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Changes in the Activities of Urea-Cycle Enzymes after the Administration of Carbon Tetrachloride

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The liver damage resulting from carbon tetrachloride poisoning leads to a number of metabolic changes among which is a profound disturbance of nitrogen metabolism. This disturbance is associated with a decline in the rate of incorporation of amino acids into protein (Smuckler, Iseri & Benditt, 1962), a decrease in amino acid activation (Rossi & Zatti, 1963*a*), an increase in the concentration of free ammonia in the liver (Gordon, 1959), and an increase in the concentrations of amino acids and ammonia in blood (Knauff & Windsheimer, 1960; Flores, Rosado, Torres & Soberón, 1962), as well as with a number of other changes in the nitrogenous composition of blood and urine.

It has also been observed that the activity of ornithine transcarbamoylase in the blood rises sharply after the administration of carbon tetrachloride (Reichard, 1959); at somewhat longer time-intervals, decreases in the activities of glutamate dehydrogenase (Rees & Sinha, 1960; Rees, Sinha &

Spector, 1961) and glutamate-pyruvate transaminase have also been observed (Bengmark & Olsson, 1962).

In the expectation that such dramatic changes in amino acid metabolism would be reflected by parallel changes in the urea-forming systems of the liver, it seemed important to examine the effects of carbon tetrachloride poisoning on the entire sequence of enzymes involved in ureogenesis. Such an approach appeared to have the advantage that measurements of a series of enzymes linked in a metabolic sequence is of greater biological significance than measurements confined to individual enzymes. Flores *et al.* (1962) have studied the effects of carbon tetrachloride poisoning at 24 hr. and after 1 month on the activity of urea-cycle enzymes, and in the latter case have found striking decreases in activity.

The present experiments are concerned primarily with the short-term effects of carbon tetrachloride

poisoning, between 1.5 and 6 hr. after administration, with the aim of studying the changes in the pattern of enzymes as a time-sequence after trauma, in order to throw more light on the nature of the initial lesion. A further point in this investigation is the nature of the intracellular distribution of the enzymes of the urea cycle, since the first two of these, carbamoyl phosphate synthetase (carbamate kinase; ATP-carbamate phosphotransferase, EC 2.7.2.2) and ornithine transcarbamoylase (ornithine carbamoyltransferase; carbamoyl phosphate-L-ornithine carbamoyltransferase, EC 2.1.3.3) are mitochondria-linked (Grisolia & Cohen, 1953), whereas the remaining three, argininosuccinate synthetase [L-citrulline-L-aspartate ligase (AMP), EC 6.3.4.5], argininosuccinase (argininosuccinate lyase; L-argininosuccinate arginine-lyase, EC 4.3.2.1) and arginase (L-arginine ureohydrolase, EC 3.5.3.1), are found in the soluble fraction of the cell (Ratner, 1955). Thus changes in the activity of these enzymes could be used to relate the known morphological and biochemical results of carbon tetrachloride poisoning. A preliminary account of some of the early changes has been published (Rossi & McLean, 1963).

METHODS

Materials. Commercial reagents were used, with the exception of argininosuccinic acid, which was a gift from Dr R. G. Westall, University College Hospital, London. α -Isonitrosopropiophenone was synthesized by the method of Claisen & Manasse (1889) by Miss M. W. Gurney.

Treatment with carbon tetrachloride. Young female albino rats with initial body weights 140–150 g. were used. The rats were starved overnight and carbon tetrachloride in liquid paraffin B.P. (1:1, v/v) was given by stomach tube (0.5 ml./100 g. body wt.) with the rat under light ether anaesthesia. The control animals were similarly treated with 0.25 ml. of liquid paraffin/100 g. body wt. Rats were killed 1.5, 3 and 6 hr. after the treatment. Rats used in the study of the effect of CCl_4 for 24 hr. were starved for only 6 hr. before the administration of carbon tetrachloride. In the study of the effects of carbon tetrachloride poisoning at 2 and 4 days young female rats of 140–150 g. body wt. were injected subcutaneously with 0.4 ml. of 20% (v/v) carbon tetrachloride in liquid paraffin B.P. Since this treatment causes some loss of appetite, control animals were pair-fed with the carbon tetrachloride-treated experimental group. Urea excretion was measured in the group of rats treated for 4 days.

The effect of carbon tetrachloride treatment on urea-cycle enzymes in adrenalectomized animals was studied in rats that had been bilaterally adrenalectomized 2 weeks previously and that were given 1% (w/v) sodium chloride solution to drink. Carbon tetrachloride in liquid paraffin (1:1, v/v) was administered by stomach tube (0.5 ml./100 g. body wt.) as described above and the rats were killed 3 hr. later.

Treatment with corticosterone acetate. Young female rats were injected with 1 mg. of corticosterone acetate intra-

peritoneally 3 hr. before the rats were killed for estimation of the activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase.

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). Except where otherwise stated, liver was homogenized in 0.1% cetyltrimethylammonium bromide solution, in the ratio of 1 g. of liver 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.

Arginase was determined by a modification of the method of Folley & Greenbaum (1948). One part of liver homogenate, prepared in cetyltrimethylammonium bromide as described above, was diluted with 9 parts of 'manganese saline' (containing 9.0 g. of sodium chloride and 8.0 g. of manganese sulphate tetrahydrate/l.) to give a final dilution of 1 part of liver in 100 ml. of medium. This was incubated for 30 min. at 37° for activation before assay of the enzyme activity.

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

Effect of calcium ions. Measurements were made of the activities of enzymes of the urea cycle in the presence of Ca^{2+} ions at concentrations ranging from 0.1 to 10 μ moles/ml. of medium. Appropriate volumes of 0.1 M- CaCl_2 were added to the normal assay medium for arginase, the arginine-synthetase system (argininosuccinate synthetase + argininosuccinase) and carbamoyl phosphate synthetase. The effect of Ca^{2+} ions on the activity of ornithine transcarbamoylase was studied by using 0.18 M-tris buffer, pH 8.3, to replace the 0.18 M-glycylglycine buffer, pH 8.3, normally used. The effect on argininosuccinase was measured by using 0.5 M-tris buffer, pH 7.3, instead of the same concentration of phosphate buffer. The effect of the addition of 1 μ mole of Ca^{2+} ion/ml. of medium on the activity of carbamoyl phosphate synthetase was studied in the presence of various concentrations (from 1.2 to 12.5 μ moles/ml. of medium) of ATP and Mg^{2+} ions which were maintained at a constant ratio of 1:1. The activity of the enzyme under these conditions was plotted according to the method of Lineweaver & Burk (1934).

Calcium content and magnesium content of liver. The concentration of these metals in the livers of normal and carbon tetrachloride-treated rats was estimated by using samples of liver homogenized in 49 vol. of 0.1 N-HCl by the flame-spectrophotometric method of Warren (1959). We are grateful to Mr R. L. Warren for these determinations.

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

The results of these experiments, summarized in Table 1 and Fig. 1, show that as early as 90 min. after treatment of the rats with carbon tetrachloride there was an increase in the two mitochondria-linked enzymes, carbamoyl phosphate synthetase and ornithine transcarbamoylase, both as units/g. of liver (Table 1) and as total activity

Table 1. *Effects of carbon tetrachloride poisoning on the activities of the urea-cycle enzymes in liver from normal and adrenalectomized rats*

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 urea/hr. under the conditions of the assay. Rats treated with carbon tetrachloride for 1.5, 3, 6 and 24 hr. were given carbon tetrachloride in liquid paraffin (1:1, v/v) by stomach tube (0.5 ml./100 g. body wt.) with the rat under light ether anaesthesia. Rats treated for 48 and 96 hr. received 0.4 ml. of 20% (v/v) carbon tetrachloride in liquid paraffin. In these latter groups the controls were pair-fed with the carbon tetrachloride-treated groups, and the values obtained were indistinguishable from the free-fed control rats. The corticosterone acetate-treated group received 1 mg. by intraperitoneal injection 3 hr. before the rats were killed for estimation of carbamoyl phosphate-synthetase and ornithine-transcarbamoylase activities. Fisher's *P* values are given in parentheses for the comparison of the experimental and control groups; N.S., not significant. Details are given in the Methods section.

		Units/g. of liver					
		Carbamoyl phosphate synthetase	Ornithine transcarbamoylase	Arginine- synthetase system	Argininosuccinase	Arginase	
(a) Carbon tetrachloride-treated rats							
Normal	0	14	261 ± 15	14 200 ± 950	57 ± 4	210 ± 11	98 400 ± 6 400
	1.5	9	350 ± 32 (<i>P</i> = 0.016)	18 900 ± 1 400 (<i>P</i> = 0.010)	66 ± 7 (N.S.)	230 ± 10 (N.S.)	108 000 ± 14 000 (N.S.)
	3	9	385 ± 30 (<i>P</i> = 0.002)	20 500 ± 900 (<i>P</i> < 0.001)	60 ± 5 (N.S.)	225 ± 8 (N.S.)	93 000 ± 7 400 (N.S.)
	6	4	353 ± 33 (<i>P</i> = 0.012)	22 860 ± 2 750 (<i>P</i> = 0.002)	63 ± 9 (N.S.)	227 ± 15 (N.S.)	98 600 ± 1 400 (N.S.)
	24	6	175 ± 14 (<i>P</i> = 0.003)	14 470 ± 910 (N.S.)	37 ± 4 (<i>P</i> = 0.009)	129 ± 4 (<i>P</i> < 0.001)	71 800 ± 5 900 (<i>P</i> = 0.022)
	48	4	162 ± 30 (<i>P</i> = 0.071)	6 650 ± 990 (<i>P</i> = 0.028)	47 ± 3 (N.S.)	—	114 800 ± 6 900 (N.S.)
	96	8	190 ± 22 (<i>P</i> = 0.049)	6 640 ± 550 (<i>P</i> < 0.001)	35 ± 5 (<i>P</i> = 0.014)	184 ± 16* (N.S.)	59 000 ± 2 900 (<i>P</i> < 0.001)
Adrenalectomized	0	7	167 ± 28	10 900 ± 1 100	31 ± 5	—	52 400 ± 6 900
	3	6	176 ± 17 (N.S.)	12 700 ± 1 070 (N.S.)	43 ± 2 (<i>P</i> = 0.063)	—	54 200 ± 5 000 (N.S.)
(b) Corticosterone acetate-treated rats							
Normal	0	9	275 ± 53	10 900 ± 1 200	—	—	—
	3	9	221 ± 36 (N.S.)	9 750 ± 450 (N.S.)	—	—	—

* 5 results only.

(Fig. 1). At 3 hr. this difference was even more striking, and 6 hr. after the administration of carbon tetrachloride the ornithine-transcarbamoylase activity was approximately 60 % greater than the control. At these time-intervals the activities of other enzymes of the urea cycle located in the soluble fraction of the cell were not significantly changed.

Fig. 1. Activities of urea-cycle enzymes and the concentration of Ca^{2+} and Mg^{2+} ions in the livers of rats at different times after the administration of carbon tetrachloride. (a) Mitochondrial enzymes: Δ , carbamoyl phosphate-synthetase activity; $\blacktriangle$, ornithine-transcarbamoylase activity. (b) Soluble-fraction enzymes: $\bullet$, activity of arginine-synthetase system; $\circ$, argininosuccinase activity; $\circ$, arginase activity. The results are given as total units in the liver expressed as a percentage of the control values. Vertical lines represent the S.E.M. values and are shown in one direction only. The horizontal dotted line represents the arbitrarily fixed control value of 100% ($\square$, with vertical line to show the S.E.M.). The times of killing the rats after the administration of a single dose of carbon tetrachloride in paraffin oil B.P. (1:1, v/v) (0.5 ml./100 g. body wt.) are shown on the heavy horizontal line between (a) and (b) of this Figure. The absolute values for the activities of the urea-cycle enzymes (total units in the liver) corresponding to 100% control activities are: carbamoyl phosphate synthetase, 1580; ornithine transcarbamoylase, 86 600; the arginine-synthetase system, 345; argininosuccinase, 1308; arginase, 622 500. (c) Ca^{2+} ion and Mg^{2+} ion content of liver. Results as $\mu\text{moles/g.}$ of liver are shown by the vertical columns: ▨ , Ca^{2+} ion concentration; $\square$, Mg^{2+} ion concentration of the liver. Details are given in the Methods section.

At a longer time-interval, 24 hr. after the administration of carbon tetrachloride, the activities of both carbamoyl phosphate synthetase and ornithine transcarbamoylase fell, both in relation to the elevated values at 6 hr. and, for carbamoyl phosphate synthetase, in relation to the control value. The specific activities (units/g. of liver) of the three urea-cycle enzymes located in the soluble fraction of the cell decreased considerably, but this was, in part, a resultant effect of a large increase in liver weight at this time after the administration of carbon tetrachloride. The total activities of these enzymes in the liver were decreased only for the arginine-synthetase system (see Fig. 1).

Chronic treatment with smaller doses of carbon tetrachloride for 4 days led to a marked decrease in the activities of all of the enzymes of the urea cycle with the exception of argininosuccinase. The pattern was essentially the same when expressed as total units in the liver and as total units/100 g. body wt. In these experiments control animals were paired with the carbon tetrachloride-treated groups since there is a small decrease in the food intake as a result of the treatment. However, the limit-fed controls were indistinguishable from the controls used in the short-term experiments.

The excretion of urea was measured in the groups of rats treated with carbon tetrachloride for 4 days. These results are summarized in Table 2, and show that there is no significant decrease in urea output as a result of this treatment. The potential rate of synthesis of urea is, presumably, governed by the

Table 2. *Actual and potential rates of urea synthesis in normal and carbon tetrachloride-treated rats*

Results are expressed as means $\pm$ S.E.M. The potential rate of urea production is calculated from the units/g. of liver (1 unit = 1 μmole of urea produced/hr.) of the rate-limiting enzyme of the arginine-synthetase system, the liver weight and the time. The rats were kept in metabolism cages. The urea output was measured by the colorimetric method of Archibald (1945). The rats in the experimental group received 0.4 ml. of 20% (v/v) carbon tetrachloride in liquid paraffin B.P. by subcutaneous injection daily for 4 days. Details are given in the Methods section.

	Control rats	Carbon tetrachloride-treated rats (4 days)
No. of animals	8	8
Body weight (g.)	138 $\pm$ 4	136 $\pm$ 3
Liver weight (g.)	6.90 $\pm$ 0.29	8.26 $\pm$ 0.21
Activity of arginine-synthetase system (units/g.)	57 $\pm$ 4	35 $\pm$ 5
Urea production, 24 hr. (μmoles)		
Potential rate (based on activity of arginine-synthetase system)	9440	6940
Actual urea excretion	6310 $\pm$ 585	6930 $\pm$ 512

activity of the rate-limiting enzyme of the arginine-synthetase system (i.e. argininosuccinate synthetase), and it has been calculated from the units/g. of liver that the maximum daily output for the group of rats chronically treated with carbon tetrachloride would be 6940 μ moles/24 hr., and for the control group 9440 μ moles/24 hr.; the rate of synthesis in the former group is just adequate to account for the daily urea production.

Effect of calcium ions on urea-cycle enzymes. The differentiation between the behaviour of mitochondria-linked and soluble enzymes of the urea cycle in carbon tetrachloride-treated rats is perhaps best seen in Fig. 1, which also shows the concentrations of Ca^{2+} and Mg^{2+} ions in the liver at these time-intervals expressed as μ moles/g. of liver. Significant changes in the concentrations of these ions were found only in the group treated for 24 hr. with carbon tetrachloride, when the Ca^{2+} ion concentration was ten times as great as the control value; the Mg^{2+} ion concentration remained unchanged. There is some parallelism between these changes and the activities of the two enzymes requiring Mg^{2+} ions and ATP, namely carbamoyl

phosphate synthetase and arginine synthetase (see Fig. 1).

In view of the changes known to occur in the concentration of Ca^{2+} ions in the cell after carbon tetrachloride poisoning, measurements were made of the effect *in vitro* of Ca^{2+} ions on the activities of all five enzymes of the urea cycle in rat liver. The results of this experiment are shown in Figs. 2 and 3. Both the arginine-synthetase system and carbamoyl phosphate synthetase are inhibited by low concentrations of Ca^{2+} ions; 50% inhibition was produced by approx. 0.5 μ mole of Ca^{2+} ion/ml. for the former enzyme and by 1.0 μ mole of Ca^{2+} ion/ml. in the latter case. This difference is probably related to the different concentrations of Mg^{2+} ions in the medium for the two enzymes, these being 5 μ moles/ml. and 10 μ moles/ml. respectively. The inhibition is competitive with respect to Mg^{2+} ions, as shown by the results in Fig. 3.

Fig. 2. Effect of Ca^{2+} ions on the activities of urea-cycle enzymes *in vitro*. Δ , Carbamoyl phosphate-synthetase activity; $\circ$, ornithine-transcarbamoylase activity; $\bullet$, activity of arginine-synthetase system; $\blacktriangle$, argininosuccinase activity; $\circ$, arginase activity. Activities in the presence of increasing concentrations of Ca^{2+} ion (1–10 μ moles/ml. of medium) are expressed as percentages of the control activity in the absence of added Ca^{2+} ions. The absolute values for the activities of the urea-cycle enzymes corresponding to 100% control activities are given in Table 1. The reaction media were modified for ornithine transcarbamoylase and argininosuccinase in that the 0.18M-glycylglycine buffer, pH 8.3, and 0.5M-phosphate buffer, pH 7.3, normally used in these assays respectively were replaced by tris buffers of the same pH and molarity. Details are given in the Methods section.

Fig. 3. Effect of Ca^{2+} ions on the activity of carbamoyl phosphate synthetase in the presence of increasing amounts of Mg^{2+} ions and ATP. The effect of Ca^{2+} ions at a concentration of 1 μ mole/ml. of medium on the activity of carbamoyl phosphate synthetase in the presence of 1–10 μ moles of Mg^{2+} ions and of ATP is shown. Results are plotted according to the method of Lineweaver & Burk (1934), with the reciprocal of substrate concentration ($1/s$), where s is expressed as μ moles/ml. of medium, plotted against the reciprocal of the initial velocity ($1/v$), where v is expressed as the change in $E^{1.0}_{490\text{ m}\mu}/15$ min. (colour reaction for citrulline; see the Methods section). $\circ$, Activity in the absence of Ca^{2+} ions; $\bullet$, activity in the presence of Ca^{2+} ions (1 μ mole/ml. of medium).

Ornithine transcarbamoylase shows a small, but constant, inhibition with low concentrations of Ca^{2+} ions; this is approx. 20 % with $1 \mu\text{mole}$ of Ca^{2+} ion/ml. of medium. This inhibition is not changed by altering the medium from one buffered with glycylglycine to one of tris of the same pH and molarity. The activity of ornithine transcarbamoylase is considerably lower (approximately half) in tris buffer compared with glycylglycine buffer. Argininosuccinase and arginase, the latter fully activated with Mn^{2+} ions, were unaffected by the Ca^{2+} ion content of the medium up to a concentration of $10 \mu\text{moles/ml.}$ of medium.

Adrenalectomy and response to carbon tetrachloride. In view of the possibility that the acute effects of carbon tetrachloride could be related to shock and to adrenal secretion, the effect of adrenalectomy, known to protect against many of the metabolic effects of carbon tetrachloride, was tested. Rats, adrenalectomized 2 weeks previously, were given the normal dose of carbon tetrachloride by stomach tube and the activities of the urea-cycle enzymes measured 3 hr. later (Table 1). The results show that there were no significant changes in the activities of these enzymes in the carbon tetrachloride-treated adrenalectomized group, in marked contrast with the response of the mitochondria-linked enzymes of the intact rats given the same treatment. The activities of all five urea-cycle enzymes are decreased in these adrenalectomized groups, in agreement with the results of McLean & Gurney (1963).

That the adrenal glands exert a 'permissive' role rather than that the effects of carbon tetrachloride are directly due to increased adrenal secretion is suggested by the experiment, also reported in Table 1, which shows that the administration of corticosterone acetate failed to elicit a response at 3 hr. similar to that produced by carbon tetrachloride. Negative results were also obtained when rats were treated with hydrocortisone (5 mg. intraperitoneally) and these enzymes, carbamoyl phosphate synthetase and ornithine transcarbamoylase, examined 1.5 hr. later.

Direct effect of carbon tetrachloride. The possibility that the changes observed in the activities of urea-cycle enzymes in carbon tetrachloride-treated rats could be due to a direct effect of carbon tetrachloride on the enzymes was examined by preparing liver homogenates in water and water saturated with carbon tetrachloride (5 mm) and comparing the activities of the enzymes in the two preparations. The liver homogenates were prepared in the way described for the cetyltrimethylammonium bromide homogenates, that is with 1 part of the liver to 9 parts of medium. Liver homogenates were centrifuged at $4000g$ for 15 min. and the activities of the enzymes in the supernatant

fraction were measured. There was no significant difference between the enzyme activities in the two preparations.

It seemed possible that the release of ornithine transcarbamoylase into the blood in carbon tetrachloride-treated animals (Reichard, 1959) might be accompanied by some release of this enzyme from the mitochondria, and an investigation on this point was undertaken to determine if there was any significant rise in the activities of the mitochondria-linked enzymes, carbamoyl phosphate synthetase and ornithine transcarbamoylase, in the soluble fraction of the cell as a result of carbon tetrachloride poisoning. Liver homogenates were prepared in 0.25 M-sucrose from control rats and from rats treated 3 hr. previously with carbon tetrachloride, conventional methods for the removal of mitochondria and cell debris were used and the homogenate (1 part of liver to 9 parts ice-cold 0.25 M-sucrose) was centrifuged at $10\,000g$ for 10 min. The carbamoyl phosphate-synthetase and ornithine-transcarbamoylase activities of the supernatant fractions were measured. In both normal and carbon tetrachloride-treated rats negligible activity was detected in the soluble fraction of the cell.

DISCUSSION

Changes after treatment for 1.5 to 6 hr. The earliest changes found in the activities of the urea-cycle enzymes in the present experiments are increases in the activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase 1.5 hr. after the administration of carbon tetrachloride; even higher activities are found at 3 hr., and these are still elevated 6 hr. after treatment. The increase in the activities of these mitochondria-linked urea-cycle enzymes, compared with the activities of those found in the soluble fraction of the cell, is of interest in that it may indicate some modification of mitochondrial structure at these very short time-intervals after treatment with carbon tetrachloride. Rossi & Zatti (1963b) have shown that the incorporation of ^{32}P into liver phospholipids *in vivo* rises at this stage, and this may be related to some change of the membrane structure of the cell (Hokin & Hokin, 1959a, b). There is, however, no evidence from electron-microscopy studies of extensive alterations to mitochondrial morphology as early as 1.5 hr. after the administration of carbon tetrachloride, and it is generally thought that this follows at a later stage, the earliest damage being found in the endoplasmic reticulum (Oberling & Rouiller, 1956; Neubert & Maibauer, 1959; Bassi, 1960; Recknagel & Lombardi, 1961; Smuckler *et al.* 1962). The present results are among the earliest changes reported in mitochondrial enzymes in carbon tetrachloride poisoning.

The mechanism by which an increase in the activities of the mitochondria-linked enzymes of the urea cycle, carbamoyl phosphate synthetase and ornithine transcarbamoylase, could occur within 1.5–6 hr. is not known. Adaptive enzyme formation, such as that proposed by Schimke (1961, 1962) to account for the changes found after feeding high-protein diets, does not seem likely at this time as there is strong evidence for an inhibition of protein synthesis (Smuckler *et al.* 1961, 1962) and of amino acid activation (Rossi & Zatti, 1963a).

Several alternative explanations may be proposed. First, the mechanism could be a direct effect of carbon tetrachloride on the activities or localization of these enzymes within the cell. We have attempted to show direct effects of carbon tetrachloride *in vitro* by homogenizing normal rat liver in water saturated with carbon tetrachloride, i.e. at a concentration of approx. 5 mM, which is the maximum concentration found in the liver by Recknagel & Litteria (1960) after oral administration of the drug, but the experiments were entirely negative and no modification of the activities of carbamoyl phosphate synthetase or ornithine transcarbamoylase could be detected. The observation that ornithine-transcarbamoylase activity in the blood rises rapidly after carbon tetrachloride poisoning (Reichard, 1959) suggested the possibility that there might be some early changes in the binding of these enzymes to the mitochondria followed by leakage from the damaged cell, but in the present experiments no evidence could be found for a change in the intracellular distribution of these enzymes in liver homogenates prepared in isotonic sucrose from control and carbon tetrachloride-treated animals.

Secondly, adrenal hormones have a profound effect on the activity of enzymes of the urea cycle (McLean, 1961; Freedland & Sodikoff, 1962; McLean & Gurney, 1963; Schimke, 1963), and it is possible the acute poisoning with carbon tetrachloride results in shock with stimulation of some nervous and hormonal functions. There is considerable evidence to show that there may be stimulation of adrenal secretion in carbon tetrachloride poisoning (Selye, 1950; Brody & Calvert, 1960); moreover, adrenergic blocking agents or adrenalectomy will prevent, to a greater or lesser extent, the changes characteristically produced by carbon tetrachloride poisoning on oxidative phosphorylation, activation of adenosine triphosphatase and lipid accumulation (Calvert & Brody, 1960; Recknagel, Stadler & Litteria, 1958). In the present experiments it was demonstrated that adrenalectomy prevented the initial rise in the activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase after treatment with

carbon tetrachloride (see Table 1), and this may suggest that this effect is mediated by adrenal-gland secretion. However, experiments in which large doses of corticosterone or hydrocortisone failed to produce any increase in carbamoyl phosphate-synthetase or ornithine-transcarbamoylase activities at 3 hr. and 1.5 hr. respectively militate against this argument, and suggest a permissive role for the adrenal glands in this effect of carbon tetrachloride (Ingle, 1954; Cuthbertson, 1960). These experiments are by no means conclusive, since it is possible that the acute effect of carbon tetrachloride may be mediated by way of the adrenal gland and that a steroid other than those tested in the present experiments is responsible for the rapid changes in the urea-cycle enzymes. The possibility that adrenaline may also have an action on these enzymes must not be overlooked.

Thirdly, the activity of carbamoyl phosphate synthetase is markedly affected by metal ions such as Mg^{2+} and Zn^{2+} ions, and by the Na^+ ion: K^+ ion quotient (see Cohen & Sallach, 1961; Caravaca & Grisolia, 1960). Unfortunately there is little information on the changes in the concentration of metal ions in the cell at very early stages of carbon tetrachloride poisoning (Thiers, Reynolds & Vallee, 1960). It would seem unlikely that the increased activity of ornithine transcarbamoylase could be related to such changes since this enzyme is relatively unaffected by metal ions (see Jones, 1962).

Changes after treatment for 24 hr. At 24 hr. after the administration of a single dose of carbon tetrachloride the pattern of the enzymes of the urea cycle changes completely from that found at the early times of treatment. On the basis of total activity, possibly the more significant in view of the changes in liver weight at this time, there is a significant decrease in the activities of both the arginine-synthetase system and carbamoyl phosphate synthetase. These results are not in complete agreement with those of Flores *et al.* (1962), who found that the total activities of the arginine-synthetase system and arginase were decreased but that the activity of carbamoyl phosphate synthetase remained unchanged. This difference between the present results and those of Flores *et al.* (1962) may be explained, in part, by the different dose and means of administration of carbon tetrachloride.

The enzyme changes found at 24 hr. are in keeping with the pathological findings. For example, it is known that, 24 hr. after the administration of carbon tetrachloride, the concentration of ammonia increases in the liver (Gordon, 1959). This rise is, presumably, related to the marked fall in the activity of carbamoyl phosphate synthetase, an enzyme of considerable importance in the utiliza-

tion of ammonia. The decrease in activity of this enzyme, together with the fall in those of glutamate dehydrogenase and glutamine synthetase observed by Flores *et al.* (1962), may well explain the observed increase in the concentration of ammonia in liver and blood.

In the present experiments only the activities of the two enzymes requiring ATP and Mg^{2+} ions, carbamoyl phosphate synthetase and the arginine-synthetase system, are decreased at 24 hr., suggesting that this is not due to some non-specific effect, such as leakage of enzymes from damaged cells (Rees & Sinha, 1960; Reichard, 1959), and this raises the question of how far the known effects of carbon tetrachloride on the metal ion content of the liver could account for this phenomenon.

The Ca^{2+} ion content of the liver is known to increase markedly 24 hr. after the administration of carbon tetrachloride (Thiers *et al.* 1960; Rees *et al.* 1961). In the present experiments an increase of approximately tenfold was observed, the concentration reaching $10 \mu\text{moles/g.}$ of liver, a value in good agreement with those of the above authors. The Mg^{2+} ion concentration in the liver remained unchanged. Since it is known that the accumulated Ca^{2+} ions are largely located in the mitochondrial fraction of the cell at this time, it is possible that this could account for some lowering of the activities of both carbamoyl phosphate synthetase and the arginine-synthetase system, as the release of Ca^{2+} ions during homogenization could yield a final concentration of $1 \mu\text{mole/ml.}$ of supernatant fraction used for enzyme assay and $0.2 \mu\text{mole/ml.}$ in the enzyme assay medium. Such a concentration could cause an approximately 20% inhibition of enzyme activity (Fig. 2). Elliot (1951) has studied the inhibition of glutamine synthetase by Ca^{2+} ions. In this latter case, as well as with the two urea-cycle enzymes, the effect is competitive and an approximately 50% inhibition of activity was found when the Ca^{2+} ion: Mg^{2+} ion ratio was approximately 1:10.

The present results, in which Ca^{2+} ions are released from the localization in the mitochondrial fraction and in which the medium is fortified with high concentrations of Mg^{2+} and K^{+} ions, may, in fact, give a very different picture of enzyme activity from that actually present in the cell. For example, the change in the K^{+} ion: Na^{+} ion ratio from 32:0.9 in normal mitochondria to 17:2.7 in mitochondria from carbon tetrachloride-treated rats (Thiers *et al.* 1960) could be of great importance in connexion with the affinity of carbamoyl phosphate synthetase for ammonia (see Cohen & Sallach, 1961).

Changes after long-term treatment. When carbon tetrachloride is administered daily for 4 days (by subcutaneous injection) the activities of all of the

enzymes of the urea cycle decreased with the exception of that of argininosuccinase. Despite the low value for the activity of the arginine-synthetase system found at this time it was calculated that this rate-limiting enzyme is still adequate to account for the observed urea production (Table 2). In the present experiments no change was observed in urea production with this treatment, and it may be that changes will only be seen if the urea cycle is forced to bear an extra load by the administration of large doses of ammonium chloride, as in the experiments of Flores *et al.* (1962). In the present experiments the food intake was controlled and the results are not, therefore, to be attributed to an adaptive change of the kind observed by Schimke (1961, 1962).

Nucleic acid synthesis and urea formation may be regarded as two opposing mechanisms, in that the former requires amino groups for purine and pyrimidine synthesis and the latter is directed to their elimination as urea. The common intermediate for the two pathways is carbamoyl phosphate, which can be incorporated either into pyrimidines via aspartate transcarbamoylase or into citrulline via ornithine transcarbamoylase. Thus the relative activities of these two enzymes could regulate the availability of the amino group of carbamoyl phosphate for either nucleic acid synthesis or urea formation. In fact, an inverse relationship has been suggested for these two enzymes (see Cohen & Sallach, 1961; Calva & Cohen, 1959).

In rats treated with carbon tetrachloride two processes are occurring. First there is the toxic effect of the drug on the enzymes of the urea cycle, and secondly there are the regenerative processes, which begin at about 24 hr., described by Cameron & Karunaratne (1936), Tsuboi, Stowell & Lee (1951), Farber, Koch-Weser, Szanto & Popper (1951), Campbell & Kosterlitz (1952), Richter, Frunder & Börnig (1958) and Leevy, Hollister, Schmid, McDonald & Davidson (1959). This latter process involves changes in the rate of nucleic acid synthesis. On the basis of the inverse relationship mentioned above it might be expected that the activity of ornithine transcarbamoylase would be decreased at this time, resulting in conditions favourable for nucleic acid synthesis. The results in Table 1 show that this is indeed the case. On the other hand no correlation can be found in the present experiments for a relationship between the activity of carbamoyl phosphate synthetase and the known changes in nucleic acid synthesis. Similar observations have been noted by Flores *et al.* (1962). Indeed, at this longer time-interval there is the situation of an unchanged urea formation and declining carbamoyl phosphate-synthetase activity associated with a high rate of nucleic acid synthesis. It would appear from this that either

carbamoyl phosphate is not rate-limiting in pyrimidine synthesis or that there is an alternative route for its synthesis.

It is not known how far the changes observed in the present experiments represent a common pattern in other types of liver damage; certain other substances such as 4-dimethylamino-3'-methylazobenzene, dimethylnitrosamine and several other liver poisons produce early alterations similar to those produced by carbon tetrachloride in the endoplasmic reticulum seen by electron microscopy (Smuckler *et al.* 1962). In parallel experiments to those reported here P. McLean, E. Reid & M. W. Gurney (unpublished work) observed that within a few days of giving 4-dimethylamino-3'-methylazobenzene there was a significant rise in the activity of rat-liver carbamoyl phosphate synthetase, followed by a marked fall in the activities of this and other enzymes of the urea cycle after prolonged treatment. This pattern is not dissimilar to that of carbon tetrachloride poisoning in acute and chronic treatment, and the possibility exists that this may prove to be a general picture in different types of liver damage.

SUMMARY

1. The activities of the urea-cycle enzymes in rat liver have been studied at different times after the administration of carbon tetrachloride, measurements being made 1.5, 3, 6 and 24 hr. after a single dose of the drug was given by stomach tube as well as 2 and 4 days after daily subcutaneous injections of carbon tetrachloride.

2. At 1.5 hr. after treatment there was an increase in the two mitochondria-linked enzymes, carbamoyl phosphate synthetase and ornithine transcarbamoylase, both as units/g. of liver and as total activity. The activities of these enzymes were further increased at 3 and 6 hr. At these times the activities of the enzymes of the urea cycle located in the soluble fraction of the cell, the arginine-synthetase system, argininosuccinase and arginase, were unchanged.

3. At 24 hr. after treatment the activities of both carbamoyl phosphate synthetase and ornithine transcarbamoylase fell, both in relation to the elevated values at 6 hr. and, for the former enzyme, in relation to the control values. The total activity of the arginine-synthetase system decreased.

4. Treatment with carbon tetrachloride for 4 days led to a marked decrease in the activities of all the enzymes of the urea cycle with the exception of argininosuccinase.

5. There were no significant changes in the activities of carbamoyl phosphate synthetase and ornithine transcarbamoylase in livers from adrenal-

ectomized rats 3 hr. after treatment with carbon tetrachloride.

6. Corticosterone acetate and hydrocortisone injections produce no short-term changes (at 3 and 1.5 hr. respectively) in the activities of these enzymes in normal rat liver.

7. The effects of Ca^{2+} ions *in vitro* on the activities of all five enzymes of the urea cycle have been measured, as well as the concentration of Ca^{2+} ion in the livers of treated animals; the pattern of change in the activities of urea-cycle enzymes after carbon tetrachloride poisoning are considered in relation to these effects.

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Action of 8-Azaguanine and 8-Azaxanthine on *Pseudomonas aeruginosa*

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Bergmann, Ungar-Waron, Kwietny-Govrin & Leon (1962) found that 6-mercaptopurine does not inhibit the growth of *Pseudomonas aeruginosa*. These bacteria oxidize the antimetabolite very rapidly, at a rate exceeding that of hypoxanthine or xanthine, and thus prevent its incorporation into nucleic acids. This observation led to a study of other growth inhibitors of the purine series which because of structural peculiarities cannot serve as substrates of the bacterial purine-oxidizing system. The present paper deals with the action of 8-azaguanine and related compounds on the growth of *P. aeruginosa* and on the production of purine-catabolizing enzymes.

MATERIALS AND METHODS

Purines. The following derivatives were synthesized: 2- and 6-thioxanthine (Beaman, 1954); 6-thiouric acid (Levin, Kalmus & Bergmann, 1960); 2- and 8-thiouric acid (Loo, Michael, Garceau & Reid, 1959); 3-methyl-2-thioxanthine

and 3-methyl-2-thiouric acid (Bergmann, Levin, Kalmus & Kwietny-Govrin, 1961); 3-methyluric acid (Bergmann & Dikstein, 1955); 8-azaxanthine (Roblin, Lampen, English, Cole & Vaughan, 1945).

6-Mercaptopurine and uracil were obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and 8-azaguanine from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Other heterocyclic compounds. Benzimidazole and benzotriazole were prepared according to *Organic Syntheses* (1948, 1955).

Bacterial cultures. The strain of *P. aeruginosa* with high xanthine-oxidase activity was that described by Dikstein, Bergmann & Henis (1957). The organism was grown in a synthetic medium similar to that described by Davis & Mingioli (1950): KH_2PO_4 , 3 g.; K_2HPO_4 , 7 g.; sodium citrate dihydrate, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g.; glucose, 5 g./l. The pH was adjusted to 7.0. The salt mixture was sterilized at 15 lb./in.² for 20 min. and the glucose passed separately through a Seitz filter.

The cells were grown by shaking at 30°. Samples were withdrawn at hourly intervals and the extinctions determined in a 1 cm. cell at 550 m μ in a Beckman spectrophotometer.