enough to drive the reaction towards the synthesis of adenosine 3'-phosphate 5'-sulphatophosphate, because owing to the presence of an excess of the ready acceptor the adenosine 3'-phosphate 5'-sulphatophosphate formed would constantly be used up.

That vitamin A is specific for these reactions is borne out by the fact that, of the many fat-soluble compounds tried, only the different forms of vitamin A were able to restore *in vitro* the loss of the activity caused by the deficiency. Different forms of vitamin A are currently being assigned different physiological functions, e.g. the aldehyde for the vision, and the acid for general growth (Wolf, 1962; Malathi *et al.* 1963). It is now shown that, in the sulphurylation of phenols by rat liver, vitamin A alcohol and acid take part in the activation and transfer steps respectively.

SUMMARY

1. Both the steps involved in the sulphurylation of phenols, namely activation and transfer of sulphate, were significantly decreased in the liver supernatant of vitamin A-deficient rats. The addition of vitamin A alcohol *in vitro* was more effective in restoring the lost activity of the first step, whereas the acid was more effective in the second.

2. Of the several fat-soluble compounds tried, only the different forms of vitamin A were, in various degrees, active in restoring the lost activity.

3. After the oral administration of three large doses of vitamin A acetate or acid to vitamin A-deficient rats, both the enzyme activities of the liver of the two groups were found to be normal.

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The Changes in some Hydrolytic Enzymes in Carbon Tetrachloride-Induced Fatty Livers

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It is known that mitochondria from fatty livers are severely damaged. They are swollen and show a high degree of uncoupling of oxidative phosphorylation, as well as of activation of adenosine triphosphatase. Their permeability is increased. This fact provokes a partial transfer of certain coenzymes or cofactors from the particles into the surrounding fluid (for references see Artizzu & Dianzani, 1962). In fatty liver the amount of soluble hydrolytic enzymes ('lysosomal' enzymes) increases. In some, such as cathepsin and ribonuclease, the specific activity of the hydrolytic enzymes is also increased

(Dianzani, 1954; Martini & Dianzani, 1958; Beaufay, Van Campenhout & de Duve, 1959b; Artizzu, 1960; Ugazio, 1960). As proteases have been found able to digest mitochondria *in vitro* (Dianzani, 1953; Koshihara, 1959), and ribonuclease treatment can produce uncoupling of oxidative phosphorylation as well as morphological changes of liver mitochondria (Hanson, 1959; Artizzu, 1961), mitochondrial changes occurring in fatty liver might be a consequence of the activation of hydrolytic enzymes. Previous work has shown that ribonuclease activation occurs after the onset of mitochondrial changes (Artizzu & Dianzani, 1962).

The present experiments studied in a more detailed way the time-course of changes in hydrolytic enzymes ('lysosomal' enzymes) occurring in the fatty liver induced by poisoning with carbon tetrachloride. Results show that the very early phases of poisoning are characterized by only slight modifications of the 'lysosomal' enzymes. The onset of mitochondrial damage seems therefore to be independent of the activation of 'lysosomal' enzymes.

MATERIALS AND METHODS

Male albino rats (Wistar strain) weighing 130–160 g. were used throughout this work. They were fed on a standard semi-synthetic diet (M. I. L. Morini, S. Polo d'Enza, Reggio Emilia, Italy). Food was withheld for 8–12 hr. before the beginning of the experiment.

Pure redistilled carbon tetrachloride (0·5 ml./100 g. body wt.) was given by stomach tube. It was a R.P. product of Carlo Erba, Milano. Animals were killed by decapitation at 0, 2, 6, 12, 24, 48, 72, 96 and 144 hr. after the administration of the poison. The removal of the liver and subsequent manipulations were done in the cold room at 4°.

Homogenates (10 %, w/v) were prepared with a Potter–Elvehjem-type homogenizer in 0·25 M-sucrose. The homogenization technique was standardized to give reproducible results. In each case three complete up-and-down runs of the Teflon pestle rotating at about 1000 rev./min. were used. When the specific activity of the enzymes with whole homogenate was to be measured, 0·25 M-sucrose contained 0·5 % of Triton X-100 (Rohm and Haas, Pa., U.S.A.).

The activity of the following enzymes was studied: arylsulphatase A, arylsulphatase B, cathepsin, β-glucuronidase, acid phosphatase, acid ribonuclease, uricase. All these enzymes, with the exception of uricase, are supposed to be 'lysosomal'. Uricase, on the contrary, seems to be located within a different type of particle, i.e. in micro-bodies (de Duve et al. 1960).

Arylsulphatases A and B were measured according to the method of Roy (1953) with dipotassium 2-hydroxy-5-nitrophenylsulphate synthesized by the method of Roy (1958). Acid phosphatase was determined according to the method of Appelmans, Wattiaux & de Duve (1955) with sodium β-glycerophosphate (E. Merck, Darmstadt) as substrate. β-Glucuronidase was determined according to the modification of Kerr & Levvy (1951) of the method of

Talalay, Fishman & Higgins (1938), with phenolphthalein glucuronide (Sigma Chemical Co., St Louis, Mo., U.S.A.). Acid ribonuclease was measured according to the method of Dubos & Thompson (1938), the yeast ribonucleic acid being purified according to the procedure of Zittle & Reading (1945). Cathepsin was determined by the modification of Gianetto & de Duve (1955) of the method of Anson (1937). Uricase was determined according to the method of Kalckar (1947) as modified by Schneider & Hogeboom (1952).

The homogenate prepared with 0·25 M-sucrose containing 0·5 % of Triton X-100 was analysed for enzymic activities as well as for nitrogen content. Nitrogen was determined by the usual micro-Kjeldahl method. As Triton X-100 solubilizes all 'lysosomal' enzymes (Wattiaux & de Duve, 1956) and produces maximum enzymic activity, the specific activities are of the fully activated enzymes.

Another portion of the liver was homogenized with 0·25 M-sucrose alone. This homogenate was centrifuged at 960 000 g-min. (16 000 g for 60 min.) in a Lourdes type A centrifuge equipped with rotor 9 RA. The centrifugal force was calculated on the average radius. The supernatant was used for the determination of the amount of the soluble enzymic activities. In several instances the homogenate prepared with 0·25 M-sucrose alone was used for the determination of the enzymic activities. In some experiments 0·25 M-sucrose homogenates were submitted to differential centrifuging to obtain 'lysosome'-rich subfractions. A 'lysosome'-rich sediment was collected by centrifuging between 33 000 (3300 g for 10 min.) and 360 000 (10 000 g for 36 min.) g-min. It was then resuspended in the original volume of 0·25 M-sucrose and submitted to three further centrifugings, respectively at 50 000 (10 000 g for 5 min.), 150 000 (10 000 g for 15 min.) and at 360 000 g-min. The sediments were resuspended in Triton-containing 0·25 M-sucrose and used for the determination of the enzymic activities. The morphological composition of the subfractions was studied by the electron microscope (Philips 100). The extent of microsomal contamination of the sediments was assessed by determining the activity of glucose 6-phosphatase, by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955).

Fat content of each liver was determined by the loss in weight of the dry powder after ether extraction in a Soxhlet apparatus. Histological specimens were fixed in 10 % (v/v) formalin, embedded in paraffin and stained with haematoxylin and eosin.

All determinations done throughout this work were made in duplicate.

The obtained results were analysed for statistical significance by determining Fisher's (1948) t value.

RESULTS

Unsedimentable activities. According to de Duve et al. (1955) a measure of 'lysosomal' damage may be obtained by determining both the unsedimentable amount of enzymic activities and the activities of unfractionated homogenates prepared with 0·25 M-sucrose. In the second case enzymic activity represents the sum of 'free' and of soluble activities. The amounts of activities found in the soluble part of the homogenate at different times after poisoning

with carbon tetrachloride are shown in Table 1, which shows that the unsedimentable amount increased for all studied enzymes at a certain time after poisoning. The times at which this increase occurred or disappeared, as well as its extent, were, however, different from one enzyme to another. At 2 hr. a significant increase occurred only in acid phosphatase. The increase remained significant for this enzyme until 72 hr. At 6 hr. a significant rise was noted for cathepsin, β-glucuronidase and acid ribonuclease, and this rise also remained significant until 48–72 hr. The unsedimentable activity of arylsulphatases A and B was increased in a significant way only at 24 hr. That of uricase showed a rather small increase only at 72 hr.

The total enzymic activities of the homogenate prepared with 0·25 M-sucrose alone were studied at 2 and 6 hr. after the administration of carbon tetrachloride to see if earlier changes could be detected (Table 2): an increase in specific activity was found at 2 hr. for acid phosphatase only, but at 6 hr. it was noted also for ribonuclease and cathepsin. With arylsulphatase A and β-glucuronidase, decrease in activity occurred at 2 hr. but not at 6 hr.

Specific activities. The behaviour of the specific activities of Triton-treated homogenates is shown in Table 3. Some heterogeneity appeared also in these experiments. Specific activities remained almost unchanged throughout the entire experimental time with β-glucuronidase and acid phosphatase. Some increase was seen at 2 and 6 hr. for acid phosphatase, and a decrease at 2 hr. occurred for β-glucuronidase, but the values returned to normal in later stages. Cathepsin, arylsulphatases A and B and ribonuclease showed a consistent increase in activity, which amounted to 100–200 % of the starting values, in later stages of poisoning. Similar findings were made by Martini & Dianzani (1958), Beaufay *et al.* (1959*b*) and Artizzu (1960) for cathepsin and ribonuclease. The present results for arylsulphatase A are, however, somewhat different from those obtained by one of us (Ugazio, 1960) in rats treated by several subcutaneous injections of a 20 % (v/v) olive oil solution of carbon tetrachloride, when no increase in the specific activity of arylsulphatase A was seen whereas a significant increase occurred for arylsulphatase B.

Differential centrifuging of 'lysosome'-rich sediments. The experiments described above showed that 'lysosomal' enzymes did not change as a uniform group. Heterogeneity in behaviour of enzymes from pathological liver was described also by Beaufay *et al.* (1959*b*). It seems to be a rather general feature of 'lysosomal' enzymes, as it occurs after other types of treatment (de Duve, 1957; Beaufay & de Duve, 1959; Beaufay, Bendall, Baudhuin,

Table 1. *Enzymic activities in the soluble phase of the liver homogenate at different times after treatment with carbon tetrachloride*

Results are given as percentages of the activity of the whole homogenate. This was prepared with 0·25 M-sucrose containing 0·5 % of Triton X-100. The number of experiments of each group is given in parentheses; s.d. is given after each average.

Time after treatment (hr.)	Arylsulphatase A	Arylsulphatase B	β-Glucuronidase	Acid phosphatase	Cathepsin	Ribonuclease	Uricase
0	11·6 ± 3·0 (6)	9·0 ± 1·5 (6)	12·3 ± 2·6 (6)	10·9 ± 4·9 (5)	4·3 ± 4·6 (6)	10·4 ± 3·1 (5)	5·0 ± 4·1 (6)
2	—	—	11·5 ± 4·2 (7)	23·7 ± 6·7 (7)*	0·1 ± 0·1 (6)	13·3 ± 0·9 (7)	—
6	9·5 ± 4·6 (4)	10·9 ± 0·8 (4)	22·2 ± 7·0 (6)**	17·3 ± 4·4 (9)***	34·2 ± 2·7 (6)*	29·8 ± 4·5 (4)*	5·1 ± 1·2 (4)
12	13·1 ± 1·9 (5)	9·9 ± 1·0 (5)	29·5 ± 2·7 (3)*	23·1 ± 7·7 (5)***	36·9 ± 23·2 (4)**	21·1 ± 4·9 (5)*	6·6 ± 4·4 (5)
24	26·4 ± 10·1 (5)*	21·9 ± 9·4 (5)**	31·9 ± 14·1 (5)*	24·4 ± 6·8 (5)**	41·6 ± 7·0 (5)*	22·8 ± 8·9 (5)*	9·5 ± 9·3 (5)
48	24·4 ± 15·1 (5)	20·1 ± 12·9 (5)	32·6 ± 17·5 (3)***	25·1 ± 5·9 (5)*	37·7 ± 13·6 (3)*	44·7 ± 17·5 (5)*	9·5 ± 6·4 (5)
72	10·2 ± 2·9 (4)	8·2 ± 2·4 (4)	20·4 ± 4·9 (4)***	23·1 ± 3·5 (4)*	44·7 ± 9·3 (4)*	9·5 ± 6·5 (4)	12·2 ± 2·1 (4)***
96	8·9 ± 2·7 (4)	8·4 ± 1·4 (4)	10·4 ± 4·2 (3)	8·2 ± 2·0 (4)	9·6 ± 9·6 (4)	17·0 ± 15·7 (4)	12·4 ± 4·8 (4)
144	12·3 ± 2·6 (4)	10·4 ± 1·1 (4)	30·5 ± 1·4 (7)*	14·9 ± 6·8 (8)	0 (6)	4·7 ± 5·5 (4)	5·4 ± 1·0 (4)

* $P < 0.01$.   ** $P < 0.02$.   *** $P < 0.05$.

Table 2. *Specific activities of some enzymes in liver homogenates prepared in sucrose solution alone*

Results are given as $\mu\mu$moles of substrate destroyed/mg. of nitrogen/min. $\pm$ s.d. For cathepsin results are given as $\mu\mu$moles of tyrosine set free from haemoglobin/mg. of nitrogen/min. and for ribonuclease as $\mu\mu$moles of inorganic orthophosphate set free from ribonucleic acid/mg. of nitrogen/min. The number of experiments in each group is given in parentheses. Times refer to the intervals after treatment with carbon tetrachloride.

Time (hr.)	0	2	6
Enzyme			
Arylsulphatase A	7.6 ± 0.6 (6)	5.6 ± 1.0 (6)*	7.0 ± 1.0 (8)
Arylsulphatase B	33.2 ± 6.3 (6)	28.5 ± 1.3 (6)	27.6 ± 3.2 (8)
β-Glucuronidase	21.0 ± 7.7 (12)	13.0 ± 4.3 (16)*	17.7 ± 7.3 (8)
Acid phosphatase	21.1 ± 7.2 (12)	44.1 ± 6.4 (16)*	52.3 ± 6.3 (8)*
Ribonuclease	3.6 ± 1.5 (6)	4.5 ± 1.4 (6)	13.9 ± 3.5 (6)*
Cathepsin	0.8 ± 0.6 (6)	0.8 ± 0.6 (6)	1.9 ± 0.6 (6)**

* $P < 0.01$.   ** $P < 0.02$.

Wattiaux & de Duve, 1959*a*; de Duve & Beaufay, 1959). The reasons for heterogeneity may be either the location of single enzymes within different types of particles, or in different sites of the same particle, or the existence of several classes of 'lysosomes' having a different quantitative enzyme composition. To obtain further data on the nature of the heterogeneity, some experiments were made in which 'lysosome'-rich sediments isolated from the liver of rats at different times after poisoning with carbon tetrachloride were submitted to differential centrifuging. Three subfractions were obtained in this way, as described above. The enzymic activities of the subfractions were studied in the presence of Triton X-100 to determine the distribution of the activities among the subfractions. Attempts to wash the sediments were avoided, to decrease the eventual damage to the particles.

The sum of the enzymic activities found in the three subfractions amounted to 70–80 % of that found in fully activated homogenates. These percentages were considerably decreased in treated animals, as a consequence of partial solubilization of the enzymes. Nitrogen contents of the individual subfractions were respectively 975 ± 147, 1232 ± 201 and $1387 \pm 263\,\mu$g./g. of wet liver in normal animals. Nitrogen content was not significantly changed at any time after treatment with carbon tetrachloride. Electron-microscopical examinations of the sediments, after embedding in methacrylate, showed that the first subfraction contained small mitochondria and dense bodies. A certain amount of osmiophilic dense granules, probably derived from the endoplasmic reticulum, were also present. The second subfraction contained a certain number of dense bodies and of membranes; small osmiophilic granules were present to a large extent. The bulk of the third subfraction was formed by small osmiophilic granules, only scanty dense bodies being present. The extent of microsomal contamination was studied also by determining the activity of

glucose 6-phosphatase. This was compared with the activity present in a microsomal fraction obtained by centrifuging normal liver homogenates at between 360 000 and 3 000 000 g-min. (20 000 g for 150 min.). It was seen that glucose 6-phosphatase activity of the first subfraction was less than 10 % of that found in microsomal sediment; the activity present within the second subfraction was about 20 % and that of the third one was about 80 % of that found in microsomes. It is clear therefore that the last subfraction contains mainly microsomes. The percentage distribution of glucose 6-phosphatase was not modified in the sediments of treated rats but the specific activity of glucose 6-phosphatase is decreased (Recknagel & Lombardi, 1961).

Table 4 shows the values for the percentage distribution of different enzymes among the 'lysosome'-rich subfractions at several times after poisoning with carbon tetrachloride. In untreated rats the bulk of the activities of uricase and of cathepsin are present in the first subfraction, only small amounts being present within the third one. A similar distribution is shown also by arylsulphatases, β-glucuronidase and acid phosphatase. In these, however, the amounts of activities present in the third subfraction were slightly higher than those seen for cathepsin and uricase; 40–50 % of the total activity was present in the first subfraction. The distribution pattern of ribonuclease was somewhat different from that seen for other enzymes; in fact, with this the highest activity was found in the last subfraction. The results, however, may have been complicated by the existence of a microsomal ribonuclease (Roth, 1960), or by different distribution among subfractions of ribonuclease inhibitors.

Treatment with carbon tetrachloride introduced further significant changes in distribution patterns. At 2 hr. a significant increase in the activity of the first subfraction was seen for ribonuclease; at the same time a decrease in the activity of the last subfraction occurred. Such changes remained significant also in later stages of poisoning. An increase in

Table 3. *Specific activities of some enzymes of liver homogenates at different times after treatment with carbon tetrachloride*

Results are given in most cases as $\mu\mu$moles of substrate destroyed/mg. of nitrogen/min. For cathepsin results are given as $\mu\mu$moles of tyrosine set free from haemoglobin/mg. of nitrogen/min. and for ribonuclease as $\mu\mu$moles of inorganic orthophosphate set free from ribonucleic acid/mg. of nitrogen/min. The number of experiments of each group is given in parentheses; s.d. is given after each average.

Time after treatment (hr.)	Arylsulphatase A	Arylsulphatase B	β-Glucuronidase	Acid phosphatase	Cathepsin	Ribonuclease	Uricase
0	16·9± 0·5 (5)	60·9±18·2 (5)	44·8±11·8 (5)	62·0±24·9 (4)	3·0±0·7 (5)	24·9± 7·2 (5)	29·1± 7·6 (7)
2	—	—	24·8± 4·5 (7)*	111·9± 3·2 (7)	2·9±0·2 (6)	30·7± 9·5 (7)	—
6	20·9± 1·6 (7)	49·9±11·6 (4)	36·4± 4·6 (6)	112·6±29·4 (10)**	2·8±0·3 (6)	39·9± 5·7 (4)**	21·0± 1·0 (4)
12	28·1± 9·2 (5)	76·2±11·0 (5)	67·7±23·9 (3)	63·9±21·3 (5)	4·2±2·3 (5)	30·8±14·9 (3)	30·7±13·3 (5)
24	18·3± 5·3 (5)	86·9±41·1 (5)	56·1±20·9 (5)	60·3±20·0 (5)	4·4±1·2 (5)	57·0±43·1 (5)	32·6±12·8 (5)
48	24·8± 2·1 (5)***	102·6± 9·2 (5)*	70·0±27·4 (3)	94·5±32·9 (5)	7·4±1·9 (3)*	41·2±13·0 (5)	14·5± 5·5 (5)*
72	35·0± 7·4 (4)*	136·1±33·9 (4)*	51·8±26·8 (4)	70·3±19·3 (4)	8·2±2·3 (4)*	47·0±15·8 (4)***	12·5± 5·0 (4)*
96	33·1±10·2 (4)	128·9±49·7 (4)***	50·0±26·9 (4)	98·7±33·5 (4)	7·3±1·1 (3)*	59·9± 9·2 (3)***	20·1± 9·7 (4)
144	28·1± 5·0 (11)*	75·5±12·9 (5)	42·3±11·2 (7)	133·2±49·7 (9)	9·5±1·7 (6)*	31·4± 5·8 (8)	11·4± 3·0 (5)*

* $P < 0.01$.   ** $P < 0.02$.   *** $P < 0.05$.

the activities present in the first subfraction was seen to occur also in arylsulphatase B and β-glucuronidase. No change was seen in acid phosphatase and arylsulphatase A. With uricase an increase in the percentage of activity present in the first subfraction occurred at 6 hr. For cathepsin the main change was an increase in the percentage of activity present within the third subfraction, as well as a decrease in the percentage present within the first one; this change mainly occurred in later stages of poisoning.

Nitrogen distribution among the subfractions did not change at any time.

Table 5 shows the values for the specific activities of enzymes within the 'lysosome'-rich subfractions. Their behaviour largely parallels that found in the unfractionated homogenates. The fact that the extent of increase in some enzymic activities in later stages of poisoning is lower in these experiments than in those made with unfractionated homogenates is because at these stages a certain amount of the enzymic activities is found in the soluble phase. This explanation can be applied also to the decrease in the specific activities of some enzymes occurring during the first 24 hr. after the administration of carbon tetrachloride.

As a difference from the behaviour observed with the whole homogenate, the activity of uricase was not decreased in a significant way at any time after the administration of carbon tetrachloride.

The morphological composition of the subfractions, as studied by the electron microscope, was somewhat modified with respect to that found in normal rats. The most evident changes were seen at 24 hr. after giving carbon tetrachloride. The most rapidly sedimenting subfraction contained mainly dense bodies and small damaged mitochondria. Small osmiophilic granules had practically disappeared and were replaced by small transparent vesicles. These may be derived from the rupture of dense bodies, but it seems very probable that the bulk of them is derived from dilated endoplasmic reticulum. In fact, many of them show the presence of small osmiophilic granules on their surface. The second subfraction contained mainly dense bodies of decreased density and transparent vesicles. The number of such vesicles showing the presence of granules on their surface was decreased. Several vesicles closely resembling smooth membranes of the endoplasmic reticulum were present. The third subfraction contained mostly smooth vesicles. A more detailed study of the electron-microscope findings will be described elsewhere.

The causes for such changes probably reside in the damage of the endoplasmic reticulum, which has been shown to occur very early after administration of carbon tetrachloride (Oberling & Rouiller, 1956; Bassi, 1960; Haba, 1960).

Table 4. *Percentage distribution of some enzymes among three 'lysosomal' subfractions at different times after treatment with carbon tetrachloride*

Subfraction 1 was collected by centrifuging between 33 000 and 50 000 g-min., subfraction 2 by centrifuging between 50 000 and 150 000 g-min., subfraction 3 between 150 000 and 360 000 g-min. Results are percentages of the activities recovered in the three subfractions. Times refer to the intervals after treatment with carbon tetrachloride.

Enzyme	Sub-fraction	Time (hr.) ... 0	2	6	12	24	48	72	96	144
Arylsulphatase A	1	49	—	50	47	51	50	47	51	51
	2	35	—	36	32	31	34	34	33	37
	3	16	—	14	21	18	16	19	16	12
Arylsulphatase B	1	46	—	53	51	49	55**	62*	57***	41
	2	38	—	34	32	31	34	22*	32	35
	3	16	—	13	17	20	11	16	11	14
β-Glucuronidase	1	39	43	45	47	51*	51*	49*	43	45
	2	34	34	34	32	33	35	33	36	34
	3	27	23	21	21	16	14	18	21	21
Acid phosphatase	1	42	58	40	47	48	47	40	30	40
	2	34	30	38	31	31	36	35	38	38
	3	24	12	22	22	21	17	25	23	22
Cathepsin	1	64	59	46*	63	60	50*	50***	46*	52*
	2	28	28	34	28	35	38*	29	35	34
	3	8	13*	20*	9	5	12	21*	19	14*
Ribonuclease	1	27	44*	44**	42	34	39	51*	45*	42**
	2	29	38	41	30	40	35	30	32	38
	3	44	18*	15*	28	26	26	19*	23*	20*
Uricase	1	54	—	71*	47	46	52	56	59	56
	2	34	—	16*	37	43	38	31	36	39
	3	12	—	13	16	11	10	13	5	5*

* $P < 0.01$.   ** $P < 0.02$.   *** $P < 0.05$.

DISCUSSION

The experiments described in this paper confirm that changes in the enzymes presumed to be located within 'lysosomes' occur in the fatty liver induced by carbon tetrachloride. Three types or degrees of changes were seen: (1) an increase in particulate activity; (2) an increase in the soluble activity; (3) an increase in the specific activity of some of the enzymes, mainly in the latest stages of poisoning.

In the liver homogenates the decrease in the total nitrogen content amounted to about 20 % at 24 hr. after the administration of carbon tetrachloride; nitrogen content returned to normal values at 72 hr. It seems that a real increase in the amount of the enzymes takes place in later stages of poisoning. This increase may be due to an increase in the number of the particles that contain the enzymes, but the possibility exists that it is due to an enrichment of particles in particular enzymes.

In our experiments with liver homogenates, the activity of uricase was decreased in later stages after the administration of carbon tetrachloride. As Table 5 shows, the decrease of uricase activity was not noted in the experiments with 'lysosome'-rich subfractions. It seems therefore possible that the decrease observed with the whole homogenates is due to the presence in these preparations of inhibiting substances.

The cell composition of the liver may change by an increase in the number of macrophages and in fact it is known that carbon tetrachloride poisoning produces a certain degree of necrosis of liver cells. Necrosis is often characterized by the migration of granulocytes and macrophages, which are known to contain a high amount of 'lysosomal' enzymes. In our experiments the number of granulocytes and macrophages present in histological specimens was not greatly altered even in the late stages after the administration of carbon tetrachloride. An increase in the number of phagocytes is unlikely therefore to account for the increases in specific activities of 'lysosomal' enzymes.

The existence of a high degree of heterogeneity in the behaviour of 'lysosomal' enzymes in different experimental conditions is well known. According to de Duve (1960), heterogeneity need not mean that single enzymes are contained in different types of particles. In fact, there may be within the same 'lysosomal' population particles having a different quantitative distribution of enzymes.

Experiments with carbon tetrachloride introduce, of course, new causes for heterogeneity. In

Table 5. *Specific activities of 'lysosome'-rich subfractions from rat liver at different times after treatment with carbon tetrachloride*

Results are given as $\mu\mu$moles of substrate destroyed/mg. of nitrogen/min. For cathepsin results are given as $\mu\mu$moles of tyrosine set free from haemoglobin/mg. of nitrogen/min. and for ribonuclease as $\mu\mu$moles of inorganic orthophosphate set free from ribonucleic acid/mg. of nitrogen/min. The number of experiments of each group is given in parentheses; s.D. is given after each average.

Time after treatment (hr.)	Arylsulphatase A	Arylsulphatase B	β-Glucuronidase	Acid phosphatase	Cathepsin	Ribonuclease	Uricase
0	433± 86 (10)	503±142 (10)	410± 98 (9)	1214±527 (17)	52±19 (10)	299±116 (10)	246± 87 (10)
2	—	—	375± 71 (7)	1318±290 (7)	47± 2 (6)	279±104 (7)	—
6	313± 61 (6)***	571±176 (6)	348± 52 (6)	845± 70 (10)	38± 5 (6)	234± 31 (5)	161± 31 (4)
12	325±126 (4)	665±145 (4)	435±105 (4)	1273± 79 (4)	43± 9 (4)	314± 70 (4)	279± 39 (4)
24	439± 96 (5)	502±206 (6)	268± 63 (5)**	1669±342 (6)	37±18 (4)	215± 61 (5)	140±105 (5)
48	380± 43 (4)	941±245 (5)*	322± 75 (5)	1397±375 (5)	89±48 (5)	404±200 (5)	218±112 (5)
72	616±162 (7)**	1157± 60 (4)*	435±109 (3)	1489±769 (4)	159±15 (3)*	397± 98 (3)	179± 78 (3)
96	688±194 (6)*	1289±246 (3)*	377±273 (3)	1138±564 (3)	168±29 (3)*	487±102 (3)***	223±107 (3)
144	572± 50 (5)*	616± 51 (5)	426±187 (6)	1031±208 (11)	86± 3 (6)*	290± 10 (6)	154± 91 (6)

* $P < 0.01$. ** $P < 0.02$. *** $P < 0.05$.

fact, it is possible that carbon tetrachloride itself has actions on single enzymic proteins, on specific inhibitors or stimulators of the enzymic activities. Enzymes such as acid phosphatase, ribonuclease, cathepsin and β-glucuronidase are affected by the treatment more than arylsulphatases. Uricase is very little affected. Arylsulphatases, cathepsin and ribonuclease increased greatly in specific activity in the later stages of poisoning, whereas other enzymes, such as acid phosphatase and β-glucuronidase, remain practically unmodified. A further example of heterogeneity of the enzymic activities of the 'lysosomes' is shown in the sub-fractionation experiments. A rather rough differential centrifuging, such as that we have used, was sufficient to show that the distribution of enzymes within the subfractions undergoes some modifications at different times after administration of carbon tetrachloride. As the morphological composition of subfractions was changed, a general modification of the distribution of enzymes could be explained by this fact. The results may have been complicated by a certain degree of readsorption of solubilized enzymes on the sediments, which has been shown to occur (Berthet, Berthet, Appelmans & de Duve, 1951). As the changes observed were different from one enzyme to another, it does not seem likely, however, that readsorption can entirely explain the changes in the patterns of distribution.

SUMMARY

1. The activities of six hydrolytic enzymes (arylsulphatases A and B, ribonuclease, acid phosphatase, β-glucoronidase, cathepsin) and of uricase were measured in liver homogenates from rats after treatment with carbon tetrachloride.

2. An increase in soluble activity was found to occur for all enzymes studied. The extent of this increase, as well as the time at which it appeared or disappeared, was different from one enzyme to another. The soluble activities of cathepsin and of ribonuclease increased only at 6 hr.

3. The specific activities of arylsulphatases, ribonuclease and cathepsin significantly increased in late stages of poisoning. The specific activity of β-glucuronidase and of acid phosphatase remained unchanged. That of uricase decreased in late stages.

4. 'Lysosome'-rich sediments were subfractionated by differential centrifuging. The distribution of enzymic activities among three subfractions was studied. Treatment with carbon tetrachloride produced significant changes in enzyme distribution, which were different from one enzyme to another. The specific activities of the enzymes were found to be increased in arylsulphatases, cathepsin and ribonuclease.

5. The causes for the observed results are discussed.

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The Phospholipids of Pigeon and Ox Skeletal Muscle

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As a preliminary to the ultimate object of determining the phospholipid composition of the subcellular particles of skeletal muscle, an investigation was undertaken of the total phospholipid of pigeon breast muscle. The phospholipids of skeletal muscle have not been studied to the same extent as the phospholipids of tissues such as liver and brain. Two recent papers report the detailed phospholipid composition of sheep skeletal muscle (Dawson, 1960) and of rabbit, pigeon and trout skeletal muscle (Gray & Macfarlane, 1961). In these investigations modern methods of phospholipid analysis were used. The results of the present investigation, which also includes a determination of the phospholipids of ox longissimus dorsi muscle, are in substantial agreement with the results of these two papers, except that the major ninhydrin-positive component in both pigeon breast muscle and ox longissimus dorsi muscle is not phosphatidylethanolamine, as was assumed, but another compound. The pigeon-breast-muscle lipid extracts contained considerable amounts of proteolipid which dissociated on silicic acid. Evidence is also presented for the possible occurrence of phenolic components in the polyglycerolphospholipid fraction.

EXPERIMENTAL

Extraction of lipid. Sample A. Three pigeons were beheaded and the breast muscles blended with chloroform–methanol (2:1, v/v) by using 20 ml. of solvent/g. of muscle. The blend was boiled for 10 min. and then kept at room temperature overnight. It was filtered through sintered glass, 0·9% sodium chloride (1 ml./5 ml. of filtrate) was

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