also been shown to have the same effect on liver lysosomal membranes.[11–13] However a recent study has shown that while hydrocortisone has an inhibitory effect on tissue plasminogen activator activity this effect is independent of lysosomal enzymes.[14] It is also possible that the inhibitory effect of salicylates may occur after plasminogen has been activated to plasmin. It is concluded from this study that salicylates are capable of inhibiting rat tissue plasminogen activator activity but the site of action has still to be elucidated.

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Activation by reductive cleavage of potentially cytotoxic azo compounds by human hepatocellular carcinoma

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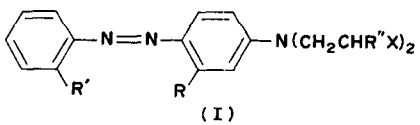
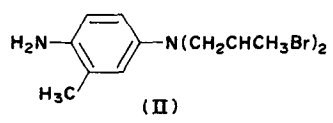
A RECENT report[1] described the synthesis of a series of substituted amino-azobenzene derivatives of the general formula (I) designed to be selective for treating hepatocellular carcinoma. One of these (R = R″ = CH_3, R′ = COOH, X = Br) had the prerequisite properties for selective action; namely absence of alkylating ability and a rapid rate of reduction by rat liver azo-reductase to the amine (II) which is an extremely reactive alkylating agent having a half-life of the same order as the circulation time in man.



(I)



(II)

This compound is undergoing cautious clinical trial in patients suffering from hepatocellular carcinoma in Kenyatta National Hospital, Nairobi, Kenya, and in Kaluva Hospital, Arua, Uganda. Results of these trials will be published elsewhere.

Since activation of this latent cytotoxic agent requires reductive cleavage of the azo linkage it was appropriate to determine whether the azo reductase system is present in hepatocellular carcinoma in man. This communication reports the rate of reduction of this compound by the 9000 *g* microsomal supernatant of primary hepatocellular carcinoma obtained by open biopsy in three Kenyan hospitals (Kenyatta National Hospital, Machakos General Hospital and Kisumu General Hospital). Samples of liver from patients not bearing hepatocellular carcinoma and from baboon, rat, mouse and rat hepatocellular carcinoma were investigated using this and other aminoazo compounds for comparison.

LIVER AND TUMOUR SAMPLES

Hepatocellular carcinoma (diagnosed by histopathology and the presence of α-fetoprotein) and small samples of normal human liver were obtained by surgical biopsy and placed immediately into ice cold 1·15% KCl in 0·01 M Tris–HCl buffer pH 7·4.

Pieces of liver were obtained from a male Olive Baboon (18 kg) immediately following death. The animal was killed by dosing with 12 mg Sernylan (Parke-Davis) in water followed by bleeding from the throat when unconscious. It had been fed a diet of bananas, pawpaw, carrots, sweet potatoes, maize and dog biscuits.

Male and female Porton–Wistar rats and C57 black mice which had been fed commercial mice pellets (Unga Ltd., Nairobi) were killed by breaking the neck and the liver immediately excised.

Primary hepatocellular carcinomas were induced in male rats by incorporating 0·06% 4-dimethylaminoazobenzene (DAB) or 4-dimethylamino-3′-methylazobenzene (3′-MeDAB) into a low protein diet[2] which was fed for approximately 6 months, and by administering diethylnitrosamine (DENA) (0·5 mg/day) by stomach tube for a similar period. [3]

CHEMICALS

Compounds of general structure I were obtained as previously described.[1] Other chemicals were obtained commercially except *N,N*-dimethylamino-3′- methylazobenzene which was made by the standard coupling method.

ENZYME ASSAYS

Sufficient liver or hepatocellular carcinoma samples were homogenised in cold 0·1 M Tris–HCl buffer pH 7·4 using ten strokes of a motor-driven glass-teflon homogeniser (850 rev/min) to make a 10 per cent

TABLE 1. RATE OF REDUCTION OF Ie (R = R″ = CH_3, R′ = COOH, X = Br) BY THE 9000 *g* MICROSOMAL SUPERNATANTS OF HUMAN CARCINOMA AND NORMAL LIVER

Type of tissue	Sex	Age	Anaesthetic	Rate of reduction (nmoles dye reduced/100 mg protein/min)	Hospital
Normal liver	F	—	Atropine, N_2O, O_2	43·7	Kiambu
	M	57	Sodium pentothal + scoline (Curare)	60·8	Kenyatta National
	M	58	Sodium pentothal + scoline (Curare)	75·9	Kenyatta National
	M	—	Sodium pentothal + scoline (Curare)	44·3	Kenyatta National
	M	33	Sodium pentothal + scoline (Curare)	81·5	Kenyatta National
Hepatocellular carcinoma	M	52	Sodium pentothal + scoline (Curare)	25·7	Kenyatta National
	M	—	Local anaesthetic	15·8	Machakos
	M	27	Atropine, pethidine N_2O, O_2	16·1	Kisumu
	M	—	Atropine, pethidine trilene	4·6	Kisumu
	M	—	Gallamine, N_2O, O_2	14·9	Kisumu

Normal liver 61·2 ± 17·4 nmoles dye reduced/100 mg protein/min. Hepatocellular carcinoma 15·4 ± 7·5 nmoles dye reduced/100 mg protein/min. Significant difference ($P < 0·001$) was revealed between reduction by normal liver and hepatocellular carcinoma when tested by Student's *t*-test. The results represent the mean ± S.E.M.

TABLE 2. RATE OF REDUCTION OF SOME AMINOAZOBENZENE COMPOUNDS BY MICROSOMES (9000 *g* SUPERNATANT) OF NORMAL LIVERS FROM RAT, MOUSE, BABOON AND FROM MALE RAT PRIMARY HEPATOCELLULAR CARCINOMA*

Compound	Reductase activity (nmoles/100 mg protein/min)						
	Rat		Mouse			Male rat primary Hepato cellular carcinoma	
	Female	Male	Female	Male	Baboon	DAB-induced‡	DENA-induced‡
Ia R= R′= R″= H, X= Br	10·5 ± 0·8	13·2 ± 1·9	10·4 ± 1·3	10·7 ± 0·2			
Ib R′ =R″= H, R= CH_3, X= Br	5·7 ± 0·8	7·3 ± 0·9	5·7 ± 0·7	7·6 ± 1·0			
Ic R= R″= H, R′= COOH, X= Br	11·9 ± 1·3	25·6 ± 2·6	11·8 ± 0·7	25·4 ± 2·7			
Id R″=H, R′=COOH, R=CH_3, X=Br	98·1 ± 3·5	99·9 ± 4·6	79·0 ± 2·0	71·2 ± 1·8			
Ie R=R″= CH_3, R′=COOH, X= Br	55·1 ± 3·1	63·3 ± 2·2	28·8 ± 2·6	29·1 ± 1·5	76·0 ± 1·4	46·1 ± 12·4	31·0 ± 6·2
DAB	31·0 ± 2·4	40·6 ± 1·9	19·2 ± 0·9	22·3 ± 2·4	11·3 ± 2·2	7·3 ± 4·8	8·3 ± 4·7
3′-MeDAB	11·8 ± 1·6	18·7 ± 1·4	10·3 ± 1·5	13·3 ± 0·7			

* The results represent the mean of six determinations (each a pool of three livers) ± S.E.M. The baboon result was determined by using pieces from five different areas in the same liver.

† Male rat primary hepatocellular carcinoma was obtained from ten different rats.

‡ Male rat primary hepatocellular carcinoma was obtained from seven different rats.

mixture (w/v). The homogenate was centrifuged at 800 *g* at 0–4° for 0·25 hr, and then at 9000 *g* for 0·5 hr using a M.S.E. Superspeed 50. The supernatant was used for the assays of the azo reductase.[4–6] The reaction mixture contained 3·5 μmoles glucose-6-phosphate, 0·2 μmoles NADP, 80 μmoles nicotinamide, 17·5 μmoles $MgCl_2$, 0·5 mM Tris-HCl buffer pH 7·4, 0·1 μmoles of azocompound (in 0·1 ml ethanol) and 1 ml of supernatant made up to 5 ml. Nitrogen was bubbled through the mixture which was incubated at 37° in a shaking water bath for 20 min (10 min for Id and Ie). The reaction was stopped by the addition of 5 ml of 18% trichloroacetic acid in acetone–ethanol (1 : 1). After 20 min the mixture was centrifuged at 2000 rev/min in a Mistral 6L. Reduction was calculated by measuring unreduced azo compound at 520 nm (Pye-Unicam SP 500) and expressed as nmoles azo compound reduced/100 mg protein/min.

Protein concentrations were measured by the method of Lowry *et al.*[7] using the concentration range 10–40 μg protein/ml.

RESULTS

Table 1 shows the rate of reduction of Ie by samples of human primary hepatocellular carcinoma and normal human liver and includes other relevant details.

Table 2 shows rates of reduction of this and other aminoazo compounds by the 9000 *g* microsomal supernatant from other species and rat primary hepatocellular carcinomas.

DISCUSSION

It was shown earlier[1] that aminoazo compounds vary in their ability to act as substrates for the azo reductase system depending on the type and position of ring substituents. There was an interesting *ortho* effect in that a carboxyl group in the R′ position enhanced reduction, a finding in keeping with earlier observations using reducing agents[8,9] other than $NADPH_2$. The present results indicate that an *ortho* substituent is not always necessary for reduction using the 9000 *g* liver microsomal supernatant.

In general rat and mouse liver 9000 *g* microsomal supernatant were similarly active in reducing the azo compounds, the major exceptions being Ie (R = R″ = CH_3, R′ = COOH, X = Br) and 4-dimethylaminoazobenzene in which case the mouse fraction was far less efficient. The baboon reduces Ie much more efficiently than the rat and mouse, but it was least able to reduce 4-dimethylaminoazobenzene. The sex differences in the reduction rates apparently disappeared for both rat and mouse when rapidly reduced compounds were substrates.

The most important result relevant to the potential usefulness of Ie as a chemotherapeutic agent for hepatocellular carcinoma in man is the ability of the 9000 *g* microsomal supernatants from all the human hepatocellular carcinoma samples to reduce it, although more slowly than normal human liver tissue. This is also reflected in the retention of azo reductase activity for Ie in chemically induced rat hepatocellular carcinomas.[10]

Further studies of drug metabolizing enzyme levels in human hepatocellular carcinoma are in progress.

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1,2-Dibromoethane—Effect on the metabolism and ultrastructure of *Escherichia coli*

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1,2-DIBROMETHANE (1,2-DBE) has a wide household and industrial distribution. It is used most extensively as a pesticide and as an anti-knock agent in leaded gasolines. Recently the mutagenicity[1–5]* of 1,2-DBE and its ability to alkylate the guanine moiety*† of DNA were demonstrated. In view of the potential health hazard created by a mutagenic environmental agent, the effects of 1,2-DBE on living cells was further investigated. The present report is concerned with the effects of this agent on the growth of *Escherichia coli*.

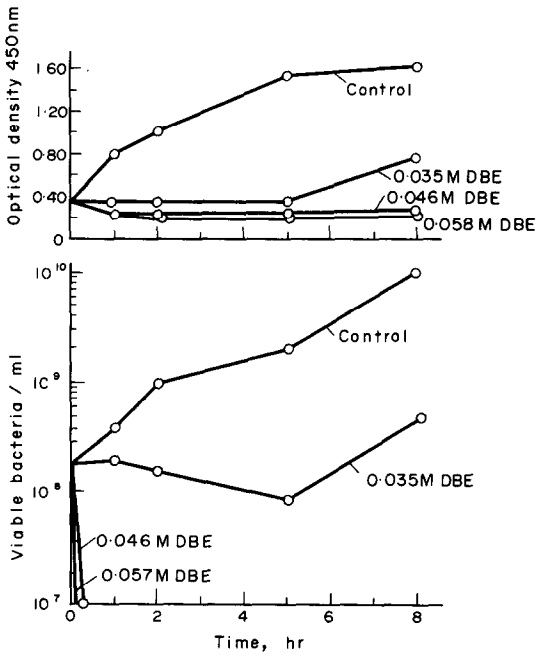

FIG. 1. Effect of DBE on growth of bacteria. Bacteria (*E. coli* K-12) in medium HA[10] were brought to the exponential growth phase, at which time portions of the cultures were distributed into flasks containing premeasured amounts of DBE. The cultures were incubated at 37° with aeration and at intervals portions of each culture were withdrawn for determination of turbidity (450 nm) and enumeration of viable cells.

* H. Brem, A. B. Stein and H. S. Rosenkranz, *Cancer Res.*, in press.
† G. A. Carden, III, H. Brem and H. S. Rosenkranz, manuscript in preparation.