Chemical and Enzymatic Changes Associated with Mouse Liver Necrosis in Vitro *

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Necrosis and cell death occur frequently in various pathological conditions, including neoplasia; many tumors are composed of mixtures of neoplastic and non-neoplastic cells, necrotic cells, stromal, inflammatory, and blood cells. These cells, depending on metabolic factors and nutrition, are in quite different phases of activity ranging from the rapid growth of the neoplastic and regenerative states to the degeneration characteristic of the necrotic state. In such mixtures of cells it is not feasible by present technics to characterize the metabolic activity of each type of cell.

The development of neoplasia and death may be regarded as progressive states which at some stages may be reversible; the exact stage of demarcation in the progression of a normal cell to the "irreversible" state of neoplasia or death cannot be defined at present to the satisfaction of everyone. Considering the universal and inevitable occurrence of cell death, it would seem to merit more study. The present study has been undertaken to obtain precise information about the chemical changes which accompany the death of mouse liver cells, and is a continuation of previous studies, in this laboratory, of liver changes under conditions of degeneration, necrosis, regeneration, and hepatoma formation following the administration of carbon tetrachloride to the mouse (22, 23, 25, 26); regeneration following partial hepatectomy in the mouse and rat (27, 28); degeneration, regeneration, and hepatoma formation following the administration of p-dimethylaminoazobenzene to rats (21); and dietary protein depletion and repletion in the rat (30). The data presented here will be compared with the findings concerned with the degenerative and necrotic phases of the above-mentioned studies.

As an initial, simple approach to this problem, pieces of mouse liver were incubated in closed, sterile containers at 37° C. in an atmosphere saturated with water vapor. Various chemical and enzymatic components were determined at intervals and related to their initial content in the liver. The authors are aware that such tissues may be considered by some investigators to be undergoing autolysis rather than necrosis, but the distinction between these terms is not precise. Autolysis and necrosis usually occur when a tissue is isolated from its normal supply of nutrient or its metabolism is interrupted by toxic physical and chemical agents. Autolysis generally refers to that phase of the process of the death of tissues and cells in which the enzymes of the tissue itself play a role in breakdown. At some phase of the process of cellular death and necrosis, autolytic changes often occur. Because of their close interrelationship, it is considered relatively unimportant whether one designates the changes these tissues are undergoing as autolysis or necrosis. The authors believe these terms designate relatively poorly understood processes and desire to obtain precise information regarding their chemical and structural changes under reasonably carefully controlled conditions in mouse liver.

The chemical changes which occur during the development of focal necrosis have been studied on a limited scale and have been concerned chiefly with liver necrosis induced by dietary means (1,
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8); studies of changes occurring in liver, designated as autolytic, have been more extensive (3). These latter investigations have been varied as to types of tissue examined and their state (2, 3, 10, 11, 18, 19). Bradley and co-workers have determined pH changes in autolysing liver brei (9), the effect of the pH of incubating brei on the rate of disappearance of proteins (2, 6), and the action of added proteolytic enzymes on the proteins of liver (2, 3).

Luck, Eudin, and Nimmo (12) have carried out similar investigations on the type of protein lysed in liver homogenates at different pH. Potter, LePage, and Klug (15) have studied changes in the oxidation of oxalacetate and coupled phosphorylation during the incubation of various rat tissues, including liver; homogenates were used and also whole tissues incubated in situ after decapitation of the animal.

Chemical changes in focal liver necrosis induced by low protein diets have been reported by Himsworth and Glyn (8) who studied changes in water content, protein, nonprotein nitrogen, and glycogen, and by Abell and Beveridge (1) who studied various liver constituents during the preneurotic period and when the livers were massively necrotic. Analyses were carried out for water, glycogen, lipid, fatty acids, total and free cholesterol, and phospholipids. Interpretation of these results is made difficult by the fact that some of the changes were based on body weight, while others were based on wet liver weight or fat-free liver weight. Of further difficulty in interpretation is the problem of determining the relative contribution of necrotic, normal, and regenerating liver cells to the total changes observed.

A somewhat different approach is that of Sperry, Brand, and Copenhagen (19), who followed changes in dry weight, cholesterol, and phospholipid in slices and pieces of rat liver incubated in the presence and absence of buffer. Changes were determined for 24 hours on an absolute basis in a manner similar to that in the present investigation, which is a more extensive study than that of Sperry, Brand, and Copenhagen and is supplementary to it. The changes in mouse liver during autolysis or necrosis in vitro will be described concern dry weight, lipid, total nitrogen and phosphorus, nitrogen and phosphorus distribution, pentose and deoxyxypentosenucleic acid, free amino acids, acid and alkaline phosphatase, succinic dehydrogenase and cytochrome oxidase, esterase, and leucylglycine peptidase.

**MATERIALS AND METHODS**

Each experiment was carried out with ten to fourteen livers obtained aseptically from normal, male strain A mice, 10–14 weeks old. The mice, which were fasted 18 hours preceding sacrifice, were killed by decapitation, and the liver was removed with sterile instruments. Each liver was divided into fourteen approximately equal pieces, and two pieces were placed into each of seven sterile, glass-stoppered weighing bottles (50 mm. diameter); fourteen approximately equivalent samples were thus obtained, seven for chemical and enzymatic analysis and seven for histological and histochemical study. The pieces of liver were placed on glass rods which rested on filter paper saturated with sterile saline, which provided an atmosphere saturated with water vapor while the tissue remained isolated. In addition, the water-saturated atmosphere prevented adhesion of the pieces of liver to the glass and facilitated their subsequent removal without mechanical injury. The initial weight of each of the seven samples for chemical and enzymatic analysis was determined, and one was analyzed immediately as a control. This was done by homogenizing with ice-cold 0.85 per cent sodium chloride to a tissue dilution of 1:10 and analyzing appropriate aliquots for the components mentioned above. The six remaining samples were incubated at 37° C. for 1, 6, 24, 50, 48, and 72 hours, respectively, after which they were removed, homogenized to a tissue dilution of 1:10 on the basis of their initial weight, and analyzed as above. This permitted calculation of absolute changes in the tissue undergoing necrosis and obviated the necessity of choosing some constituent such as dry or wet weight or some other component as a relative base-line. All samples were checked for bacterial contamination, and data from those samples which were later shown to be infected were discarded.

Since this study concerned the changes in the tissue undergoing necrosis, only the intact pieces of liver were analyzed. In the earlier stages of incubation these represented all the material on the glass rods, while in the longer incubation experiments a small amount of material was found to have leaked out of the tissue following the onset of cellular disintegration. Since this material was no longer a part of the necrotic tissue, it was not added to the intact pieces of liver.

Aliquots of homogenized liver were assayed for acid and alkaline phosphatase (84), esterase (14), succinic dehydrogenase and cytochrome oxidase (17), and L-leucylglycine peptidase. The latter was determined in a manner similar to that described by Schwartz and Engel (18) for mouse serum peptidase. For normal mouse liver the assay was carried out at 37° C. with equal volumes of homogenate (1/400) and 0.01 M L-leucylglycine in 0.02 M veronal buffer of pH 8.0, containing 0.001 M MnSO₄. Appropriate tissue and substrate blanks were run. After an incubation period of 0.5 hour, 20 ml. of 5 per cent trichloroacetic acid was added to the digest, which was shaken and filtered. Then 0.1- or 0.5-mL aliquots of the filtrate were analyzed by the ninhydrin procedure of Moore and Stein (13), and the amount of leucylglycine hydrolyzed was calculated.

An aliquot of each homogenate was fractionated into acid-soluble, lipid, nucleic acid, and protein components successively, according to the method of Schneider (16), and each fraction was analyzed for nitrogen (microl-Kjeldahl) or phosphorus (7) or both. Lipid was determined on an aliquot of the second fraction of Schneider's procedure (16). The aliquot was evaporated to dryness, extracted with petroleum ether (80°–100°) which was subsequently removed and the residue weighed. This method was found to give somewhat higher and more consistent results than the successive treatment of a dried aliquot of homogenate with ethanol, ethanol-diethyl ether, and petroleum ether. Free amino acids were determined by the ninhydrin procedure of Moore and Stein (13) on the filtrate of an aliquot of homogenate after treatment with 5 per cent trichloroacetic acid. The nucleic acid fraction was ana-

¹ To be published.
lyzed for total nucleic acid by ultraviolet spectrophotometric measurement at 260 µm and differentially analyzed for pentose-nucleic acid (PNA) by the method of Brown (4) and for deoxy-pentosenucleic acid (DNA) by the method of Dische (5). Moisture or dry weight was determined after heating an aliquot of homogenate to constant weight at 105°C.

RESULTS

Cytological alterations.—The detailed histological and histochemical studies carried out concurrently with the chemical and enzymatic analyses are to be published elsewhere, but some of the pertinent data are given below. The tissues were stained with hematoxylin and eosin after freezing-drying or fixing in Stieve fluid. After 1 hour's incubation the pieces of liver showed little morphological change. The nuclei and cytoplasm stained almost as well as the control tissue and were essentially intact.

Chemical changes.—The changes which occurred in the various constituents analyzed are recorded in Table 1. The most dramatic changes occurred in the content of PNA and DNA, which respectively decreased to a constant value of 6–7 and 12 per cent of their original content in 48 hours. This rapid reduction in precipitable nucleic acid of both types reflects high activity of their respective nucleases in the dying tissue, and the rapid disappearance of DNA agrees with the histological observations of the tissue (1), which show that disintegration of the nuclear membrane and nuclear material is not evident until 24 hours and is essentially complete in 48 hours.

Dry weight, total nitrogen, and lipid were reduced at a slower rate than protein nitrogen or total phosphorus. Acid-soluble nitrogen and free amino acids increased, largely at the expense of disintegrating protein, being retained by the cells for a time, but decreased again when the cells began to disintegrate completely. The same explanation is applicable to the rise and decline of acid-soluble phosphorus, which increased largely as a result of disintegrating nucleic acid. Here, too, the chemical changes coincide with the morphological alterations of the livers which showed cellular disintegration to be almost complete in 48 hours, as was the over-all histological picture of necrosis. The changes in dry weight and lipid found here are similar to those described for dry weight, phospholipid, and cholesterol by Sperry, Brand, and Copenhaver (19).

Enzyme alterations.—Of the six enzymes studied, all except alkaline phosphatase and esterase showed a fairly regular pattern of decline (Table 2). These latter two enzymes exhibited a slight rise in activity at 1 hour and thereafter showed a pattern of decline similar to that of the four other enzymes studied. The explanation of this is not evident, although one might assume the

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td><strong>CHEMICAL ALTERATIONS ASSOCIATED WITH MOUSE LIVER NECROSIS IN VITRO</strong></td>
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</table>

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<thead>
<tr>
<th>Analysis</th>
<th>0 hr.</th>
<th>1 hr.</th>
<th>6 hrs.</th>
<th>24 hrs.</th>
<th>48 hrs.</th>
<th>72 hrs.</th>
</tr>
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<tbody>
<tr>
<td>Dry weight</td>
<td>324 ± 88</td>
<td>92 ± 4</td>
<td>100 ± 5</td>
<td>86 ± 5</td>
<td>88 ± 4</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>Lipid</td>
<td>88 ± 18</td>
<td>98 ± 9</td>
<td>89 ± 3</td>
<td>81 ± 6</td>
<td>80 ± 5</td>
<td>58 ± 2</td>
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<tr>
<td>Total nitrogen</td>
<td>56.5 ± 0.5</td>
<td>99 ± 2</td>
<td>96 ± 2</td>
<td>94 ± 1</td>
<td>98 ± 2</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Acid-soluble nitrogen</td>
<td>2.4 ± 0.1</td>
<td>103 ± 0.9</td>
<td>150 ± 2</td>
<td>270 ± 6</td>
<td>298 ± 10</td>
<td>274 ± 6</td>
</tr>
<tr>
<td>Protein nitrogen</td>
<td>24.0 ± 1.0</td>
<td>96 ± 4</td>
<td>92 ± 3</td>
<td>70 ± 2</td>
<td>69 ± 1</td>
<td>46 ± 3</td>
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<tr>
<td>Free amino acids</td>
<td>9.2 ± 0.8</td>
<td>110 ± 8</td>
<td>16 ± 6</td>
<td>580 ± 48</td>
<td>598 ± 14</td>
<td>604 ± 40</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>3.7 ± 0.2</td>
<td>99 ± 4</td>
<td>97 ± 1</td>
<td>85 ± 5</td>
<td>79 ± 5</td>
<td>62 ± 12</td>
</tr>
<tr>
<td>Acid-soluble phosphorus</td>
<td>0.26 ± 0.02</td>
<td>104 ± 7</td>
<td>147 ± 7</td>
<td>205 ± 15</td>
<td>190 ± 21</td>
<td>134 ± 10</td>
</tr>
<tr>
<td>Protein phosphorus</td>
<td>0.20 ± 0.02</td>
<td>105 ± 7</td>
<td>79 ± 6</td>
<td>64 ± 8</td>
<td>59 ± 10</td>
<td>40 ± 9</td>
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<tr>
<td>PNA</td>
<td>9.2 ± 0.4</td>
<td>99 ± 4</td>
<td>76 ± 3</td>
<td>72 ± 2</td>
<td>71 ± 2</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>DNA</td>
<td>5.3 ± 0.3</td>
<td>95 ± 4</td>
<td>94 ± 1</td>
<td>50 ± 5</td>
<td>50 ± 5</td>
<td>12 ± 6</td>
</tr>
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* Mg/gm wet weight. Each value in the succeeding column, expressed as per cent of the control value, is the mean ± standard deviation from the mean for at least four separate experiments.

After 6 hours' incubation some hepatic nuclei were pyknotic, and occasional nuclear membranes showed evidence of disintegrating. Some of the endothelial and Kupffer cells were hyperchromatic. The cytoplasm was still homogeneous and stained well although somewhat paler than the control tissue.

Marked changes occurred by 24 hours. Necrosis appeared to start in the central area of the tissue, where karyolysis and karyorrhexis were prominent. Hyperchromatic hepatic cell nuclei were found throughout the tissue; endothelial and Kupffer cells also showed a fair proportion of hyperchromatic nuclei. Most of the hepatic cells, however, contained reduced amounts of chromatin, and their nuclear membranes were indistinct; nuclear fragments were visible in the sinusoids. Nevertheless, most of the cells still stained with hematoxylin and eosin. By 48–72 hours the pieces of liver tissue became completely necrotic in appearance, with the cells either isolated or loosely attached, and with only traces of nuclear material being visible.
release of physiologically inhibited enzyme, since it is unlikely that synthesis of enzyme protein would occur under the experimental conditions used here. Alkaline phosphatase appears to be the most stable enzyme during the onset of necrosis, followed by esterase and leucylglycine peptidase.

DISCUSSION
A comparison of the changes observed here with those found in mouse liver during the degenerative phases following the feeding of carbon tetrachloride (22, 23, 25, 26) is complicated by the fact that in the latter studies there was considerable overlap between the processes of initial necrosis and subsequent regeneration which was cytologically apparent in 24 hours (26). Nevertheless, some similarities were noted. With a single dose of carbon tetrachloride there was a loss of DNA and PNA for 24 and 48 hours, respectively (26), and these losses were associated with necrosis, which appeared to encompass about 40 percent of the liver in 48 hours. Similar reductions of PNA and DNA were observed in the present in vitro study, although the decrease was much greater for both types of nucleic acid.

The changes in total nitrogen, acid-soluble nitrogen, and phosphorus found in the present study are different from those obtained for the same constituents during the degenerative stages following the administration of carbon tetrachloride (22, 26). In the latter studies these constituents all decreased during the first 24 hours, while in the present study acid-soluble nitrogen and phosphorus increased greatly, while total nitrogen decreased slightly. The differences, however, are more apparent than real and are explainable by the greater ease of diffusion of the acid-soluble degradation products of nucleic acid and protein out of the dying cells in vivo, as compared with the isolated tissue in the present investigation.

The extremely rapid decrease in total cytochrome oxidase and succinoxidase activities, which are essential in the energy-providing systems of the cells, probably initiates the other changes which contribute to the death of the cell. The decreased rate of removal of protein after 48 hours reflects decreased activity of proteolytic enzymes, and this is indicated to some extent by the fairly rapid disappearance of L-leucylglycine peptidase.

This may provide some explanation why necrotic tissue in vivo is often not completely dissolved, since without proteolytic activity soluble products of protein degradation will not be formed. Further studies are in progress on the death of tissue in vivo, and preliminary data indicate the changes are similar to those observed in the above in vitro study.

SUMMARY
Chemical and enzymatic changes have been studied in mouse liver undergoing necrosis or autolysis in vitro by incubating pieces of liver under controlled conditions of temperature, humidity, and sterility. Succinoxidase and cytochrome oxidase activity disappeared in 24 hours, while loss of activity of acid and alkaline phosphatase, esterase, and peptidase continued over a period of 72 hours. Free amino acids, acid-soluble nitrogen, and acid-soluble phosphorus increased up to 48 hours and then declined. Protein nitrogen, PNA, and DNA decreased steadily up to 48 hours and then remained relatively constant. PNA and DNA fell to much lower levels than the other constituents studied; the lipid content changed the least.

REFERENCES
5. DISCHE, Z. Some New Characteristic Color Tests for Thymonucleic Acid and a Microchemical Method for
Determining the Same in Animal Organs by Means of These Tests. Mikrochemie, 8:4–8, 1930.