Tissue Fractionation Studies

11. INFLUENCE OF VARIOUS HEPATOTOXIC TREATMENTS ON THE STATE OF SOME BOUND ENZYMES IN RAT LIVER

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The studies described in the preceding paper (de Duve & Beaufay, 1959) having revealed, amongst other enzymic alterations, a rapid release of lysosomal enzymes in ischaemic liver, it appeared of interest to perform similar determinations on animals subjected to various other treatments reputed to cause liver damage. The results of these investigations are reported in this paper, together with similar data obtained on a few animals treated with hydrocortisone. The results of an extensive investigation of the same kind performed on animals fed with carcinogenic dyes have been published elsewhere (Deckers-Passau, Maisin & de Duve, 1957).

METHODS

The experiments were performed on rats of either sex, weighing from 100 to 150 g. The animals were divided into groups A–G.

(A) Controls, fed *ad lib.* a standard diet containing 45% of ground wheat, 40% of ground oats, 8% of fish meal, 3% of skimmed milk, 2% of cod-liver oil, 1% of dry yeast and 1% of salt.

(B) Animals fasted for 6 days but supplied with plenty of drinking water.

(C) Animals fasted as in group B, and then given access to food 8 hr. before being killed.

(D) Animals on diet A, treated for 5 days with a daily subcutaneous injection of 0·2 ml. of carbon tetrachloride in 0·8 ml. of olive oil, and killed on the following day.

(E) Animals receiving 8 g. daily of a necrogenic diet (containing 73% of ground maize, 18% of dry bakers' yeast, 5% of arachis oil, 1% of cod-liver oil and 3% of salt) and supplemented with a daily dose of 20 μg. of thiamine hydrochloride, 25 μg. of riboflavin, 20 μg. of pyridoxine and 100 μg. of calcium pantothenate (Glynn & Himsworth, 1944).

(F) One animal on diet A, killed 8 days after ligation of the main bile duct.

(G) Animals on diet A, treated for 3 days with a daily subcutaneous injection of 12·5 mg. of hydrocortisone and killed on the following day.

In addition, a more limited experiment was performed on two groups of animals subjected to a prolonged period of starvation (6–8 days) without preliminary treatment (H) or after intoxication with carbon tetrachloride as in group D (J).

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As a rule, three animals belonging to three different groups and one control were killed on the same day. Their livers were homogenized, centrifuged and analysed as described in the preceding paper (de Duve & Beaufay, 1959). Samples of liver were also fixed in Bouin's fluid for histological examination.

This design made it possible to assign to each group a set of comparable control animals and to take into account eventual variations due to seasonal effects or other uncontrolled factors. However, subsequent statistical analysis revealed no significant trend amongst the control animals and these were therefore treated as a single group, against which all others were compared.

RESULTS

In Table 1 are summarized the results of the analyses performed on the animals in groups A–G. Total enzymic activities are expressed in units/g. of liver nitrogen and can be related to the wet weight of the tissue or to the weight of the animal by means of the corresponding data on liver nitrogen. The unsedimentable activities of the lysosomal hydrolases, on the other hand, are given as percentages of the corresponding total activities, a mode of expression which provides a direct estimate of the state of the lysosomes in the preparations. To assist in the perusal of the table, all values differing significantly from the control values are indicated by suitable signs.

As shown by the headings of Table 1, not all animals resisted the treatment to which they were subjected. Some were found dead and were discarded; others were killed in a comatose or precomatose state. This fact turned out to be of great interest, since typical biochemical signs were found to be associated with coma. Consequently, the results obtained on the comatose and precomatose animals will be examined separately, together with those of groups H and J.

Results on non-comatose animals

Starvation (*groups B*1 *and C*1). Except for a certain degree of shrinking of the cells, there were no consistent histological changes in the livers of the starved animals. After refeeding, however, the cells appeared swollen and vacuolated in several

Table 1. *Summary of biochemical results*

All values are means±S.E.M. Injections (D and G) were given subcutaneously.

Group	A	B				C		
Treatment	None	Complete starvation				Complete starvation+subsequent meal 8 hr. before killing		
Days of treatment	—	6				6		
Subgroup	—	1	2	3	4	1	2	3
State of animals	Normal	Non-comatose	Pre-comatose	Comatose	Dead	Non-comatose	Pre-comatose	Dead
No. of animals	20	8	1	3	1	8	1	1
Liver								
Weight (g./100 g. of animal)	4·13±0·13	3·37±0·10***	3·04	3·04±0·39*	—	3·96±0·10†††	2·81	—
Nitrogen (mg./g. of liver)	32·0±0·50	32·5±1·5	23·1	32·0±2·7	—	29·2±1·0*	30·9	—
Nitrogen (mg./100 g. of animal)	130±4·2	107±7·4*	70·0	95·0±3·9***	—	113±4·7*	87	—
Total enzymic activities (units‡/g. of N)								
Cytochrome oxidase	616±26	541±46	—	240±72***	—	496±69	256	—
Glucose 6-phosphatase	627±33	719±27*	—	398±114	—	732±69	521	—
Acid phosphatase	189±6·0	199±9·0	386	339±17***	—	174±6·0 †	301	—
β-Glucuronidase§	18·5±1·6	20·2±1·5	34·9	38·9±4·3***	—	16·8±1·3	65·0	—
Acid ribonuclease	68·3±4·3	71·1±3·0	115·5	101±25	—	62·6±5·1	78·6	—
Acid deoxyribonuclease	20·0±1·5	21·9±2·6	58·9	40·8±7·5*	—	19·7±2·2	35·3	—
Cathepsin	38·0±1·3	46·5±2·6**	64·9	85·0±5·8***	—	34·6±2·5††	78·6	—
Unsedimentable activities (% of total)								
Acid phosphatase	7·18±0·18	10·9±0·71***	16·3	32·3±3·3***	—	8·80±0·60*	40·3	—
β-Glucuronidase	5·78±0·53	8·96±0·47***	25·0	46·0±3·8***	—	7·80±0·30**	27·3	—
Acid ribonuclease	9·74±0·69	15·5±1·6**	28·0	24·8±4·4**	—	13·8±0·55***	33·3	—
Acid deoxyribonuclease	2·82±0·23	3·80±1·3	24·0	17·2±2·6***	—	6·20±0·54***	11·0	—
Cathepsin	4·94±0·32	8·58±1·0**	16·5	19·3±1·3***	—	8·10±0·57***	15·4	—

Group	D	E					F	G
Treatment	0·2 ml. of CCl_4 injected daily	Deficient diet					Ligation of main bile duct	12·5 mg. of hydrocortisone injected daily
Days of treatment	5	53±7·2	33	46±6	68	64±7	8	3
Subgroup	—	1	2	3	4	5	—	—
State of animals	Non-comatose	Non-comatose	Comatose, gastr. perf.	Comatose	Comatose	Dead	Non-comatose	Non-comatose
No. of animals	9	7	1	3	1	7	1	4
Liver								
Weight (g./100 g. of animal)	6·36±0·41***	4·12±0·20	4·25	2·79±0·28***	3·93	—	3·67	6·15±0·25***
Nitrogen (mg./g. of liver)	24·2±0·84***	27·3±1·2**	29·5	27·6±0·70***	30·8	—	42·1	27·7±0·59***
Nitrogen (mg./100 g. of animal)	149±10·5	112±4·5**	125	77·0±7·3***	121	—	155	170±10**
Total enzymic activities (units‡/g. of N)								
Cytochrome oxidase	552±47	604±44	411	332±39***	584	—	501	369±14***
Glucose 6-phosphatase	227±26***	453±57*	390	641±28	438	—	262	660±49
Acid phosphatase	204±8·0	238±13**	321	345±28***	254	—	194	158±11*
β-Glucuronidase	17·9±2·5	14·9±1·4	23·9	17·4±3·3	17·6	—	14·9	14·4±1·4
Acid ribonuclease	84·2±9·7	74·7±5·0	136	141±38	54·9	—	100	52·9±5·3*
Acid deoxyribonuclease	26·1±3·7	38·2±2·5***	45·0	70·7±18**	25·3	—	56·2	17·9±1·3
Cathepsin	73·8±5·0***	59·8±6·6**	85·0	75·0±4·1***	54·2	—	67·1	30·9±0·54
Unsedimentable activities (% of total)								
Acid phosphatase	9·88±0·61***	9·12±0·63**	22·2	26·1±6·7*	9·70	—	9·84	7·43±0·50
β-Glucuronidase	14·9±1·6***	9·98±1·3**	29·5	41·4±1·3***	11·2	—	30·2	5·93±0·61
Acid ribonuclease	17·4±1·5***	17·5±2·5**	21·1	23·7±8·2	18·4	—	20·3	7·20±1·1*
Acid deoxyribonuclease	7·65±0·75***	7·65±0·75***	9·8	15·5±6·0	8·20	—	8·4	0·80±0·30***
Cathepsin	9·28±0·74***	9·17±1·2**	16·8	26·0±2·0***	4·90	—	13·8	3·78±0·58

* Difference from control values (A) significant at $P<0·05$.
** Difference from control values (A) significant at $P<0·01$.
*** Difference from control values (A) significant at $P<0·001$.
† Difference from B1 significant at $P<0·05$.
†† Difference from B1 significant at $P<0·01$.
††† Difference from B1 significant at $P<0·001$.
‡ Units are those defined by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955).
§ Uncorrected for inhibition by sucrose (approx. 28%).

though not all organs. The loss in liver weight and nitrogen was proportionately greater than the total loss of weight of the animal. Refeeding caused a considerable gain in liver weight and a smaller rise in liver nitrogen. The latter change is not significant when related to the weight of the animal, but is clearly shown by the decrease in the specific activity of most enzymes.

Cytochrome oxidase appeared to be somewhat more affected by starvation than the total proteins, whereas glucose 6-phosphatase was relatively spared. Lysosomal hydrolases tended to lag behind the average proteins, with the result that their specific activity increased slightly during starvation and decreased upon refeeding. These changes were statistically significant only for cathepsin. The unsedimentable activities of the acid hydrolases were significantly increased in both groups.

Carbon tetrachloride poisoning (*group D*). Despite the high doses of carbon tetrachloride administered, the animals appeared to remain in an excellent state of health. As shown by the results of Table 2, they were even more resistant to starvation than untreated animals. Their livers were considerably enlarged, owing mostly to the accumulation of fat, and showed extensive centrilobular fatty degeneration.

Most notable amongst the enzymic modifications observed in this group were the considerable dedecrease in glucose 6-phosphatase and increase in total cathepsin, as well as the moderate rise in the unsedimentable fraction of the lysosomal hydrolases.

Deficient diet (*group E* 1). The livers of the seven animals of the group which were killed in apparent health did not differ morphologically from those of the control group except in one case where a few zones of swollen or lysed cells were seen. The liver weight was normal, but its nitrogen content was low. This relative loss of protein was paralleled by cytochrome oxidase and exceeded by glucose 6-phosphatase and possibly also β-glucuronidase, though in the latter case the change was not significant. On the other hand, acid phosphatase and ribonuclease were essentially preserved, with a consequent moderate rise in specific activity, and cathepsin and especially acid deoxyribonuclease were distinctly increased. The unsedimentable activities of all lysosomal hydrolases were significantly augmented.

Bile-duct ligation (*group F*). This animal showed typical signs of obstructive jaundice, but tolerated this condition with no outward sign of ill-being. Histologically, the liver showed a remarkable multiplication of the bile canaliculi, which were also greatly distended. The biochemical state of the liver was characterized by a high nitrogen content, a very low glucose 6-phosphatase activity and high levels of ribonuclease, cathepsin and especially deoxyribonuclease. In terms of animal weight this last enzyme reached almost four times its normal level. The unsedimentable activities of lysosomal hydrolases were all distinctly elevated. Although such results obtained on a single animal furnish only little information, it should be pointed out that several of the observed differences exceed by far the standard deviation of the normal population and are probably significant.

Hydrocortisone treatment (*group G*). The livers of these animals were enlarged considerably and showed the typical pale colour associated with a high glycogen content. The cells were hypertrophied and appeared vacuolated, presumably by glycogen deposits. The latter could not, however, be revealed histochemically, owing to improper fixation. The nitrogen content was decreased, but not in proportion to the increase in liver weight, so that the total liver nitrogen showed a 30 % increase, when related to the animal weight. Glucose 6-phosphatase was the only enzyme to have participated in this hypertrophy, whereas lysosomal hydrolases appeared to exhibit a corresponding dilution, which, however, was significant only for acid phosphatase and ribonuclease. Cytochrome oxidase was lowered more than could be accounted for simply by dilution and showed a significant decrease even when related to animal weight. The unsedimentable activities of lysosomal hydrolases were either unchanged or lower than normal.

Results on comatose animals

Deaths, comatose and near-comatose states were recorded only amongst the starved animals and amongst those fed on a deficient diet. In the former group, one animal died after 4 days and another after re-feeding; three were killed on the sixth day of fasting, in a state of deep coma characterized by complete immobility, hypothermia and reduced cardiac and respiratory activity; another animal was already very weak when it was killed. Such was also the case for one of the re-fed animals. Histologically the livers of these comatose animals did not show any greater evidence of damage than the organs of the non-comatose animals in the same group.

The mortality was particularly high in group E. Out of nineteen animals subjected to the deficient diet, seven were discovered dead and five others were found in a comatose state at times between 33 and 87 days after the regimen was started. Both by the suddenness of onset and by the absence of premonitory symptoms, coma and death resembled closely the phenomena described by Glynn & Himsworth (1944). However, in no case were the macroscopic or microscopic signs of massive

hepatic necrosis reported by these authors observed. In several cases, coma or death occurred in animals which had been left without food for a short period of time (less than 24 hr.), and one of the main characteristics of the animals in this group may therefore have been an extreme lack of resistance to fasting. Amongst the five animals which were killed in a comatose state, one was found to have a perforated gastric ulcer, and one other showed none of the biochemical changes observed in all other comatose and precomatose animals. The results obtained on these two animals (E 2 and E 4) have been presented separately.

In order to find out whether liver damage might decrease the resistance to starvation (a hypothesis suggested by the observations on group E), a number of additional animals were deprived of food, either without pretreatment (group H) or after carbon tetrachloride poisoning (group J). The animals were watched at least every 4 hr. and killed when in a comatose state. For those which were found dead, the time of death was estimated with an error which could not have exceeded 1–2 hr. As shown by Table 2, this experiment revealed that the poisoned animals were actually more resistant to food deprivation than normal ones. This may possibly have been due to their large stores of hepatic fat, which were found to disappear almost entirely during fasting.

The livers of comatose animals in groups H and J were weighed and analysed for cytochrome oxidase, glucose 6-phosphatase, total and unsedimentable acid phosphatase. The results of these determinations, related to animal weight, are shown in Fig. 1, together with the similarly expressed results obtained on the comatose animals of groups B 3 (which was combined with group H) and E 3, as well as on comparable non-comatose animals. The biochemical picture of the liver of these fourteen comatose animals is strikingly similar: the liver weight was reduced to about two-thirds, cytochrome oxidase to one-third and glucose 6-phosphatase to approximately one-half of their normal values. These changes appear to be largely unaffected by the previous state of the animals. In particular, the animals poisoned with carbon tetrachloride and those on a deficient diet, which presumably already had low glucose 6-phosphatase activities before falling in coma, did not show an additional loss of this enzyme after becoming comatose. Acid phosphatase, on the other hand, was slightly elevated, presumably owing to relative conservation with respect to loss of total animal weight, and was transferred to the extent of about 35 % to the unsedimentable fraction of the homogenates (as compared with 7 % in normal controls).

As shown by Table 1, the behaviour of acid phosphatase is typical of all lysosomal hydrolases, with the exception of β-glucuronidase in group E, which, though similarly released, did not become relatively concentrated during coma. The results observed on the two precomatose animals (B 2 and

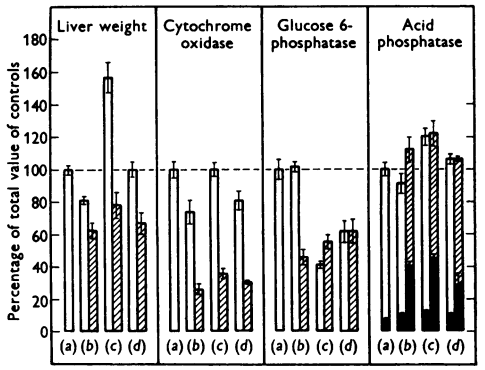

Fig. 1. Biochemical changes associated with coma. Blocks represent means ± S.E.M. All values are related to animal weight and then expressed as percentage of control values. Unfilled blocks, non-comatose animals; cross-hatched blocks, comatose animals; filled-in areas, unsedimentable activity. (*a*) Controls (twenty animals); (*b*) starved animals (eight non-comatose and seven comatose); (*c*) animals poisoned with carbon tetrachloride (eight non-comatose and four comatose as result of subsequent starvation); (*d*) animals on a deficient diet (seven non-comatose and three comatose).

Table 2. *Influence of carbon tetrachloride poisoning on survival during starvation*

Group	H		J	
Treatment	Complete starvation		CCl_4 poisoning as in D, followed by complete starvation	
State of animals	Comatose	Dead	Comatose	Dead
No. of animals	4	4	4	2
Days between beginning of starvation and death or killing in comatose state	6·25 ± 0·15		7·0 ± 0·18	
Difference	0·75 ± 0·23 ($P < 0·01$)			

C2) and on the comatose animal with gastric perforation (E2) also follow the same general pattern. However, animal E4, though killed in what appeared to be a state of deep coma, was biochemically indistinguishable from the non-comatose animals of the same group (E1).

DISCUSSION

The most striking observations concern the comatose and precomatose animals, of which seventeen out of eighteen exhibited the same general enzymic pattern, which was closely reminiscent in its most important aspects of that found by de Duve & Beaufay (1959) after 5–6 hr. of complete ischaemia. As in the latter case, cytochrome oxidase and glucose 6-phosphatase are reduced to respectively one-third and one-half their normal value, whereas all five acid hydrolases are present in unsedimentable form to an extent corresponding to a considerable disruption of the lysosomes. In contrast with the ischaemic livers, the organs of the comatose animals lose weight and nitrogen, presumably by circulatory removal of the products of autolysis, and do not seem to suffer any inactivation of cathepsin and of the nucleases. Consequently, they exhibit a relative concentration of the lysosomal hydrolases, a phenomenon which could not be observed in the ischaemic livers. In this respect, as in their apparent release from the particles with which they are normally associated, the lysosomal enzymes thus show a type of behaviour entirely compatible with their assumed role as agents of the autolytic processes.

In two cases (B2 and C2), the biochemical symptoms preceded those of coma. From this it may be deduced that the severe hepatic lesions revealed by the enzymic assays may take place at a time when the general state of the animal is still relatively unaffected, and could in fact in such cases be responsible for the subsequent onset of coma. Whether this actually did happen in some of the comatose animals cannot be decided, but the case of E4 does at least show that it did not happen in all, since in this animal coma was completely established without appreciable biochemical alterations of the liver.

A slight but significant increase in the amount of lysosomal enzymes released was also observed in all non-comatose animals except in those treated with hydrocortisone. This phenomenon, which corresponds to the situation obtaining after a short period of complete ischaemia, has also been observed in mild post-operative shock (de Duve & Beaufay, 1959) and during treatment with carcinogenic dyes (Deckers-Passau *et al.* 1957). It may therefore be taken as a feature common to all situations where some degree of injury, either associated with localized necrosis or likely to develop into necrosis, is imposed on the tissue. On the other hand, treatment with hydrocortisone, which has been applied successfully in some cases of hepatic injury, appeared to decrease the release of lysosomal enzymes.

As pointed out before (de Duve & Beaufay, 1959), we do not know the exact nature of the intracellular changes responsible for the observed alterations of the lysosomes, but the pathological context with which they are generally associated makes it likely that they correspond to an actual release of hydrolytic activity within the cells. Another problem of interest is that of the mechanism whereby the lysosomes become damaged under the various circumstances studied here. According to Himsworth (1947), obstruction of the intrahepatic circulation may play an important role in the production of necrosis under the influence of toxic or dietetic factors and it is therefore possible that the mechanism of lysosomal damage operative in ischaemic liver may also be involved in some of the present experiments. Other factors could be at work also, for instance a direct injury of the lysosomal membrane by carbon tetrachloride in the experiments with this poison, and by the accumulated bile salts in obstructive jaundice. It is certainly suggestive that glucose 6-phosphatase, which like lysosomes is very sensitive both to carbon tetrachloride and to bile salts (Beaufay & de Duve, 1954), is considerably lowered in both these cases.

An interpretation of the other enzymic changes observed cannot be attempted at the present time. Attention should, however, be drawn to the frequency with which liver tissue exposed to unfavourable conditions responds by an increased content of cathepsin and of acid nucleases. This has been noticed after feeding with carcinogenic dyes or a low-protein diet (Deckers-Passau *et al.* 1957), as well as in several of the present experiments. It should also be noted that the total activities of the various lysosomal enzymes vary in what appears to be a completely independent manner, but that their unsedimentable activities show essentially parallel changes as has also been found to be the case *in vitro*.

It should finally be noted that there was little correlation between the histological and the biochemical findings. Especially where dietary influences were studied (groups B, C and E), few manifest alterations were observed and these were definitely not more pronounced in the livers of comatose animals, despite the dramatic biochemical changes which accompanied the onset of coma. This lack of visible necrosis is perhaps not as surprising as may appear at first sight. As was pointed out above, the enzymic pattern observed

in the comatose animals was similar to that found after 5–6 hr. of complete ischaemia, and this is a state where according to Berenbom, Chang, Betz & Stowell (1955) microscopic alterations are still very slight. There is little reason therefore to question the validity of the biochemical findings, and the conclusion must rather be drawn that enzymic disorganization precedes by probably as much as several hours the appearance of the typical structural features of necrosis. This explanation could also account for our failure to observe, on a limited number of animals, the characteristic signs of dietary necrosis described by Glynn & Himsworth (1944) and by other authors. It is, of course, possible, in view of the observations of Gillman, Gilbert, Gillman & Spence (1952), that the yeast included in the diet may not have been of sufficiently low nutritional value to be necrogenic. However, the high mortality and incidence of coma observed in group E rather suggests that a severe dietary lesion was indeed inflicted but was followed by death too rapidly to express itself morphologically.

Some of the enzymes studied in this work have been previously investigated by other authors under similar conditions, especially after prolonged fasting. The most extensive survey has been made by Allard, de Lamirande & Cantero (1957), who have followed the fate of eleven enzymes during starvation. As in the present work, they have observed a small increase in the specific activity of acid phosphatase and acid ribonuclease, but contrary to the findings of Miller (1948) and to our own they have noted a decrease in the specific activity of cathepsin. Our results on glucose 6-phosphatase confirm those obtained by Weber & Cantero (1954, 1958), Langdon & Weakley (1955) and Ashmore, Hastings, Nesbett & Renold (1956) on fasted animals, and by Weber, Allard, de Lamirande & Cantero (1955) and Ashmore *et al.* (1956) on animals treated with cortical hormones. The only paper relating to the state of the lysosomes is that of Dianzani (1954), who has described what amounts to a complete release of acid phosphatase in hepatic homogenates from animals treated with carbon tetrachloride. In our experiments, this release was much less marked and became essentially complete only in the animals which had been rendered comatose by a subsequent period of prolonged fasting.

SUMMARY

1. A number of properties of the liver have been studied in: (A) normal rats; (B) animals starved for 6 days; (C) animals starved for 6 days and then fed for 8 hr.; (D) animals poisoned with carbon tetrachloride; (E) animals fed on a deficient diet; (F) one animal with ligated bile duct ; (G) animals treated with hydrocortisone; (H) animals rendered comatose by starvation; (J) animals rendered comatose by starvation after carbon tetrachloride poisoning.

2. Microscopic appearance, weight, nitrogen content, cytochrome oxidase and glucose 6-phosphatase activity, total and unsedimentable activities of five lysosomal hydrolases (acid phosphatase, β-glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease) were investigated.

3. The most striking biochemical changes occurred in the animals which were killed in a comatose or precomatose state. Out of eighteen such animals, belonging to groups B, C, E, H and J, seventeen showed the same typical picture characterized by a 30 % greater loss of liver weight and nitrogen than of body weight, an even greater fall of cytochrome oxidase (70 %) and glucose 6-phosphatase (50 %), a conservation with corresponding concentration of the lysosomal hydrolases, accompanied by a considerable transfer of these enzymes from the particulate to the soluble phase of the homogenates.

4. In all non-comatose animals, except those treated with hydrocortisone (group G), there was a significant increase in the proportion of lysosomal hydrolases found in unsedimentable form. Several other significant changes were also found to be associated with the various treatments applied.

5. Few morphological findings of interest were made. In particular, there was no obvious correlation between microscopic appearance and enzymic pattern, even in the livers of comatose animals, which did not show more pronounced alterations than the organs of the non-comatose animals in the same groups.

6. The biochemical results are interpreted as further confirmation of the hypothesis that the opening of lysosomes and consequent release of their internal enzymes plays an important role in the initiation of autolytic and necrotic phenomena, and the lack of correlation between biochemical and morphological findings is taken as an indication that enzymic alterations may precede by as much as several hours the appearance of well-defined microscopic lesions.

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Tissue Fractionation Studies

12. INTRACELLULAR DISTRIBUTION OF SOME DEHYDROGENASES, ALKALINE DEOXYRIBONUCLEASE AND IRON IN RAT-LIVER TISSUE

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In a previous publication from this Laboratory, a new scheme of fractionation of rat-liver homogenates permitting a more detailed analysis of the large granules has been described (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955). In the present investigations, this scheme has been applied to a study of the intracellular distribution of a few enzymes which were either known or suspected to be associated with the large granules: the diphosphopyridine nucleotide-linked dehydrogenases of glutamic acid, malic acid and β-hydroxybutyric acid and alkaline deoxyribonuclease (deoxyribonuclease I). In addition, we have studied the distribution of iron to verify the possibility, suggested by electron-microscope observations, that particles rich in ferritin might be concentrated in the same rat-liver fraction as the lysosomes (Novikoff, Beaufay & de Duve, 1956). In all these experiments, cytochrome oxidase, acid phosphatase and glucose 6-phosphatase were measured simultaneously to serve as reference enzymes. Measurements were also made of acid deoxyribonuclease (deoxyribonuclease II), which has been studied before (de Duve *et al.* 1955) but was assayed here by an improved technique. Some of the results obtained have been reported previously in preliminary communications (de Duve & Beaufay, 1957; Baudhuin, 1959).

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METHODS

The tissue fractionations and determinations of reference enzymes were performed according to the methods described by Appelmans, Wattiaux & de Duve (1955), de Duve *et al.* (1955) and Wattiaux & de Duve (1956), except that cytochrome oxidase was assayed at 25° instead of at room temperature.

Glutamic dehydrogenase was measured by the following modifications of the methods described by Hogeboom & Schneider (1953). For the forward reaction [diphosphopyridine nucleotide (DPN) reduction] the final reaction mixture contained 0·02 M-potassium phosphate buffer, pH 7·7, 0·03 M-nicotinamide, 0·4 mM-sodium cyanide, 0·1% of Triton X-100, mM-cysteine, 13 mM-potassium glutamate, 1·4 mM-DPN and enzyme in a total volume of 3 ml. The reaction was started by the addition of enzyme and the increase in extinction at 340 mμ and 25° was followed in a Beckman model DU spectrophotometer with a reference cell containing all components of the system except glutamate. Triton X-100 was used to release the enzyme; it also causes a reversible inhibition (about 28%) which was ignored in these experiments since the conditions were the same for all fractions. Cysteine could be replaced by mM-ethylenediaminetetra-acetic acid (EDTA).

For the back reaction the reaction mixture contained 0·02 M-potassium phosphate buffer, pH 7·7, 0·03 M-nicotinamide, 0·4 mM-sodium cyanide, 0·1% of Triton X-100, mM-EDTA, 0·01 M-sodium α-oxoglutarate, 0·05 M-ammonium chloride, 0·28 mM-reduced DPN (DPNH) and enzyme in a total volume of 1·8 ml. In order to avoid an initial lag-phase in the reaction, it was found necessary to pre-incubate the enzyme with all components of the reaction mixture except α-oxoglutarate at 25° for 15 min. The reaction was then started by the addition of 0·18 ml. of