Effect of Azo-Dye Carcinogenesis on Enzymes Concerned with Urea Synthesis in Rat Liver

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(Received 6 November 1963)

Dickens & Weil-Malherbe (1943) first demonstrated that slices of liver tumours, induced by giving DAB* in the diet, when incubated *in vitro* with ammonium salts and alanine in the presence of trace amounts of ornithine, were unable to synthesize urea and that in this respect the tumour had lost one of the specialized functions of normal liver. The work of Greenstein (1942), who used transplanted rat hepatomas, showed that slices of this tissue also failed to synthesize urea from citrulline and ammonia, whereas urea was rapidly formed under the same conditions by liver slices.

These results may, in part, be explained by the decrease in arginase activity that has been reported in azo-dye-induced hepatomas (Greenstein, Jenrette, Mider & White, 1941; Takahasi, 1954), and decreases in the activities of transaminases and glutamate dehydrogenase may also be of importance in this context (Cohen, Hekhuis & Sober, 1942; Greenstein, 1943; Pitot, 1960; see also Weber, 1961; Reid, 1962*a*).

Burke & Miller (1956, 1959*a, b*, 1960) and Burke (1962) have shown that the total urea nitrogen produced during the perfusion of rat livers with various amino acids, including arginine and citrulline, is considerably decreased in rats fed on a diet containing 3'-Me-DAB for 2 months, although the rate of production from ammonia and glutamine was unchanged.

Ono, Blair, Potter & Morris (1963) have measured the activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase in a series of 'minimal-deviation' hepatomas (i.e. those which possessed significant, though decreased, activities of the liver 'marker' enzymes such as glucose 6-phosphatase) as well as in Novikoff hepatoma which contained little of these 'marker' enzymes. The activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase were subnormal but showed wide variations that could not readily be correlated with the rate of growth of the tumour. These studies, together with those of Jones, Anderson, Anderson & Hodes (1961) on the activity

of ornithine transcarbamoylase in a series of rat and mouse hepatomas, were concerned largely with a survey of a wide variety of liver tumours to determine whether the loss of one of these enzymes is specific in the induction of tumours.

The present work is concerned with the pattern of change in the activities of all five enzymes of the urea cycle in rat liver in both the 'precancerous' stage and in the tumours resulting from giving the carcinogenic azo-dyes 3'-Me-DAB, DAB and 4'-F-DAB in the diet. Information on the overall pattern of change of these enzymes might be of value, not only in determining the sequence of events preceding the loss of the ability to synthesize urea, but also in relation to the apparent fall in the number of mitochondria during azo-dye carcinogenesis (Striebich, Shelton & Schneider, 1953; Novikoff, 1957; Allard, de Lamirande & Cantero, 1957), the first two enzymes of the urea cycle being located in the mitochondrial fraction.

METHODS

Materials. Commercial reagents were used, with the following exceptions: argininosuccinic acid, which was a gift from Dr R. G. Westall, University College Hospital, London; phosphoenolpyruvic acid, which was synthesized by Dr P. N. Campbell; carbamoyl phosphate, which was synthesized by the method of Spector, Jones & Lipmann (1957); α -isonitrosopropiophenone, which was synthesized by the method of Claisen & Manasse (1889). Pyruvate kinase and arginase were commercial preparations from Boehringer und Soehne G.m.b.H. and Sigma Chemical Co. respectively. A purified preparation of ornithine transcarbamoylase was obtained as described below.

Animals. From the age of 7 weeks male albino rats were fed on a 20% protein diet containing the azo-dye (750 mg./kg. of diet), as described in the experiments of Griffin, Nye, Noda & Luck (1948). The diet was given in weighed amounts and the controls were restricted to the food intake of the experimental group. The rats kept for tumours were given the diet containing 3'-Me-DAB for 90–110 days, and the stock diet was then given *ad lib.* until tumours arose approx. 7–12 months from the time of starting the treatment. The gross and histological appearance of such tumours has been described by Nodes & Reid (1963). In experiments on the early changes in the activities of urea-cycle enzymes in rats given azo-dyes the experimental groups received 3'-Me-DAB or 4'-F-DAB (600 mg./kg. of

* The abbreviations used are: DAB, 4'-F-DAB, 2-Me-DAB, 3'-Me-DAB and 4'-Me-DAB, 4-dimethylaminoazobenzene and its 4'-fluoro, 2-methyl, 3'-methyl and 4'-methyl derivatives respectively.

diet), whereas controls were given the same synthetic diet without azo-dye or with the addition of the non-carcinogenic derivatives 4'-Me-DAB or 2-Me-DAB. These diets were replaced by stock diet on the day before the rats were killed. The rats were kept without food overnight before being killed for estimation of the activities of the urea-cycle enzymes. It was necessary to store some of the tissue samples for up to 1 hr. in ice before extraction and estimation of the urea-cycle enzymes, but this had no appreciable effect on the enzyme activities (see Table 1).

The groups of rats treated with DAB for 4 weeks were kindly supplied by Dr B. Ketterer. The carcinogenic diet was removed and groups of the azo-dye-treated rats were transferred to the stock diet for either 36 hr. or 11 days before the rats were killed for estimation of the activities of the urea-cycle enzymes in liver. These rats were also kept without food overnight. During the 36 hr. period the free dye in the liver has largely disappeared, although there is still a considerable amount of bound dye; after 11 days the concentration of bound azo-dye is low since the half-life of the protein-bound dye is about 3-4 days (Gelboin, Miller & Miller, 1958).

Urea-cycle enzymes. The activities of the urea-cycle enzymes were measured essentially according to the methods of Brown & Cohen (1959) as modified by McLean & Gurney (1963). There are certain special problems concerned with the estimation of the activities of these enzymes in tumours, and these are discussed under the individual enzyme assays given below. Except where otherwise stated, liver or tumour was homogenized in 0.1% cetyltrimethylammonium bromide solution in the ratio of 1 g. of tissue to 9 ml. of solution, and then centrifuged at 4000g for 15 min. at 5°. The supernatant fraction was used in the assay of all urea-cycle enzymes except arginase.

Carbamoyl phosphate synthetase. The measurement of the activity of carbamoyl phosphate synthetase (ATP-carbamate phosphotransferase; EC 2.7.2.2) depends on the formation of citrulline from the combined activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase. This latter enzyme is very active in normal liver. In the present experiments it was found that ornithine-transcarbamoylase activity was low in tumours and consequently it was necessary to supplement with excess of ornithine transcarbamoylase. A simple and rapid method of obtaining a suitable ornithine-transcarbamoylase preparation for supplementation was to homogenize isolated washed mitochondria from normal livers with 0.1% cetyltrimethylammonium bromide. Liver mitochondria were prepared by the usual method from an homogenate in 0.25M-sucrose, and after separation of the nuclei and cell debris by centrifugation at 600g for 10 min. the mitochondria were isolated by centrifugation at 8000g for 15 min. The mitochondria were washed by resuspension in 0.25M-sucrose, and after centrifugation the drained pellet from 10 g. of liver was homogenized in 10 ml. of 0.1% cetyltrimethylammonium bromide and this preparation centrifuged for 15 min. at 4000g. The supernatant fraction had no carbamoyl phosphate-synthetase activity but had a high ornithine-transcarbamoylase activity. This preparation could be stored at -15° without appreciable loss of activity for several weeks. This illustrates the extremely labile nature of carbamoyl phosphate synthetase.

Since the liver tumours showed a very low carbamoyl phosphate-synthetase activity (Table 1) the enzymic

activity of tumour extracts prepared with 0.1% cetyltrimethylammonium bromide solution (used by Brown & Cohen, 1959) was compared with that of extracts prepared with 1 mM-GSH (used by Jones & Spector, 1960), since it was possible that GSH might protect the enzyme against inactivation. In each case the assay mixture was supplemented with the following: 0.05 ml. of mitochondrial ornithine-transcarbamoylase preparation, 10 μ moles of phosphoenolpyruvic acid in 0.1 ml., and 0.05 ml. of a solution containing 1 mg. of pyruvate kinase/ml. The final volume of the reaction mixture was 1.0 ml. and the time of incubation 15 min. No significant differences in the activity of carbamoyl phosphate synthetase of tumours were found between extracts prepared in these two media. Supplementation of the assay mixture with ornithine transcarbamoylase and an ATP-generating system (phosphoenolpyruvic acid and pyruvate kinase as above) did not increase the activity of normal rat-liver extracts prepared in 0.1% cetyltrimethylammonium bromide. The activities were 190 units in the absence and 214 units in the presence of the ATP-generating system.

Ornithine transcarbamoylase. The activity of ornithine transcarbamoylase (carbamoyl phosphate-L-ornithine carbamoyltransferase; EC 2.1.3.3) was estimated by the method of Brown & Cohen (1959).

The breakdown of carbamoyl phosphate in the presence of liver and tumour extracts was measured by estimating the release of inorganic phosphate from this substrate when incubated with liver and tumour extracts in amounts normally used for the assay of ornithine-transcarbamoylase activity. The method of inorganic phosphate determination in the presence of carbamoyl phosphate was that described by Spector *et al.* (1957).

Arginine-synthetase system. The activity of the arginine-synthetase system, i.e. argininosuccinate synthetase [L-citrulline-L-aspartate ligase (ATP); EC 6.3.4.5] plus argininosuccinase (L-argininosuccinate arginine-lyase, EC 4.3.2.1), was measured with a 0.1% cetyltrimethylammonium bromide extract as described by Brown & Cohen (1959). This preparation was supplemented with excess of purified arginase to prevent this enzyme becoming a rate-limiting step in the assay of the arginine-synthetase system in tumour preparations. In certain experiments the activity of the arginine-synthetase system was measured in the presence of an ATP-generating system; the concentrations of phosphoenolpyruvic acid and pyruvate kinase were similar to those employed in the carbamoyl phosphate-synthetase system described above. The addition of this ATP-generating system did not increase the measured activity of either normal or tumour extracts. Comparison of 0.1% cetyltrimethylammonium bromide with 1 mM-GSH showed that the latter gave only 30% of the arginine-synthetase activity found in the former extracting medium (see Table 1). For this reason all estimations were carried out with 0.1% cetyltrimethylammonium bromide extracts.

In certain experiments an attempt was made to estimate the activity of the condensing enzyme of the arginine-synthetase system which catalyses the reaction:

by measurement of the disappearance of citrulline. This is only possible when separate experiments show that argininosuccinase and arginase are absent, since the

formation of urea from argininosuccinase by these later enzymes would seriously interfere with the colour reaction for citrulline (Archibald, 1944). The method described by Ratner (1955) was used and the disappearance of citrulline in the presence of tumour extracts was measured with and without the addition of aspartic acid. The disappearance of citrulline in the absence of aspartic acid was used to correct for the removal by reactions other than by condensation with aspartic acid.

Argininosuccinase. Argininosuccinase activity was measured as described by Brown & Cohen (1959) with the addition of excess of purified arginase (Sigma Chemical Co.).

Arginase. The activity of arginase (L-arginine ureo-hydrolase, EC 3.5.3.1) was low in supernatant fractions from homogenates prepared in 1 mM-GSH, possibly due to adsorption of the enzyme on particulate material. Whole homogenates were used and fully activated with Mn^{2+} ions, as described by Polley & Greenbaum (1948) and McLean & Gurney (1963).

Stability of urea-cycle enzymes. Since it was necessary to store tumour material in ice for periods of up to 1 hr. before extraction and estimation of the enzymes, the stability of these enzymes was tested by measuring the activities in liver samples immediately after death and after keeping samples of liver in ice for 2½ hr. before

extracting with 0.1% cetyltrimethylammonium bromide. There was no loss of enzymic activities (Table 1).

Supernatant fractions from 0.1% cetyltrimethylammonium bromide homogenates could be stored at -15° for several days without loss of ornithine-transcarbamoylase, arginine-synthetase or argininosuccinase activity. Carbamoyl phosphate synthetase was inactivated during prolonged storage at 0° or by freezing and thawing, and this enzyme was measured first as soon as possible after preparing the extracts.

Enzyme units. The activities of the urea-cycle enzymes were all expressed in terms of a unit defined as the amount of enzyme catalysing the formation of 1 μ mole of urea or citrulline/hr. under the conditions of the assay.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The activities of glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate-NADP oxidoreductase; EC 1.1.1.44) were measured by the method of Glock & McLean (1953). A unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 μ mole of $NADPH_2$ /hr. at 20° , based on the initial velocity measured during the first 5 min.

Calcium and magnesium contents of liver. The concentration of these metal ions in the livers of normal and

Table 1. *Stability of urea-cycle enzymes and the effect of different extracting media on the activities of the arginine-synthetase system and arginase*

A unit of enzyme activity is defined as the amount catalysing the formation of 1 μ mole of citrulline or urea/hr. under the conditions of the assay. Experimental details are given in the Methods section. (a) The liver was removed immediately after the rat had been killed and one part was extracted immediately with 0.1% cetyltrimethylammonium bromide (CTB); the enzyme activities were estimated immediately (control group) and after storing for 24 hr. at -15° . A second part was extracted with 0.1% cetyltrimethylammonium bromide after storing on ice for 2.5 hr. The supernatant fraction after centrifugation at 4000g for 15 min. was used for the assay of all enzymes except arginase which was determined on the whole homogenate after activating with Mn^{2+} ions as described by McLean & Gurney (1963). (b) The activities of the arginine-synthetase system in the supernatant fraction from 0.1% cetyltrimethylammonium bromide and 1 mM-GSH homogenates were compared as well as the activity in the whole homogenate prepared in 1 mM-GSH. The activity of arginase was measured after homogenizing liver in 0.1% cetyltrimethylammonium bromide, 1 mM-GSH or Mn^{2+} -saline (containing 9 g. of NaCl and 8 g. of $MnSO_4 \cdot 4H_2O$ /l.). In each case the Mn^{2+} ion concentration was brought to the same final concentration and arginase was fully activated for 30 min. at 37° before determining the activity. Comparison was made between the whole homogenate and supernatant fraction.

		Enzyme activity (units/g. of liver)		
(a) Stability of enzymes in liver and liver extracts		Control	Liver kept at 0° for 2.5 hr.	Extract kept at -15° for 24 hr.
Enzyme	Medium			
Carbamoyl phosphate synthetase	0.1% CTB	263	244	102
Ornithine transcarbamoylase	0.1% CTB	11 900	14 900	12 100
Arginine-synthetase system	0.1% CTB	68	75	65
Argininosuccinase	0.1% CTB	126	128	130
Arginase	0.1% CTB	102 000	100 000	—
(b) Comparison of extracting media		Supernatant fraction	Whole homogenate	
Arginine-synthetase system	0.1% CTB	66	—	
	1 mM-GSH	23	< 5	
Arginase	0.1% CTB	41 700	110 000	
	1 mM-GSH	18 600	91 000	
	Mn^{2+} -saline	—	113 400	

Table 2. *Activities of urea-cycle enzymes in normal rat liver and in primary hepatomas induced with 4-dimethylamino-3'-methylazobenzene*

The results are expressed as means $\pm$ s.e.m. A unit of enzyme activity is defined as the amount catalysing the formation of 1 μ mole of citrulline or urea/hr. under the conditions of the assay. Experimental details are given in the Methods section.

Tissue	No. of samples assayed	Enzyme activity (units/g. of tissue)				
		Carbamoyl phosphate synthetase	Ornithine trans-carbamoylase	Arginine-synthetase system	Arginino-succinase	Arginase
Control liver	5	252 $\pm$ 51	10 400 $\pm$ 780	70 $\pm$ 14	112 $\pm$ 13	69 300 $\pm$ 5 460
Primary hepatoma	9	5 $\pm$ 3	1 220 $\pm$ 290	< 5	12 $\pm$ 4	3 200 $\pm$ 780
Adjacent host liver	5	145 $\pm$ 31	8 060 $\pm$ 513	47 $\pm$ 6	100 $\pm$ 20	49 000 $\pm$ 10 500
Nodule with bile-duct hyperplasia	1	26	4 350	< 5	20	6 850

'precancerous' rats was estimated by the flame-spectrophotometric method of Warren (1959), with samples of liver homogenized in 49 vol. of 0.1N-HCl. We are grateful to Mr R. L. Warren for these determinations.

Significance of results. Fisher's *t* test was used, a difference between two mean values being regarded as significant if *P* was no greater than 0.05.

RESULTS

Urea-cycle enzymes in tumours. The activities of the urea-cycle enzymes in hepatoma, adjacent host liver and normal rat liver is shown in Table 2. There is a very low, and in some cases almost negligible, activity of carbamoyl phosphate synthetase in the tumours. The activity of the arginine-synthetase system is also greatly decreased, being too low to detect in most tumours. In view of the very low activity of the cleavage enzyme (arginino-succinase) in tumours, measurements were made of the disappearance of citrulline in the presence of aspartic acid in an attempt to measure the activity of argininosuccinate synthetase (the rate-limiting condensing enzyme) directly. In certain hepatomas, there was some residual arginino-succinate-synthetase activity. Since the range of activity for this latter enzyme was 2–45 units/g. in different tumours, the cleavage enzyme may have become rate-limiting in some instances. Ornithine-transcarbamoylase and arginase activities are both considerably lower in hepatomas than in either normal liver or the adjacent host liver, the mean values being about 10 and 5 % of the control values respectively. It is difficult to assess the exact significance of the ornithine-transcarbamoylase and arginase activities of these tumours, since both these enzymes are extremely active in normal liver and a very small contamination with normal liver cells could contribute significantly to this activity.

There were differences among the hepatoma nodules in histological features, such as the extent of fibrosis and of necrosis and the predominating type of tumour (adenocarcinoma or trabecular carcinoma), but no corresponding variations in enzymic pattern were evident.

The host liver adjacent to the tumour has a very active urea-cycle system which, although tending to be lower than the control animals, was not significantly different (Table 2).

It seemed possible that the very low activities of carbamoyl phosphate synthetase and arginine synthetase might be ascribed to one of the following causes: instability of the enzymes on storage or extraction, breakdown of ATP or, in the case of carbamoyl phosphate synthetase and ornithine transcarbamoylase, to destruction of carbamoyl phosphate before conversion into citrulline. The storage of portions of liver in ice for up to 2.5 hr. had no appreciable effect on the activities of the urea-cycle enzymes (Table 1), but the stability of these enzymes in tumours may be somewhat different. Carbamoyl phosphate synthetase is a very labile enzyme and, as shown by the destruction of carbamoyl phosphate-synthetase activity in isolated mitochondria by 0.1 % cetyltrimethylammonium bromide (see the Methods section), might be markedly altered by the extracting medium. A comparison was made between the activity of extracts prepared in 0.1 % cetyltrimethylammonium bromide and 1 mM-GSH. The latter did not give a higher activity of carbamoyl phosphate synthetase and, in fact, yielded low activities for other urea-cycle enzymes (see Table 1) which could only in part be explained by the inhibition of colour development by GSH (see McLean & Gurney, 1963).

The supplementation of the tumour extracts with phosphoenolpyruvic acid and pyruvate kinase did not enhance the activity. The values for carbamoyl phosphate-synthetase activity in one of the hepatomas were 5 and 6 units/g. with and without the addition of an ATP-generating system respectively; and for the hyperplastic nodule, which had a slightly higher activity, the values were 26 and 22 units/g. respectively. The very low arginine-synthetase activity of tumour extracts was also unaffected by the addition of an ATP-generating system.

Attempts were made to compare the breakdown of carbamoyl phosphate by tumour and liver

extracts by measurement of the release of inorganic phosphate as described by Spector *et al.* (1957). This method presented some difficulties owing to the instability of carbamoyl phosphate and the high blank values obtained, but there was no appreciable increase in the breakdown of carbamoyl phosphate by the tumour extracts. Breakdown thus could not account for the low carbamoyl phosphate-synthetase and ornithine-transcarbamoylase activities in the present experiments.

Urea-cycle enzymes in precancerous liver. The effect of feeding various carcinogenic azo-dyes to rats for periods up to 6 weeks on the activities of urea-cycle enzymes are shown in Tables 3 and 4 and in Fig. 1. The results in Table 3 show that 4'-F-DAB produces a much greater effect after 6 weeks than does 3'-Me-DAB, the former producing almost complete loss of activity of the two ATP-requiring enzymes, carbamoyl phosphate synthetase and arginine synthetase. After 6 weeks' treatment with the carcinogenic azo-dyes there is a great decrease in the activities of all the urea-cycle enzymes, whereas treatment for a similar period with the weakly carcinogenic or non-carcinogenic derivatives 4'-Me-DAB and 2-Me-DAB did not cause any significant decrease in the activities of these enzymes.

The precancerous changes were further studied by measuring the activities of urea-cycle enzymes in rat liver after giving 3'-Me-DAB to rats for periods of 5 and 8 days and 2, 4 and 6 weeks. The results of this experiment are shown in Fig. 1. There is an initial rise in carbamoyl phosphate-synthetase activity after 5 days' treatment (+64%; P 0.008). This is followed by a fall in activity which, after 4 weeks' treatment, resulted in an enzyme activity less than half the control value. The activity of the

other mitochondria-linked urea-cycle enzyme, ornithine transcarbamoylase, does not show this pattern of change and falls progressively from the commencement of the treatment, the decline being statistically significant after 2 weeks. The urea-cycle enzymes in the soluble fraction of the cell also behave somewhat differently from each other: the activities of the arginine-synthetase system and argininosuccinase are relatively well maintained throughout the period of treatment and, indeed, after 6 weeks' treatment with this azo-dye it would appear that the carbamoyl phosphate-synthetase activity becomes as low as that of the rate-limiting enzyme, arginine synthetase (see Table 3). Values for argininosuccinase activity (not shown in Fig. 1) after 2 and 4 weeks' treatment are 97 and 123% of the control values respectively. Arginase activity shows a pattern of change similar to that of carbamoyl phosphate-synthetase activity in that there is an initial increase followed, after 6 weeks of treatment, by a significant fall in activity.

Preliminary experiments on the comparison of the short-term effects of 3'-Me-DAB (600 mg./kg. of diet) with ethionine (2.5 g./kg. of diet) on the activities of the urea-cycle enzymes after 2 weeks' treatment reveals that in one respect they have a similar effect: in each case there is a decrease in the activity of ornithine transcarbamoylase. The activities, as units/g. of tissue, for controls, 3'-Me-DAB-treated and ethionine-treated rats are 11700 ± 1370 , 6330 ± 890 ($P < 0.01$) and 6960 (two values only) respectively. The activities of the other urea-cycle enzymes were not altered by giving the diet containing ethionine for this time.

The concentration of bound azo-dye in the liver decreases when the azo-dye is withdrawn, the half-life of the bound form being about 3-4 days

Table 3. *Activities of urea-cycle enzymes in normal and precancerous rat liver*

The results are expressed as means $\pm$ S.E.M. A unit of enzyme activity is defined as the amount catalysing the formation of 1 μ mole of citrulline or urea/hr. under the conditions of the assay. Fisher's P values are given for the comparison of the group maintained on the control diet and the experimental groups; they are regarded as significant if less than 0.05. (N.S. indicates that the P values were greater than 0.1.) Experimental details are given in the Methods section.

Diet and time of treatment	No. of animals	Enzyme activity (units/g. of tissue)				
		Carbamoyl phosphate synthetase	Ornithine trans-carbamoylase	Arginine-synthetase system	Argininosuccinase	Arginase
Control diet	8	278 ± 25	11700 ± 1370	64 ± 7	213 ± 9	117000 ± 6500
Carcinogenic diets						
3'-Me-DAB (2 weeks)	6	218 ± 26	6300 ± 890	53 ± 6	207 ± 17	158000 ± 15200
P		N.S.	0.01	N.S.	N.S.	0.014
3'-Me-DAB (6 weeks)	4	45 ± 15	3760 ± 990	39 ± 11	—	76000 ± 6600
P		< 0.001	0.003	0.085	—	0.006
4'-F-DAB (6 weeks)	2	< 4	2140	8	—	60600
Diets containing non-carcinogenic derivatives of DAB						
4'-Me-DAB (6 weeks)	2	207	9160	50	—	111600
2-Me-DAB (6 weeks)	3	274 ± 39	9510 ± 1170	59 ± 5	202 ± 20	125000 ± 9000

(Gelboin *et al.* 1958). The activities of the urea-cycle enzymes were studied after rats had been given the diet containing DAB for 4 weeks followed by periods of 1.5 or 12 days on stock diet (see Table 4). As expected, 1.5 days after removal of the azo-dye the activities of carbamoyl phosphate synthetase, ornithine transcarbamoylase and arginine synthetase were decreased, both as units/g. of

liver and as total units/100 g. body wt. But, 11 days later, the activities of two of these enzymes were restored practically to control values and only that of ornithine transcarbamoylase was significantly lower. In contrast, arginase activity showed no change at 1.5 days but was significantly decreased (units/g. of liver) after the 12 days on the stock diet.

In certain pathological conditions there may be considerable accumulation of Ca^{2+} ions in the liver, e.g. in carbon tetrachloride poisoning (Thiers, Reynolds & Vallee, 1960; Rees, Sinha & Spector, 1961), and this may have a marked inhibitory effect on ATP-requiring enzymes. Measurements of the Ca^{2+} ion and Mg^{2+} ion concentrations in the liver in the 'precancerous' stage, that is after giving the diet containing DAB for 4 weeks, revealed no changes in the content of these inorganic ions (Table 4).

An early and very striking change was found in liver glucose 6-phosphate-dehydrogenase activity, which almost doubled after rats were given 3'-Me-DAB for 2.5 weeks, whereas that of 6-phosphogluconate dehydrogenase remained completely unchanged. These results are summarized in Table 5.

DISCUSSION

The present results show that there is a marked decrease in the activities of all the enzymes of the urea cycle in primary liver tumours induced with 3'-Me-DAB. This is completely in accordance with the work of Dickens & Weil-Malherbe (1943), who first described the loss of urea-synthesizing ability of primary liver tumours, and with the work of Greenstein *et al.* (1941) and Takahasi (1954), who observed a decreased activity of liver arginase. Among transplanted liver tumours, some decreases in the activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase have been found with most of the tumours studied (Jones *et al.* 1961; Ono *et al.* 1963), including some 'minimal-deviation' tumours such as Morris 5123, although the fall in activity was more striking in hepatomas deviating widely from normal liver such as the Novikoff hepatoma (Ono *et al.* 1963). Ono *et al.* (1963) considered that the deletion or diminution of these enzymes could not be considered essential to the malignant process in hepatoma cells. The present experiments show that all the enzymes of the urea cycle are markedly depleted in the primary tumour and that this is not a change occurring on successive transplantation (see Greenstein, 1954, p. 350; Novikoff, 1957); the results are in accordance with the view that specific and specialized functions of a tissue may be lost during the process of carcinogenesis (Greenstein, 1954, p. 362).

Fig. 1. Effect of feeding 3'-Me-DAB on the activities of urea-cycle enzymes in rat liver. Rats were given a diet containing 3'-Me-DAB (600 mg./kg. of diet) for periods up to 6 weeks, or for 12-15 weeks in the case of the tumours. Control animals received the same diet without the azo-dye. The enzyme activities of the experimental groups as units/g. of tissue are expressed as percentages of the corresponding control value. Each point represents the mean value of not less than five experimental animals, and tumour values are the means of nine values. The vertical lines represent the s.e.m.; where no vertical line is shown the s.e.m. is too small to show. Details of the assays are given in the Methods section. (a) Mitochondria-linked enzymes: ○, carbamoyl phosphate-synthetase activity; ●, ornithine-transcarbamoylase activity; ●, control values arbitrarily fixed at 100%. (b) Enzymes in soluble fraction of cell: ▲, activity of arginine-synthetase system; △, arginase activity; ▲, control values arbitrarily fixed at 100%.

Table 4. *Activities of urea-cycle enzymes in normal rat liver and in livers from rats given 4-dimethyl-aminoazobenzene (for 4 weeks) followed by different periods on control diet (1.5 and 12 days)*

The results are expressed as means $\pm$ s.e.m. A unit of enzyme activity is defined as the amount catalysing the formation of 1 μ mole of citrulline or urea/hr. under the conditions of the assay. Fisher's *P* values are given for the comparison of the experimental group with the group of animals maintained throughout on the control diet; they are regarded as significant if less than 0.05. (N.S. indicates that the *P* values were greater than 0.1.) The experimental animals were fed on the diet containing DAB for 4 weeks followed by periods of 1.5 or 12 days on the control diet.

	Control diet	Time on control diet after giving DAB for 4 weeks	
		1.5 days	12 days
No. of animals	5	5	5
Body wt. (g.)	206 $\pm$ 9	192 $\pm$ 10	259 $\pm$ 8
Liver wt. (g.)	9.84 $\pm$ 0.60	10.97 $\pm$ 0.73	12.83 $\pm$ 0.46
Metal ions (μ g./g. of liver)			
Ca ²⁺ ion	57 $\pm$ 2	63 $\pm$ 4	—
<i>P</i>		N.S.	
Mg ²⁺ ion	190 $\pm$ 4	198 $\pm$ 11	—
<i>P</i>		N.S.	
Enzyme activity (units/g. of liver)			
Carbamoyl phosphate synthetase	288 $\pm$ 41	110 $\pm$ 42	260 $\pm$ 38
<i>P</i>		0.016	N.S.
Ornithine transcarbamoylase	13 860 $\pm$ 1 600	7 980 $\pm$ 1 100	9 500 $\pm$ 630
<i>P</i>		0.016	0.041
Arginine-synthetase system	64 $\pm$ 5	35 $\pm$ 9	58 $\pm$ 6
<i>P</i>		0.027	N.S.
Argininosuccinase	126 $\pm$ 6	155 $\pm$ 12	—
<i>P</i>		0.078	
Arginase	120 000 $\pm$ 7 100	118 800 $\pm$ 23 000	84 100 $\pm$ 6 960
<i>P</i>		N.S.	0.007
Total liver enzyme activity (units/100 g. body wt.)			
Carbamoyl phosphate synthetase	1 360 $\pm$ 210	624 $\pm$ 235	1 300 $\pm$ 204
<i>P</i>		0.048	N.S.
Ornithine transcarbamoylase	64 400 $\pm$ 6 800	44 600 $\pm$ 4 850	47 800 $\pm$ 3 970
<i>P</i>		0.045	0.068
Arginine-synthetase system	295 $\pm$ 16	197 $\pm$ 33	290 $\pm$ 33
<i>P</i>		0.027	N.S.
Argininosuccinase	594 $\pm$ 73	905 $\pm$ 131	—
<i>P</i>		0.094	
Arginase	565 000 $\pm$ 52 000	644 600 $\pm$ 86 800	418 400 $\pm$ 39 800
<i>P</i>		N.S.	0.054

Direct mitochondrial counts have indicated that in primary tumours induced by DAB the number of mitochondria/g. wet wt. is decreased to half the normal value (Allard *et al.* 1957). This decrease only partially explains the marked fall in the activity of the mitochondria-linked urea-cycle enzymes, carbamoyl phosphate synthetase and ornithine transcarbamoylase, in primary hepatomas.

The almost total absence of carbamoyl phosphate synthetase from the tumours is unexpected in view of the dual role of carbamoyl phosphate as a precursor for both pyrimidine synthesis and urea formation. Perhaps during the preparation of the tissue extracts this very labile enzyme might be destroyed, although no evidence for such destruction could be obtained. There may be alternative pathways for carbamoyl phosphate synthesis (see Cohen, 1962), and even for urea synthesis (Burke & Miller, 1959*b*; Cedrangolo, Della Pietra, Cittadini, Papa & De Lorenzo, 1962). Whatever the role of

such pathways, the results now obtained for the classical pathways suggest that in the hepatomas the supply of carbamoyl phosphate may be diverted from urea synthesis to pyrimidine synthesis, the rate of which in normal liver and hepatomas is so severely restricted by the orotate-forming enzyme system (Reid, 1962*b*; Reid & Morris, 1963) that even in hepatomas the supply of carbamoyl phosphate may be adequate. Such a diversion of carbamoyl phosphate in hepatomas would be favoured by the marked rise in the activity of aspartate transcarbamoylase relative to that of the competing enzyme ornithine transcarbamoylase (Calva, Lowenstein & Cohen, 1959; Jones *et al.* 1961; Reid, 1962*b*; Ono *et al.* 1963; see also Cohen & Sallach, 1961).

There is evidence that urea-cycle enzymes alter in an adaptive manner to the level of amino acid catabolism, as shown by giving a high-protein diet (Schimke, 1962) and by hormonal conditions

Table 5. *Effect of giving a diet containing 4-dimethylamino-3'-methylazobenzene for 2.5 weeks on the activities of rat-liver glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase*

Results are expressed as units which are defined as the amount of enzyme catalysing the reduction of 1 μ mole of NADP/hr. at 20°. The experimental animals were given a diet containing 3'-Me-DAB (600 mg./kg. of diet), and the controls received the same diet without the addition of the azo-dye. The rats were kept without food overnight. The results are given as means $\pm$ S.E.M. Fisher's *P* values are given and are regarded as significant if less than 0.05 (N.S. indicates that the *P* values were greater than 0.1).

Diet	Control	3'-Me-DAB added
No. of animals	5	5
Body wt. (g.)	156 $\pm$ 6	153 $\pm$ 5
Liver wt. (g.)	7.63 $\pm$ 0.36	8.93 $\pm$ 0.64
Glucose 6-phosphate-dehydrogenase activity		
Units/g.	71 $\pm$ 13	118 $\pm$ 4
<i>P</i>		0.016
Total units/100 g. body wt.	364 $\pm$ 99	682 $\pm$ 35
<i>P</i>		0.023
6-Phosphogluconate-dehydrogenase activity at pH 9.0		
Units/g.	101 $\pm$ 6	97 $\pm$ 2
<i>P</i>		N.S.
Total units/100 g. body wt.	480 $\pm$ 36	564 $\pm$ 21
<i>P</i>		0.10

leading to changes in the breakdown of amino acids (Freedland & Sodikoff, 1962; McLean & Gurney, 1963; Schimke, 1963). It is possible that the changes in urea-cycle enzymes may be secondary to alterations in other enzymes controlling the catabolism of amino acids, the concentrations of many of which have been shown to be decreased in hepatomas (Auerbach & Waisman, 1958; see also Weinhouse, 1960; Weber, 1961; Reid, 1962*a*). Moreover, one of the first changes induced by azo-dyes seems to be located in the endoplasmic reticulum (Porter & Bruni, 1959; Pitot, 1962), a site associated with protein synthesis, providing further evidence that alterations in urea-cycle enzymes may be secondary to earlier changes.

The relative activities of the urea-cycle enzymes vary markedly with different periods of giving azo-dye in the diet (Fig. 1 and Table 3). The earliest change found in the present experiments was a transient increase in carbamoyl phosphate-synthetase activity after giving the diet containing azo-dye for only 5 days. (Fig. 1). This change was somewhat reminiscent of the early changes in acute carbon tetrachloride poisoning (Rossi & McLean, 1963), and in this context the similarity of the electron-microscopic picture in the two pathological conditions is noteworthy (Smuckler, Iseri &

Benditt, 1962). This increase in carbamoyl phosphate-synthetase activity does not seem to be related to the number of mitochondria, since that of the related enzyme ornithine transcarbamoylase remained unchanged, whereas that of arginase, which is located in the soluble fraction of the cell (Rosenthal, Gottlieb, Gorry & Vars, 1956), also showed a transient increase after 1-2 weeks' treatment.

A significant decrease in ornithine-transcarbamoylase activity was seen as early as 2 weeks after commencement of treatment with diet containing the azo-dye (Fig. 1 and Table 3) or ethionine. As in hepatomas, conditions more favourable for pyrimidine synthesis may result from this early change, which should be considered in conjunction with the results obtained by Reid (1962*b*) for enzymes of the pyrimidine pathway in precancerous liver.

Despite the known effect of ethionine in inhibiting protein synthesis (Farber, 1963) it is noteworthy that, in preliminary experiments, only ornithine-transcarbamoylase activity was decreased after 2 weeks' administration, a pattern of change very like that found with a similar period of treatment with 3'-Me-DAB (Table 3). Farber (1963) has noted that the changes in the ultra-structure of hepatic cells after the administration of ethionine closely resembles those reported for rats given azo-dyes.

The urea-cycle enzymes of the soluble fraction of the cell, arginine synthetase, argininosuccinase and arginase, retain their activities during the precancerous stage to a greater extent than do the mitochondria-linked enzymes (see Fig. 1). This may be related in part to the decrease in the number of mitochondria as a result of the treatment with azo-dye (Allard *et al.* 1957). There does appear to be some relationship between the carcinogenic activity of the azo-dyes and the decrease in the activities of the two ATP-requiring enzymes of the cycle, carbamoyl phosphate synthetase and arginine synthetase; the results in Table 3 show that these are considerably lower in rats treated with 4'-F-DAB compared with those treated for a similar period with 3'-Me-DAB.

Evidence that the results now obtained *in vitro* for the activities of urea-cycle enzymes are valid and may be related to metabolic events in the intact liver stems from the experiments of Burke (1962), who has shown a marked fall in the urea production from many amino acids, including citrulline, arginine and aspartic acid, in a perfused liver system in which control and 3'-Me-DAB-treated rats were compared, the latter having received the azo-dye for 2 months.

Alterations in enzyme pattern that are evident both during the administration of azo-dye and in the hepatoma may regress at an intermediate stage

even if the treatment with azo-dye is continued. Reid (1962*a*) has reviewed examples of such changes occurring in a wide variety of enzymes. The experiments shown in Table 4 provide yet another example of such reversibility. The pattern of change after giving the azo-dye in the diet for 4 weeks already tends towards the low values found in the hepatomas, and yet removal of the azo-dye at this point results in almost complete reversal of these changes within 12 days.

There is considerable evidence that the pentose phosphate pathway of glucose metabolism is active in precancerous liver and in a wide variety of hepatomas, from experiments in which the activities of the enzymes of this pathway have been measured and from experiments with specifically labelled glucose. These experiments have been reviewed by Weber (1961) and Aisenberg (1961). The present finding that glucose 6-phosphate-dehydrogenase activity doubles while that of 6-phosphogluconate dehydrogenase remains unchanged after giving the diet containing 3'-Me-DAB for 2 weeks represents a notably early change among the enzymes associated with this pathway of metabolism. Nodes & Reid (1963) have shown that not only 3'-Me-DAB but also 4'-F-DAB gives, when administered for 4 weeks, an increase in glucose 6-phosphate-dehydrogenase activity. However, the overall effect of this increase in glucose 6-phosphate-dehydrogenase activity is difficult to evaluate in view of the evidence that the concentration of NADP plus NADPH₂ is decreased by the administration of azo-dye (Nodes & Reid, 1963).

Experiments of Elwood, Lin, Cristofalo, Weinhouse & Morris (1963) indicate that glucokinase activity in primary hepatomas induced with azo-dyes is within the range of values for normal liver. If this is true for the precancerous stage, then, together with the fall in glucose 6-phosphatase activity (Weber, 1961; Reid, 1962*a*), the overall effect would be an increase in the availability of glucose 6-phosphate. Dickens (1959) has reviewed many of the factors controlling the alternative pathways of glucose metabolism, and has pointed out the very important role of substrate concentration and K_m values for competing enzymes in control mechanisms at branch points of metabolism and has calculated that the proportion of glucose utilized by the glycolytic route may be increased fivefold by a change in glucose 6-phosphate concentration from 0.1 to 1 mM.

SUMMARY

1. The activities of the urea-cycle enzymes (carbamoyl phosphate synthetase and ornithine transcarbamoylase, located in the mitochondria,

and arginine synthetase, argininosuccinase and arginase, located in the soluble fraction of the cell) have been measured in rat liver in both the precancerous stage and in the tumours resulting from giving carcinogenic azo-dyes in the diet.

2. The activities of all the urea-cycle enzymes are very low in primary liver tumours induced with 4-dimethylaminoazobenzene and its derivatives.

3. The relative activities of the urea-cycle enzymes have been studied after different periods of giving azo-dyes. The earliest change found was a transient increase in the activity of carbamoyl phosphate synthetase after giving the diet containing azo-dye for 5 days; after 2 weeks' treatment arginase activity was also increased but ornithine transcarbamoylase activity was decreased significantly.

4. With longer periods of treatment with azo-dyes a gradual decline in the activities of the urea-cycle enzymes was noted, although those of the enzymes located in the soluble fraction of the cell decreased less rapidly. Removal of the azo-dye after 4 weeks' treatment results in almost complete reversal of these changes within 12 days.

5. The activity of liver glucose 6-phosphate dehydrogenase was almost doubled when rats were given 4-dimethylamino-3'-methylazobenzene for 2.5 weeks; in contrast, the activity of 6-phosphogluconate dehydrogenase was unchanged.

6. These results are discussed in relation to control at branch points of metabolism; in particular the very low activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase in primary hepatomas are considered in relation to the requirements for carbamoyl phosphate for both urea production and pyrimidine synthesis.

We are indebted to Professor F. Dickens, F.R.S., for his interest and advice. This work was in part financed by a grant to Middlesex Hospital Medical School from the British Empire Cancer Campaign and by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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The Effect of Cupric Oxyacetate on the Binding of Azo-Dye by Protein during the Induction of Liver Tumours in the Rat

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(Received 25 September 1963)

Miller & Miller (1947) concluded that the binding of azo-dyes to rat-liver proteins that occurs when they are given in the diet may be of fundamental importance in carcinogenesis by these agents. In subsequent work by them and by other workers, good correlations were obtained between the maximum level of binding by protein, or the rapidity of its attainment, and the percentage of the animals developing tumours (Miller, Miller, Sapp & Weber,

1949). For example, in the rat liver, a potent carcinogen would give a higher degree of binding than a weaker one at the same dose level. The same carcinogen would be bound to a greater extent in the rat than in the mouse, which is less susceptible to azo-dye carcinogenesis. In the rat, a particular carcinogen would be bound strongly to protein in the liver but in no other organ, with subsequent tumour development only in the liver.