The GSH levels quoted in Tables 1, 2 and 3 have been calculated on the assumption that in every case the mercaptide consisted of GSCu 1·5 H_2O. Allowance has been made for the 1 ml aliquots of TCA extract removed for chromatography from each liver extract prior to pooling of pairs of extracts. From the weight of mercaptide obtained, we calculated the amount expected from 2 ml TCA extract and added this value to the mercaptide weight to obtain an estimate of the total mercaptide per 2 × 5 g liver and hence of GSH per 100 g liver.

Paper chromatography

Samples of liver TCA extracts (1 ml) were extracted 6 times with 1 ml portions of ether to remove excess TCA. The last traces of ether were removed in a stream of nitrogen and the aqueous residues were stored at −15° until required.

0·01 samples were chromatographed in the phenol–ammonia and butanol–methyl-ethylketone-dicyclohexylamine–water solvents proposed by Bowden[5] and used by us[1] in earlier studies.

RESULTS

GSH levels

In Tables 1, 2 and 3 are presented the GSH levels of livers of male albino rats injected with 3′-MeDAB, 4′-MeDAB, 2-MeDAB and with arachis oil only. We have included values for the change in body weight of the animals which occurred between time of injection of the dye and death and we also give liver weights expressed as a

TABLE 1. GSH LEVELS IN COMBINED LIVER SAMPLE (2 × 5 G) FROM PAIRS OF MALE ALBINO RATS AT VARIOUS TIMES AFTER INTRAPERITONEAL INJECTION OF 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE IN ARACHIS OIL (RATS 1–8) OR OF ARACHIS OIL ONLY (RATS 9–16)

Rat No.	Time elapsed since injection (hr)	Total body weight at death (g)	Weight change since injection (g)	Liver weight % of total body weight at death	Cuprous mercaptide mg/10 g liver wet weight	GSH level mg/100 g liver wet weight
1	24	233	−5	3·4	32·5	250
2		295	−5	3·1		
3	48	317	−11	3·0	24·0	185
4		332	−10	3·0		
5	72	274	−8	3·5	20·4	157
6		285	−20	3·1		
7	96	300	−10	3·7	19·3	149
8		286	−9	3·5		
9	24	269	+6	3·7	13·5	104
10		256	+3	3·9		
11	48	341	+1	3·5	13·2	102
12		365	−1	3·7		
13	72	295	+5	3·9	15·6	120
14		371	+5	3·8		
15	96	290	+20	3·6	11·5	89
16		323	−7	3·7		

percentage of the total body weight at death. There is no direct relationship between the change in liver GSH levels and, for example, drop in body weight which may be taken as a guide to the toxicity of the injected substance. In general, it would appear that 4′-MeDAB is more toxic than 3′-MeDAB, while 2-MeDAB is of intermediate toxicity.

There was a tendency for liver percentage weight to be lower in the 3′-MeDAB group than in controls (Table 1). From Table 3 it can be seen that the livers of rats injected 24 hr previously with 2-MeDAB are smaller than those of rats injected with 3′-Me-, 4′-Me-DAB or arachis oil alone. However the percentage of dry matter

(portions of liver were dried to a constant weight in an oven at 110°) in the 2-MeDAB livers was the same as in the other livers.

TABLE 2. GSH LEVELS IN COMBINED LIVER SAMPLE (2 × 5 G) FROM PAIRS OF MALE ALBINO RATS AT VARIOUS TIMES AFTER INTRAPERITONEAL INJECTION OF 4′-METHYL-4-DIMETHYLAMINOAZOBENZENE IN ARACHIS OIL (RATS 1–8) OR OF ARACHIS OIL ONLY (RATS 9–16)

Rat No.	Time elapsed since injection (hr)	Total body weight at death (g)	Weight change since injection (g)	Liver weight % of total body weight at death	Cuprous mercaptide mg/10 g liver wet weight	GSH level mg/100 g liver wet weight
1	24	252	−13	3·1	11·8	91
2		274	−26	3·3		
3	48	272	−33	3·5	14·3	110
4		281	−14	3·5		
5	72	246	−9	3·5	11·5	89
6		299	−6	4·1		
7	96	232	−8	3·7	15·8	122
8		269	−11	4·3		
9	24	281	−5	4·0	8·2	63
10		244	−6	3·3		
11	48	298	+5	3·6	9·9	76
12		261	−4	3·6		
13	72	256	+4	3·7	6·5	50
14		255	+5	3·7		
15	96	257	+12	3·5	7·5	58
16		286	+1	3·7		

TABLE 3. GSH LEVELS IN COMBINED LIVER SAMPLE (2 × 5 G) FROM PAIRS OF MALE ALBINO RATS INJECTED INTRAPERITONEALLY WITH AZO DYE (16·5 MG/100 G BODY WEIGHT) IN ARACHIS OIL (0·6 ML/100 G BODY WEIGHT) OR WITH ARACHIS OIL ONLY (0·6 ML/100 G BODY WEIGHT) AND KILLED 24 HR AFTER INJECTION

Rat No.	Material injected	Total body weight at death (g)	Weight change since injection	Liver weight % of total body weight at death	Dry weight % of liver	Cuprous mercaptide mg/10 g liver wet weight	GSH level mg/100 g liver wet weight
1	3′-MeDAB	330	−4	3·5	26·3	22·0	169
2		286	−9	3·3	26·1		
3	4′-MeDAB	285	−13	3·3	25·2	13·5	104
4		340	−23	3·0	26·6		
5	2-MeDAB	323	−7	2·3	26·9	4·6	35
6		358	−19	2·6	25·7		
7	Arachis oil	256	−4	3·6	26·1	11·1	86
8	control	322	+7	3·7	26·8		

The trends of change in liver GSH levels as a result of injections of 3′-MeDAB and 4′-MeDAB are shown in Fig. 1 which has been prepared from data in Tables 1 and 2. In each experiment, the mean control liver GSH value was calculated and setting this value equal to 1 we recalculated the GSH levels for livers of dye-treated rats on this basis.

It is interesting to compare the trends shown in Fig. 1 with plots of amount of protein-bound dye in livers of rats at various times after dye-feeding as determined by Miller and Miller.[6] For 3′-MeDAB, the most extensive dye binding occurs early in the feeding experiment after which the level declines as dye-feeding is continued. With 4′-MeDAB, dye-binding is at first less than with 3′-MeDAB but it increases with time of feeding reaching a maximum at a later time than with 3-′MeDAB and at a time when 3′-MeDAB binding is already in decline. Thus there is a parallel between the binding capacity of these dyes and their ability to cause accumulation of liver GSH.

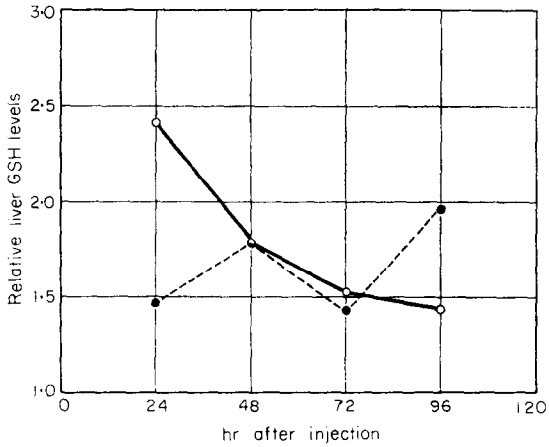

FIG. 1. Changes in the relative levels of GSH with time in livers of rats injected with 3′-MeDAB in arachis oil (○——○) or with 4′-MeDAB in arachis oil (●——●).
(For explanation see text and Tables 1 and 2.)

On the other hand, the Millers' results[6] for the binding of the non-carcinogenic dye 2-MeDAB indicate that at an early stage in the feeding experiment when 3′-MeDAB binding is high, the level of binding attained by 2-MeDAB is lower than that attained by 3′-MeDAB but greater than the level reached by 4′-MeDAB. Thus the parallel between extent of dye-binding and increasing GSH level breaks down at this point since we have found (Table 3) that 2-MeDAB causes a drop in liver GSH levels below the level for 4′-MeDAB liver (and for control livers) 24 hr after injection.

Recently a group of South African workers reported[7] that after a single dose (feeding) of azo-dye the protein-binding of the dye "is accompanied by a large increase in the level of trichloracetic acid soluble sulphydryl groups in the liver. The magnitude of the increase after 40 hr is related to the extent of binding in the case of the various carcinogenic and non-carcinogenic azo dyes". Our results at 24 hr after dye injection seem to support this statement at least in part.

We can give no explanation at present for the lower control liver GSH values found in the 4′-MeDAB experiment (Table 2) as compared with the 3′-MeDAB experiment (Table 1). Beck and co-workers[8] have shown that there is a marked diurnal variation in rat liver GSH levels. Because of these findings care was taken in the present study to ensure that as far as possible livers were collected at the same time of day during 11 a.m.–1 p.m.

With regard to GSH recovery, we have not studied recovery of authentic GSH added to liver extracts. We have found that there are reasonably consistent low recoveries of GSH when its TCA solution is treated with cadmium chloride etc. exactly as for liver extracts. GSH recovery experiments were not run with the 3′-MeDAB experiment (Table 1) but pairs of recovery experiments were performed at the same time as the animal experiments described in Tables 2 and 3. The recovery percentages were 49·4, 40·2 and 39·9, 41·5 respectively.

Paper chromatography

3′-MeDAB series. A sharp rise in liver GSH occurred 24 hr after injection of 3′-MeDAB and the levels of phosphoethanolamine (PE), glutamic acid (glu) and aspartic acid (asp) were also higher as compared with 24 hr control livers, judging from the intensity of reaction of the spots with ninhydrin. We have shown[9] previously that intraperitoneal injections of carcinogenic azo dyes increase the level of rat liver PE. After 24 hr, GSH declined but at 96 hr after injection its level was still higher in 3′-MeDAB livers than in controls. Glu and asp levels were higher in 3′-MeDAB livers at 24 hr (not seen clearly in Fig. 2) and more so at 48 hr after injection than in control livers but at 72 and 96 hr after injection the levels of these amino acids in the dye-treated livers were approaching those of the control livers.

Figure 2 shows chromatograms of TCA extracts of some 3′-MeDAB livers and of control livers. Chromatograms of aqueous solutions of GSH (E) and GSSG (F) are given for comparison. The spots marked 2 and 3 in liver extract chromatograms appear to be due to GSSG and that marked 1 to GSH which has survived oxidation in solution and during the run on the paper. 3′-MeDAB extract chromatograms A and C show large No. 1 spots (A > C) due to GSH but only traces of this spot can be seen in control liver extract B and none at all in D.

In the 3′-MeDAB series, GSSG spots Nos. 2 and 3 had about the same intensity from 24 to 96 hr after injection but this was not so in the control series where spots Nos. 2 and 3 became more intense at 72 and 96 hr than at 24 and 48 hr after injection. Possibly there is a slow build-up in the level of liver GSSG following arachis oil injection or the liver extracts of oil-injected rats contain some material which promotes oxidation of GSH to GSSG.

The spot marked No. 2 in liver extract chromatograms is shaped like an S lying on its side and quite distinct from the corresponding spot due to authentic GSSG (see Fig. 2F). Whether this S-shaped spot is due solely to GSSG distorted through interactions with other components of the TCA extract or to GSSG plus some other ninhydrin-positive material remains to be determined.

Other trends were seen in chromatograms of extracts of 3′-MeDAB livers. At 24 and 48 hr after injection, the taurine (tau) levels of these livers appeared to be greater than in control livers but at 72 and 96 hr tau levels were less than in control liver extracts. Throughout the 3′-MeDAB series, the glutamine (glu-NH_2) levels seemed in general to be less than in control livers though it was rather difficult to judge the intensity of this spot as it overlaps a spot due to α-ala.

4′-MeDAB series. The GSH levels in all dye-treated livers were greater than in control livers and the levels increased from 24 hr up to 96 hr following 4′MeDAB injection. It appears from Fig. 3 which shows chromatograms of TCA extracts of some

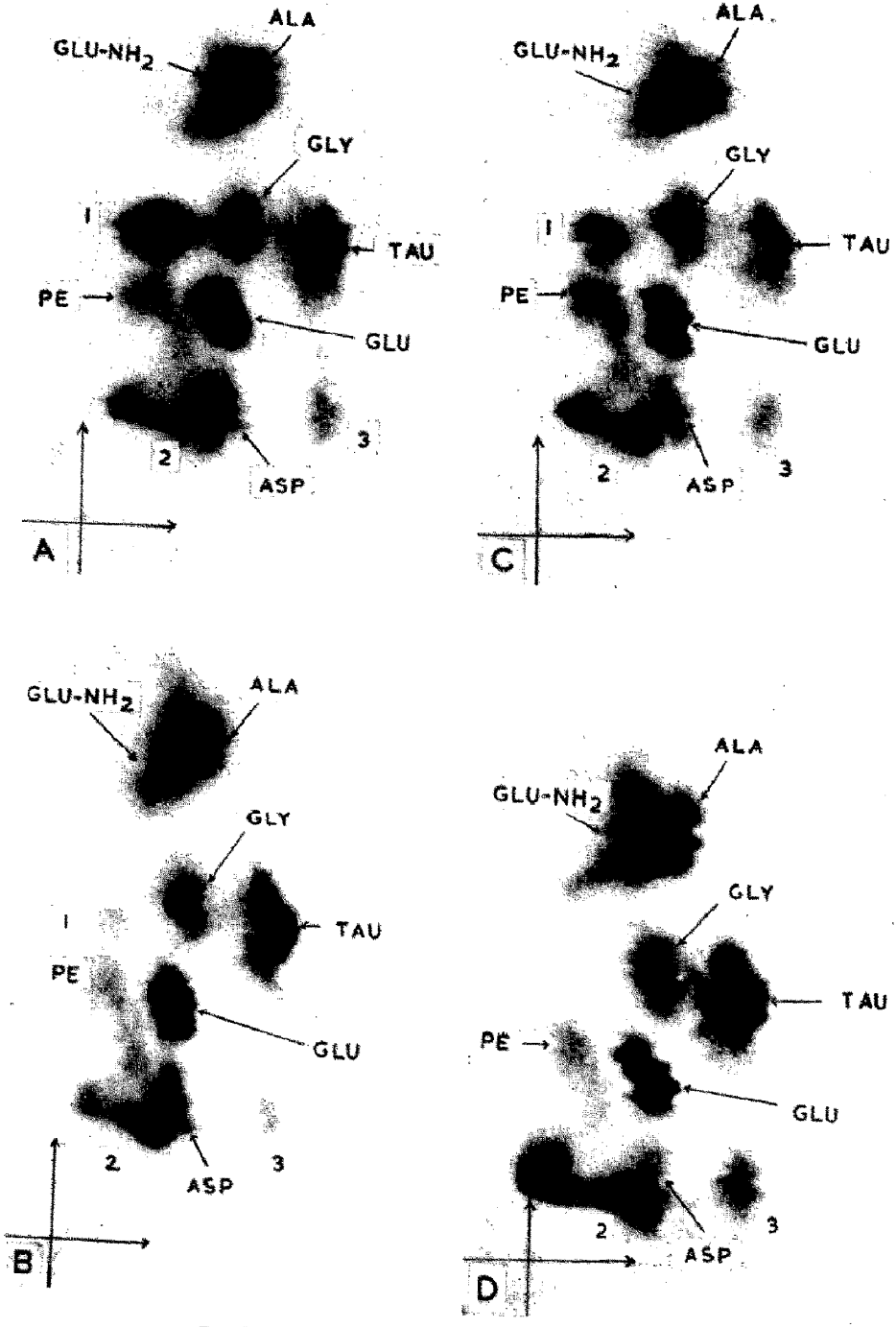

FIG. 2. Remainder of illustration and caption overleaf.

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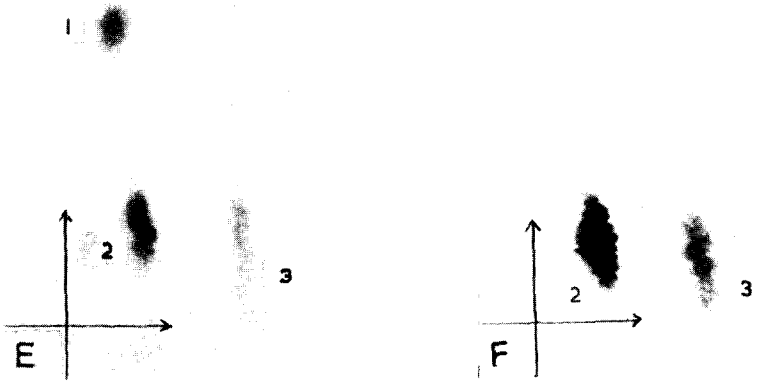

FIG. 2. Chromatograms of TCA extracts (0·01 ml) of rat livers
A and C: 24 and 74 hr respectively after injection of 3′-MeDAB in arachis oil.
B and D: 24 and 72 hr respectively after injection of arachis oil.
Chromatograms E and F: 0·01 ml of solutions of GSH and GSSG in water (1 mg/ml) respectively.
Axes cross at point of application of extract
Vertical axis = direction of phenol solvent
Horizontal axis = direction of butanol–methylethyl ketone solvent
Chromatograms developed with a mixture of 0·2% ninhydrin in n-butanol (100 ml) and glacial acetic acid (4 ml). Dried at room temperature for 24 hr and photocopied on Kodak autopositive paper.
Numerals 1, 2 and 3 represent spots due to GSH and CSSG (see text).
ala = α-alanine; asp = aspartic acid; glu = glutamic acid; glu-NH_2 = glutamine; gly = glycine; PE phosphoethanolamine; tau = taurine.

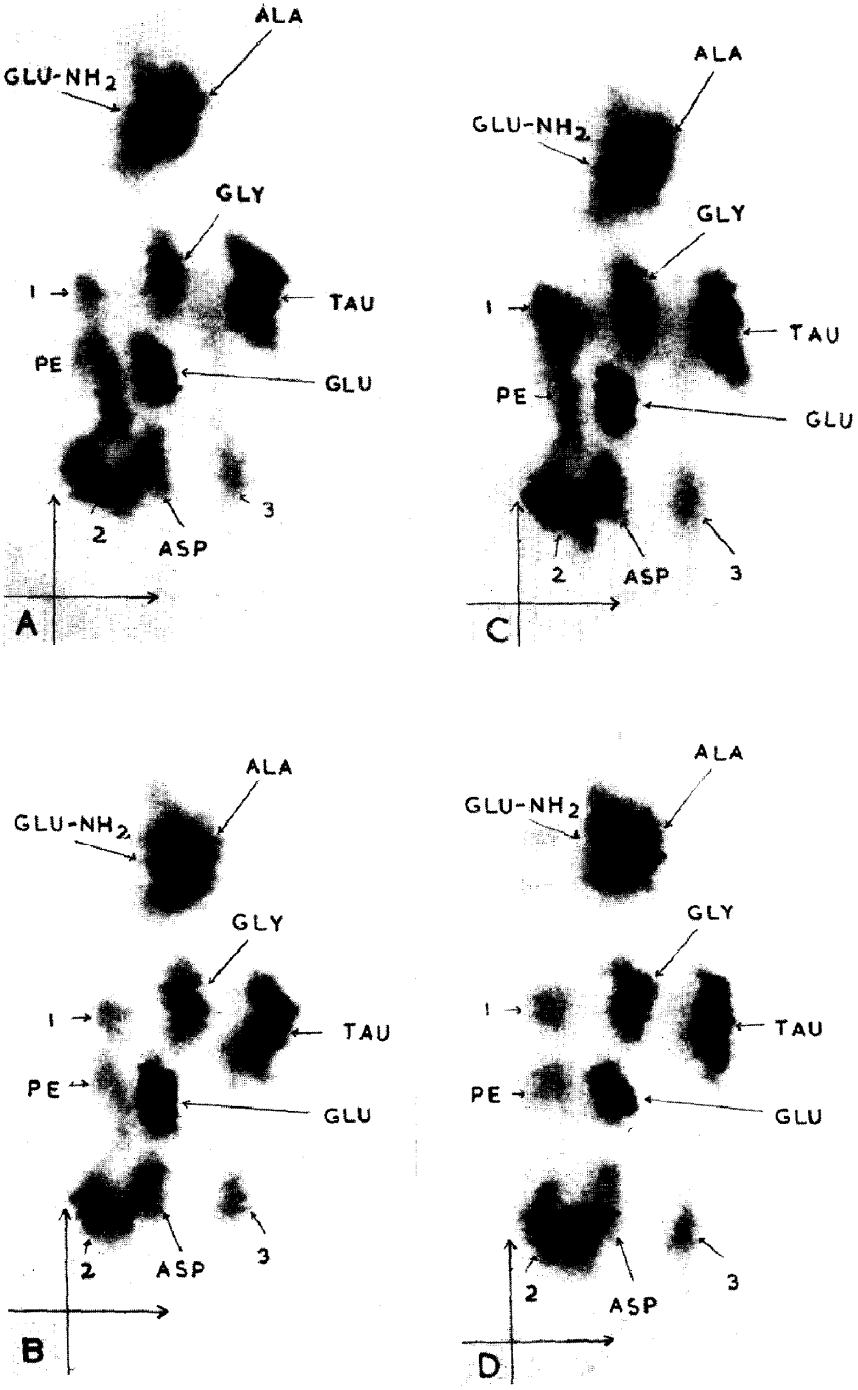

FIG. 3. Chromatograms of TCA extracts (0·01 ml) of rat livers.
A and C: 24 and 96 hr respectively after injection of 4′-MeDAB in arachis oil.
B and D: 24 and 96 hr respectively after injection of arachis oil.
(For explanation of symbols see Fig. 2 legend and text).

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4′-MeDAB livers and of control livers that this increase can be related mainly to a steady increase in the GSH No. 1 spot.

As with control livers of the 3′-MeDAB series, GSSG spots Nos. 2 and 3 again showed a tendency to increase with time after arachis oil injection. PE seemed to increase up to 96 hr as the result of 4′-MeDAB injection but the spot was sometimes not too well-defined and part of it might be due to tailing from spots 1 and 2 (see Fig. 2 E). There was little or no change in the glu levels of 4′-MeDAB livers as compared with controls and the level remained constant throughout the experiment. There may be some increase in asp due to 4′MeDAB injection. The tau spot was rather erratic but there was no marked difference between dye-treated and control livers in respect of it. No noticeable difference was detected in the glu-NH_2 levels of 4′-MeDAB and control livers throughout the experiment.

2-MeDAB experiment. Besides the drop in liver GSH level, marked decreases in the levels of glycine and of the glutamine plus α-alanine spot as compared with controls were the most noticeable features in TCA extracts of the livers of rats injected 24 hr previously with 2-MeDAB.

General. It is interesting that all the prominent changes which we have observed in livers of rats injected with 3′-MeDAB have also been noted in extracts of regenerating rat liver following partial hepatectomy. It has been estimated[4] that regenerating rat liver (24 hr after partial hepatectomy) contains nearly twice the amounts of GSH, PE, glu and asp and only about half the amounts of tau and glu as livers of control fasted rats. The level of lysine was also stated[4] to increase in regenerating rat liver.

Essentially the same changes were observed by Sano and Roberts[3] in regenerating rat liver 12 hr to 6 days after partial hepatectomy but contrary to Ferrari and Harkness,[4] Sano and Roberts[3] failed to detect a change in the lysine level at any time after hepatectomy. In the present work we have been unable to detect lysine at all in our rat liver chromatograms.

DISCUSSION

Elevation of rat liver GSH by carcinogenic azo dyes

Because of the ability of the powerful hepatocarcinogen 3′-MeDAB and of the weaker 4′-MeDAB to increase the level of male rat liver GSH, the literature has been examined for reports of other substances able to influence liver GSH. From this survey, some facts have emerged which may point the way towards a better understanding of the mechanism of carcinogenesis.

Boyland and Mawson found that intraperitoneal injection of 3,4:5,6-dibenzcarbazole, carcinogenic for mouse liver, led to an elevation of liver GSH in this species whereas 1,2:5,6-dibenzanthracene and 20-methylcholanthrene had no effect on mouse liver GSH levels. Kuriaki and Hiyoshi[11] stated that when nitromin (nitrogen mustard N-oxide) was injected intravenously (10 mg/kg) into albino rats, the liver GSH level increased by 72 per cent at 4 hr and by 20 per cent at 24 hr after injection. The level returned to normal values 72 hr after injection. Nitromin, like other tumour-inhibiting alkylating agents may well be carcinogenic for rats but no information could be found on this point.

Calcutt, Doxey and Coats[12] found that *o*-aminoazotoluene and carbon tetrachloride, both hepatocarcinogenic for mice, produced a rise in sulphydryl levels of mouse

livers and the same workers[13] showed that the livers of rats fed 4-dimethylaminoazobenzene had an elevated total sulphydryl content. It was concluded[12] (see also Calcutt[14]) that "an elevation of tissue —SH levels is an essential prerequisite for tumour formation". Fiala and Fiala[15] found that 3′-MeDAB caused an elevation in rat liver GSH level during the 30th to 80th day of dye-feeding.

Some interesting observations on anti-rheumatic agents are due to Sproull.[16] He found that 17 hr after intraperitoneal injection of sodium salicylate the level of GSH in the livers of male rats* increased from 258 mg per cent to 363 mg per cent while the GSH level of female rat liver was unchanged by salicylate injection. On the other hand, intraperitoneal injection of antipyrine raised the level of female rat liver GSH from 264 to 332 mg per cent but had no effect on the GSH level in male rat liver.

Consideration of Sproull's results and our own experiments raises the question of possible carcinogenic activity of salicylic acid towards male rat liver and of antipyrine towards female rat liver. According to surveys prepared by Hartwell[17] and by Shubik and Hartwell[18] salicylic acid has not been examined for carcinogenicity in rats. It it said to be non-carcinogenic for mice and rabbits. Hartwell[17] records that acetylsalycilic acid (not examined by Sproull) has not produced tumours in rats. In one experiment with rats (sex unstated), antipyrine elicited no tumours.

Recently, Sherman and Friedell[19] found that intraperitoneal injections of sodium salicylate produced considerable inhibition and even regression of a transplantable fibrosarcoma in hamsters. The fact that sodium salicylate has tumour-inhibitory properties indicates that it may well have carcinogenic activity also.

The anti-inflammatory properties of salicylic acid are thought by Whitehouse and Bostrom[20] to be connected in some way with its ability to form complexes with metal ions.

It is interesting that another metal-chelating agent, 8-hydroxyquinoline has been found to possess anti-inflammatory properties[21] and to be carcinogenic for mouse bladder.[22]

That an association between anti-inflammatory activity and carcinogenicity may not be uncommon is suggested by other data in the literature. Carcinogenic polycyclic hydrocarbons (but not non-carcinogenic ones) have been found by Saliamon[23] to have anti-inflammatory activity. Later, Saliamon[24] drew attention to the possession of this same property by many other carcinogenic agents including ionizing radiations, metals, radiomimetic substances, urethane, etc., all of which can simultaneously damage tissue and weaken its reaction to injury through inhibition of inflammation. Cocarcinogenesis is explained by the damaging action of the cocarcinogen on tissues rendered hyporeactive by previous applications of carcinogen.

It would appear to be of interest to determine whether the carcinogenic azo dyes (or their metabolites) have anti-inflammatory activity and whether they can chelate with metals. Neish[25] suggested that they might interfere with the formation of rat serum caeruloplasmin through chelation with liver copper.

Recently Miller, Enomoto and Miller[26] have reported that the cupric chelate of N-hydroxy-2-acetylaminofluorene (N-hydroxy-2-acetylaminofluorene is a proximate carcinogenic metabolite of the carcinogen, 2-acetylaminofluorene) is highly active in

* We have confirmed Sproull's observations with male rats and have shown that besides the increase in liver GSH produced by sodium salicylate there is also an increase in phosphoethanolamine.

producing sarcomas at the site of injection in rats whereas comparable doses of the parent organic compound failed to elicit tumours under similar conditions.

We should like to suggest that carcinogenic activity should be looked for in substances which possess some or all of the following properties:

(i) antirheumatic or anti-inflammatory activity.

(ii) metal ion complexing ability.

(iii) ability to produce a rise in the soluble thiol components (e.g. GSH) of tissues.

(iv) ability to bind to tissue constituents.

Should there prove to be a definite association between anti-inflammatory and carcinogenic activity, it may be possible to arrive at a better understanding of tumorigenesis and inflammation through a study of reactions (in both fields) in which metal-chelating, sulphydryl-stimulating and tissue-binding properties of the compounds in question are involved.

Because of the widespread use of anti-inflammatory substances (e.g. aspirin, phenylbutazone, chlorpromazine, etc.) it would appear that all should be screened for carcinogenic or cocarcinogenic activity. Already, the suggestion has been made[27] that there may be a connection between the development of leukaemia in a group of elderly male patients and the recent administration of phenylbutazone to these patients.

Liver regnerative phenomena following the action of carcinogenic azo dyes

We have found that changes in the levels of some TCA-extractable ninhydrin-positive substances from the livers of rats injected with 3′-MeDAB are very similar to those observed in regenerating liver of normal rats subjected to partial hepatectomy. It is almost as if 3′-MeDAB has induced "chemical hepatectomy" of rat liver.

Increases in liver GSH and PE may be critical changes for hepatocarcinogenesis. On the other hand these changes may simply be the reflection of a regeneration process greater with 3′-MeDAB than with 4′-MeDAB which forms the background for the operation of an unrecognized tumorigenic stimulus. Unfortunately no information could be found as to the possible incidence (if any) of tumours in normal rat livers which have regenerated after partial hepatectomy.

Some studies of Blomqvist[28] are of interest for the topic of liver regeneration and carcinogenesis. She found that dry powders prepared from regenerating rat liver (after partial hepatectomy) but not those obtained from normal rat liver or from livers of rats fed DAB are able to produce tumours when they are injected intraperitoneally into rats.

According to Smith[29] a peak of mitotic activity coinciding with a peak in the non-protein sulphydryl level occurred in regenerating rat liver 36 hr after partial hepatectomy. Cell division is apparently associated with high sulphydryl levels. Jaffe[30] observed a diurnal periodicity of mitosis in regenerating rat liver with high and low levels at 6 a.m.–10 a.m. and 6 p.m.–10 p.m. respectively. It is interesting that high and low levels of mitosis occur at these very times when the GSH levels of normal rat liver are high and low respectively.[8] If GSH elevation promotes increased cell proliferation then 3′-MeDAB and to a lesser extent 4′-MeDAB ought to be active in this respect whereas 2-MeDAB should be inert. In fact, Maini and Stich[31] have found that 3′-MeDAB and 4′MeDAB both cause proliferation of rat liver cells but 2-MeDAB fails to do so,

although it is as capable as the powerful carcinogen 3′-MeDAB in producing mitotic abnormalities in the liver.* For a substance to be hepatocarcinogenic, it must apparently cause not only chromosomal damage but also cellular proliferation which, acting together, produce a genetically heterogeneous cell population from which tumour cells arise.[31]

It seems possible that the increased level of liver GSH induced by hepatocarcinogens is the impetus for liver cell proliferation, but the mechanism for chromosomal damage remains obscure. Possibly this factor could be recognised from a study of changes induced in rat liver by the non-carcinogen, 2-MeDAB.

In a later paper Maini and Stich[32] studied the response of carcinogen-treated liver to the mitotic-stimulus of partial hepatectomy. In the course of carcinogenesis, liver tissue ceased to respond to this stimulus and in the developed tumours partial hepatectomy failed to alter their mitotic rate.

General

It should be of interest to examine the mechanisms and consequences of an increase in liver GSH for hepatocarcinogenesis.

GSH may well be a critical factor in cell division as discussed by Needham[33] and more recently by Mazia[34] and Stern.[35] Its overproduction in rat liver through the action of hepatocarcinogenic azo dyes ought to be considered from this view point and the consequences resulting from change in cellular pH (autolysis?), increased glycolysis etc. should be examined.

Fiala[36] has obtained evidence that a sharp decrease (after the early initial rise in liver GSH[15]) in GSH occurs in the precancerous rat liver after 100 days of 3′-MeDAB feeding. In the developed hepatoma the level of GSH is only 20 per cent that of the normal rat liver level. As we have already seen, there is some parallel between the extent of azo-dye binding and the level of TCA-soluble sulphydryl in the liver.[7] Miller and Miller[6] found that as dye-feeding is continued the dye-binding capacity of the liver decreased, until in the developed liver tumour, dye-binding was no longer detectable. It was thought that this "deletion" of the dye-binding site (protein) is critical for hepatocarcinogenesis. It should be useful to determine the significance and connection (if any) between the apparently parallel changes in dye-binding capacity and the GSH level of the liver up to their final deletion or low level in the hepatoma.

If a low level of GSH has been attained by a tumour cell race arising from a normal tissue as a response of that tissue to chronic injurious over-production of GSH, we might expect the tumour cells to be susceptible to some control when the level of GSH in the tumour environment is increased artificially. In fact, Heise and Lührs[37] have been able to bring about appreciable retardation of the growth of a transplantable rat tumour by intraperitoneal injections of GSSG alone, or, better, of GSSG plus dehydroascorbic acid.

It seems to us that the role of GSH in carcinogenesis and in tumour inhibition deserves intensive study.

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* It should be of interest to determine whether simulataneous application of 2-MeDAB (mitotic abnormalities) and salicylic acid (GSH elevation; increased liver cell proliferation?) to rats would lead to hepatocarcinogenesis.

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