STUDIES ON NECROSIS OF MOUSE LIVER *IN VITRO*

ALTERATIONS IN SOME HISTOCHEMICALLY DEMONSTRABLE HEPATOCELLULAR ENZYMES

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This report represents part of a correlative morphologic, histochemical and biochemical study of the cytologic changes associated with the cellular death and necrosis of the mouse liver *in vitro* [1-8] and *in vivo*.[9,10] The present series [1,2,5-8] has emphasized earlier time intervals, ultrastructural studies and different histochemical and biochemical methods. The alterations in some histochemically demonstrable enzymes, chosen because of their known association with certain organelles or subcellular systems,[11-21] and the correlation of these changes with the biochemical and electron microscopic observations are reported here.

Terms such as "necrosis" and "autolysis" are subject to widely different interpretation, and could be the subject of extensive semantic discussion. Our present lack of precise knowledge of the processes of cellular death and disintegration makes such discussion relatively unproductive and we prefer to avoid it at this time. It can be assumed, however, that under the conditions of the present experiments the cells "die" at some time between the onset of incubation and 24 hours, the longest time interval studied. Other studies indicate that mammalian liver cells can survive 30 to 60 minutes of total ischemia at 37° C.[22,23]

The changes that are presumed to occur prior to the irreversible loss of essential vital functions are referred to here by the phrase "the process of cellular death"; those that occur from this point to dissolution and debris, are termed "necrosis."[24]

MATERIAL AND METHODS

Experimental System

Male white Swiss mice, 10 to 14 weeks of age, fed *ad libitum* on a normal laboratory diet were used in all experiments. Food was removed from the cages in the after-

noon preceding the experiment and the animals were allowed only water for a period of 18 hours prior to death by cervical dislocation at approximately the same hour each morning. The livers were quickly removed under sterile conditions and cut into slices 1 to 2 mm thick on sterile dental wax plates. The slices were placed in large sterile glass weighing bottles with tightly fitting glass stoppers. In the weighing bottles the tissues rested on glass rods beneath which was a filter paper moistened with sterile isotonic saline. The rods prevented the tissue from coming into direct contact with the filter paper. The slices were oriented so that a capsular surface rested on the glass rods. The weighing bottle was then closed and transferred to an incubator at 37° C. Tissue slices were removed for study after 30 minutes, and 1, 2, 4, 8, 12 and 24 hours and fixed or quenched in the same manner as controls. Liver slices from at least 6 animals were examined at each interval.

In the subsequent discussion, tissues fixed or quenched immediately will be referred to as "control tissues" while those incubated at 37° C *in vitro* will be termed "experimental tissues." When the word "control" is used alone, it will refer to procedures used to ascertain whether the histochemical reaction is taking place. The word "incubation" will be used to indicate the length of time the sections were allowed to react in the specific histochemical procedures.

For determinations requiring fresh tissue, slices of control and experimental tissues were trimmed at the ends to make rectangular slices measuring approximately 10 × 4 × 1 mm. These slices were placed on aluminum foil and plunged into isopentane cooled to −155° C by liquid nitrogen. The frozen tissues were then quickly transferred to the cryostat where they were allowed to warm to −17° C for at least 15 minutes prior to sectioning.

For histochemical procedures employing fixed tissue, fixation was allowed to proceed for 6 hours at 0 to 4° C in 4 per cent formaldehyde buffered to pH 7.4 with 0.067 M phosphate buffer containing 7.5 per cent sucrose.[25] The tissue was then washed overnight in 1 M sucrose-gum acacia solution [25] at 0 to 4° C. After washing, fixed tissues were trimmed, quenched, and transferred to the cryostat in the same manner as fresh tissue.

For the demonstration of acid phosphatase, additional slices were fixed at 0 to 4° C for 4 hours in 6.25 per cent glutaraldehyde buffered to pH 7.4 with 0.067M phosphate or 0.08M cacodylate buffer.[26] They were then washed overnight in chilled buffer and frozen with circulating liquid freon on the stage of a sliding microtome. For general morphologic observations, control and experimental tissues were fixed in phosphate-buffered formaldehyde (pH 7.4), Carnoy's or Stieve's fixatives and embedded in paraffin.

Microtomy and Mounting. All sections except those for demonstration of acid phosphatase and paraffin-embedded material were cut at 6 to 8 μ in the Pearse cryostat (Bright's Refrigerator Service Ltd., 399 Portobello Road, London W. 10, England) equipped with a Cambridge rocking microtome. The tissue blocks were oriented on the cryostat chuck so that the sections were cut at right angles to the surface that had rested on the glass rods. Since the outer zone, one to two cell layers thick (15 to 20 μ), reacts differently from the remainder of the block, perhaps due to diffusion of oxygen, the first few sections were always discarded until it was certain that a sufficient depth had been reached to avoid this zone. The sections were picked up directly from the microtome knife on clean, warm cover slips. Sections were always cut within 4 hours after quenching and stored in the cryostat only briefly before incubation in the reaction mixtures.

For the acid phosphatase determination, frozen tissues were prepared on a sliding microtome at 8 to 10 μ and free-floated in the various solutions. The sections were picked up from the surface of the last solution on glass slides and dried in air.

Histochemical Reactions. With the exception of the acid phosphatase as noted, the cover slip-mounted sections were immersed in the reaction mixtures in Columbia

staining dishes and placed in an incubator at 37° C. Buffer solutions were checked and prepared at frequent intervals. Lead nitrate solutions were never kept for more than 2 days, as results were found to be variable if they had been stored. At the completion of the enzyme procedures, the cover slips were mounted in glycerogel or polyvinyl pyrrolidone. Some of the acid phosphatase preparations were given a light nuclear counterstain with purified methyl green. Reagent grade chemicals and buffers were obtained from the Fisher Scientific Co., sodium β-glycerophosphate adenosine triphosphate, adenosine-5-phosphate, DPN (NAD), and glucose-6-phosphate (Sigma Chemical Co., St. Louis, Mo.) and tetrazolium salts (Nutritional Biochemicals Corp., Cleveland, Ohio), (Dajac Laboratory, Division of Borden Chemical Co., Philadelphia, Pa.), and (Sigma Chemical Co.).

The histochemical methods were adapted from standard procedures,[27-34] the only modification being that the volumes and concentrations were adjusted to a total reaction mixture of 5 ml. Control tissues were incubated in the reaction mixtures with experimental tissues, except at the longer time intervals. All reaction mixtures were made up immediately prior to use, and warmed at 37° C in the incubator prior to immersing tissue sections in them.

The following histochemical methods were used:

1. Adenosine triphosphatase (ATPase) (method of Wachstein and Meisel[27]): Formalin fixed tissues were incubated in the reaction mixture for 15 minutes. Histochemical controls consisted of the reaction mixture without adenosine triphosphate.

2. 5′ Nucleotidase (method of Wachstein[28]): Formalin fixed tissues were incubated for 45 minutes; histochemical controls were immersed in the reaction mixture without substrate.

3. Glucose-6-phosphatase (method of Chiquoine[29] as modified by Wachstein and Meisel[30]): Cryostat sections of fresh tissue were incubated for 10 minutes at 37° C; controls were incubated in the reaction mixture without substrate.

4. Succinic dehydrogenase: A wide variety of reaction mixtures with and without phenazine methosulphate, were tried, based primarily on methods of Nachlas and associates[31] using nitro-BT (NBT) and of Pearse[32] using MTT-cobalt tetrazoliums as electron receptors. Also, the method of Walker and Seligman[35] using small blocks of tissue fixed in graded concentrations of formalin, with tetra-nitro BT (TNBT)[36,37] as acceptor, was used. The method utilized primarily was that of Pearse,[33] using cryostat sections of fresh tissue, and NBT as acceptor without phenazine methosulphate. Sections were incubated for 30 minutes at 37° C, and histochemical controls consisted of the reaction mixture without substrate, and of the reaction mixture without substrate, but with added cysteine (0.5 ml of a 0.05 M solution) to produce non-enzymatic reduction of the tetrazolium to the formazan. Sections were postfixed in formalin and mounted in glycerogel.

5. Glutamic dehydrogenase: The method of Pearse[33] using NBT as acceptor was finally used after attempting variations similar to those used in the succinic dehydrogenase reaction. Sections of fresh tissue were incubated for 15 minutes and postfixed. Controls were similar to those used in the demonstration of succinic dehydrogenase.

6. Acid phosphatase: A more complete description of this method, as applied both to light and electron microscopy has been published elsewhere.[38] Essentially, Miller's[39] modification of the Gomori technique[34] was used, employing sections of glutaraldehyde- or formalin-fixed tissue cut on the freezing microtome and free-floated in the reaction mixture. Sections were incubated for from 5 to 20 minutes, mounted, and given a light nuclear counterstain with purified methyl green. Cover slips were mounted with polyvinyl pyrrolidone. Control sections were incubated in the reaction mixture without substrate. In addition, sections were incubated in the complete reaction mixture with sodium fluoride added to inhibit the enzymatic reaction.[33]

RESULTS

A summary of the results is presented in Table I. The earliest changes were seen in the ATPase preparations, and these were of a sequential nature. Definite alterations in the reaction for the other enzymes tested did occur, but at a later time interval. The changes will be discussed under the respective enzyme.

TABLE I

HISTOCHEMICAL ALTERATIONS DURING IN VITRO NECROSIS OF MOUSE LIVER

ENZYME	CONTROL	30 MINUTES	1 HOUR	2 HOURS	4 HOURS	8 HOURS	12 HOURS	24 HOURS
Adenosine Triphosphatase	Strong in bile canaliculi, sinusoids, and vessels.	Granular appearance of bile canaliculi; sinusoids, vessels unchanged.	Beaded reaction in canaliculus some spreading of sinusoidal reaction to adjacent cytoplasm.	No reaction in canaliculi, some decrease, some spreading of sinusoidal reaction; vessels and bile ducts, unchanged.	Focal reaction in sinusoids, none in bile ducts. Vessels still react as does bile duct.	Slight vessel reaction; non-specific deposition in cytoplasm.	--	--
5' Nucleotidase	Strong reaction sinusoids and vessels; inconstant reaction in bile canaliculi.	No change.	No convincing change.	Slight spread of sinusoidal reaction, vessels unchanged.	Reduction in all activity; slight in Kupffer cells and vessels.	Non-specific deposition in cytoplasm.	--	--
Glucose-6-phosphatase	Diffuse cytoplasmic reaction, stronger around portal zones.	No change.	No change.	No change.	No change.	Slightly diminished in central areas.	Markedly diminished in central area, moderate reaction in portal zone.	Non-specific deposition in cytoplasm.
Succinic dehydrogenase	Diffuse cytoplasmic granules suggesting mitochondria; stronger around portal zones.	No change.	Reaction more intense in all zones.	Reaction more intense in all zones.	Reaction more intense in all zones.	Reaction still more intense than controls in portal zones diminished slightly in central areas.	Active in portal zone, diminished in central areas.	Negative
Glutamic dehydrogenase	Diffuse cytoplasmic granules; reaction stronger around portal zones, occasional large deposits of formazan.	No change.	Large deposits of formazan not seen. General increase in intensity of reaction.	Increased intensity of reaction in all zones.	Reaction more intense than controls in all zones.	Reaction remains more intense than controls.	General diminution in reaction.	Negative
Acid Phosphatase	Focal deposits most numerous around bile canaliculi. Strong in Kupffer cells.	--	No change.	--	No change.	Negative	--	--

General Morphologic Features

The tissue fixed in formalin and embedded in paraffin was stained by a variety of techniques. These will be dealt with in other reports of our

findings, but for orientation a brief description of the changes seen in hematoxylin and eosin stained tissues is included here.

The histologic characteristics of the normal mouse liver have been adequately described previously, and they do not differ significantly from those of other mammalian species (Fig. 1).

Significant changes were not discernible in the earlier time periods. After 4 hours incubation at 37° C (Fig. 2) there were only subtle changes, consisting of slight widening of sinusoidal spaces, rounding of the nuclei of sinusoidal lining cells, and some separation of lining cells from parenchymal cells. The nuclei of hepatic cells appeared unaltered except for a tendency toward a coarsening of the chromatin aggregations. After 12 hours many of the hepatic cells had lost their polygonal outline, tending to be round or oval, and their cytoplasm was more homogeneous. Sinusoidal lining cell nuclei were still evident. Some hepatic cell nuclei had lost their granular character, tending to be homogeneously basophilic with dispersed chromatin while in some cells nuclear definition was lost and the chromatin was dispersed in the cytoplasm. Nucleoli were difficult to identify (Fig. 3). After 24 hours *in vitro* the hepatic cells were strikingly changed (Fig. 4). Cellular outlines were distinctly round or oval with definite densely eosinophilic plasma membranes. The sinusoids were widened, containing a moderate amount of eosinophilic amorphous debris resembling cytoplasmic fragments. Sinusoidal lining cell nuclei were virtually absent. The nuclei of parenchymal cells were discernible only as relatively clear round or slightly oval areas surrounded by a delicate faintly basophilic nuclear membrane on which there were minute clumps of basophilic chromatin. The cytoplasm was rather densely eosinophilic and coarsely granular. Bile canaliculi could not be identified with certainty.

Adenosine Triphosphatase (ATPase)

Control. In sections of tissue fixed immediately (Fig. 5) the lead sulphide deposits might be seen to stain bile canaliculi, bile ducts, and the endothelium and medial coat of arteries and veins. The sinusoidal margins and Kupffer cells of the mouse liver also exhibited strong activity unlike those of the rat.[27] Controls incubated without substrate were brown, but there was no specific localization of deposits.

Experimental. At one-half hour, the reaction became granular in many of the finer bile radicles, though it was not altered along sinusoidal margins or in vessels (Fig. 6). At one hour, this granularity or clumping of reaction product in bile canaliculi was more marked, and gave them a "beaded" appearance, since there were segments of the canaliculi that did not react between the dense deposits. At this time too, sinusoidal mar-

gins appeared less sharply defined, and there was some spreading of re-
action product into the adjacent cytoplasm. Vessels and bile ducts were
little changed (Fig. 7). At 2 hours, the bile canaliculi did not react, with
the exception of an occasional small deposit, and some sinusoidal margins
showed diminished staining while others showed spreading to adjacent
cytoplasm, whereas large blood vessels and bile ducts still exhibited a
fairly strong reaction (Fig. 8). After 4 hours there was a slight granular
deposition of reaction product in hepatic cell cytoplasm; vessels still
stained, but sinusoids only stained focally, and bile canaliculi were not
stained. (At 4 hours there were some nuclear deposits, but these were
inconstant.) (Fig. 9). After 8 hours there was only a weak reaction, con-
fined to the endothelium of the larger arteries and veins, but there was
a rather marked nonspecific deposition of lead sulphide similar to that
of control sections incubated without substrate (Fig. 10).

5' Nucleotidase (AMPase)

Control. In tissues fixed immediately the lead sulphide reaction prod-
uct stained sinusoids and the endothelium of arteries and veins. The re-
action along bile canaliculi was inconstant in the mouse as well as the
rat,[27] but occasional deposits in the area of bile canaliculi, particularly
in portal zones, could be seen. Since this was variable, however, it was
difficult to draw conclusions about this part of the reaction in the experi-
mental system. Controls incubated without substrate showed diffuse
brown deposits without specific localization.

Experimental. No appreciable change was evident in sections of tissue
incubated for one-half hour prior to fixation, nor was it possible to be
certain of any alteration in this enzyme reaction at 1 hour. After 2 hours,
the reaction product had spread, somewhat into the adjacent cytoplasm
of hepatic cells along their sinusoidal margins, but the endothelium of
blood vessels appeared unchanged. After 4 hours, the reaction along
sinusoids was greatly diminished and only occasional Kupffer cells con-
tained deposits of reaction product. There was reduction in activity of
the endothelium of both arteries and veins at this time. At 8 hours there
was a diffuse, nonspecific deposition of lead reaction product, similar to
that of sections incubated in the reaction mixture without substrate.

Glucose-6-Phosphatase

Control. Liver quenched immediately after sacrifice of the animal gave
a strong reaction in the cytoplasm of hepatic parenchymal cells. The
reaction product was deposited as a finely granular precipitate through-
out the cytoplasm of all parenchymal cells, but this was much heavier
in portal zones as has been found in the rat (Fig. 11).[29,30] Control sec-

tions incubated in the reaction mixture without substrate had a light brown tinge after development in ammonium sulphide, but there was no difference between central areas and portal zones, and definite cytoplasmic granules were not seen.

Experimental. No difference in reaction from the control tissues could be seen in tissues incubated at 37° C up to 4 hours prior to the histochemical determination (Figs. 12 to 14). At 8 hours there was a slight reduction in activity in central areas (Fig. 15) and at 12 hours the reaction in the cytoplasm of cells in the centrilobular area was negative, but a narrow zone surrounding the portal areas was still moderately reactive (Fig. 16). At 24 hours, however, the reaction was negligible, and sections incubated in the complete reaction mixture were indistinguishable from the histochemical control sections incubated in the reaction mixture without substrate.

Succinic Dehydrogenase

Control. Sections quenched immediately and incubated in the substrate containing NBT as electron acceptor showed bluish granular deposits approximating the size of mitochondria in the cytoplasm of hepatic parenchymal cells, as well as in the epithelium of bile ducts and the endothelium of larger vessels. The reaction was stronger in portal areas. In addition the cytoplasm contained a diffuse reddish background stain, and there were occasional accumulations of bluish-black formazan near lipid droplets. Nuclei stood out as clear zones. (Fig. 17). Histochemical controls showed little or no deposition of formazan even after prolonged incubation. In sections incubated in the reaction mixture without substrate, but with 0.5 cc of 0.05 M cysteine added to produce non-enzymatic reduction of the tetrazolium, the deposition might be slightly increased, but there was no increased deposition around portal zones, and the deposits were very sparse with no regular distribution.

Experimental. There was no obvious change in the reaction at 30 minutes, but at 1 hour, the intensity of the reaction was uniformly increased, although portal zones continued to give a greater reaction than central areas (Fig. 18). At 2 and 4 hours (Fig. 19) this increased reaction was still apparent, but at 8 hours (Fig. 20) although the portal zones were still more reactive than those of the control tissue, there was a diminution in the reaction in central areas. This greater differential between central and portal areas was also apparent at 12 hours (Fig. 21). At 24 hours the reaction was negative (Fig. 22) except in a narrow zone 1 to 2 cells in width immediately beneath the capsular surfaces of some of the tissue blocks. This zone of reaction was inconstant, being absent in half of the specimens tested, but was apparently due to enzymatic ac-

tivity, since sections incubated in the reaction mixture with added cysteine did not show this zone.

In tissue fixed by the method of Walker and Seligman [35] and incubated in the reaction mixture containing TNBT as electron acceptor, deposits closely resembling mitochondria in distribution and structure were seen. At 4 hours these deposits appeared enlarged, perhaps indicating mitochondrial swelling. At 8 hours there were still discrete deposits, but the definite outline of mitochondrion-like deposits was lost, the borders being less distinct.

Glutamic Dehydrogenase

Control. In fresh sections incubated in the reaction mixture with NBT as acceptor (Fig. 23) the sections appeared similar to those of succinic dehydrogenase. In these mice the portal zone showed greater activity of this enzyme than did central areas. Jones has observed that the differential distribution of enzyme activity might vary with the age of the mouse.[40] The dark blue deposits were discrete and similar to those described in the succinic dehydrogenase reaction. In addition to the discrete "mitochondrial" deposits, there were large accumulations of formazan in what could represent lipid droplets. These were more numerous in the more heavily stained areas of the portal zone. The formazan of NBT is said to be sparingly soluble in lipid [33] and that of TNBT is allegedly less so [36] but these large accumulations appeared in control tissues when either tetrazolium was used. In the first 24 hours of fasting, mice increase their liver lipid two-fold to five-fold, and though it is diffusely deposited throughout the lobule, it may be heavier in portal zones. It was not possible to be certain that these large accumulations were always associated with lipid, although Sudan black B stains on parallel sections showed some sudanophilic material in these areas. In our hands, attempts to extract the lipid with cold acetone prior to the histochemical reaction, as done by Hitzeman,[42] so reduced the enzyme activity that it was not possible to be certain that the accumulations had been removed. These accumulations were not as marked in sections from the same blocks incubated in the succinic dehydrogenase reaction mixture. They did not stain excessively when these sections were incubated in the reaction mixture with added cysteine. Their exact nature, therefore, remained undetermined.

Experimental. No change was evident at 30 minutes, but at 1 hour the intensity of the reaction was uniformly increased, as was that for succinic dehydrogenase, and the portal zones were still more intensely stained (Fig. 24). The large formazan deposits seen in control sections, were not present at 1 hour. At 2 hours the reaction remained more intense than that of control tissue, and this increased activity persisted at 4 and 8

hours (Figs. 25 and 26). At 12 hours, however, the reaction was greatly diminished, though portal zones still gave a stronger reaction (Fig. 27). At 24 hours no activity was demonstrated (Fig. 28).

Acid Phosphatase

Control. In fixed control tissues acid phosphatase activity in hepatic cells was indicated by small discrete deposits of lead sulphide reaction product which were more numerous along the canalicular border of hepatic parenchymal cells (Fig. 29). The cytoplasm of Kupffer cells contained closely packed brownish-black deposits. In histochemical controls incubated in the reaction mixture without substrate and in the complete reaction mixture with sodium fluoride added to inhibit the reaction, no deposits were present.

Experimental. The size, number and arrangement of the discrete droplets indicating enzyme activity were unchanged after 1 (Fig. 30) and 4 hours (Fig. 31). At 8 hours no activity was seen (Fig. 32).

DISCUSSION

The enzymes selected were chosen on the basis of their known intracellular distribution, or histochemical localization in the liver, to demonstrate a variety of cellular structures. A previous histochemical study of the same experimental system by Chang, Stowell, Betz and Berenbom [4] demonstrated alterations in non-specific alkaline phosphatase activity at 1 hour in acetone fixed or frozen-dried tissue, as well as an apparent increase in activity of acid phosphatase after 24 hours' incubation *in vitro*. They noted little change in non-specific esterase activity at 1 or 6 hours, and only slightly decreased reaction for this enzyme after 24 hours.

Our findings with the alkaline phosphatases, ATPase and 5' nucleotidase, confirmed the above authors' findings with non-specific alkaline phosphatase, but suggested that alterations might begin to be apparent at one-half hour, a time interval which they did not investigate. The alternation of dense accumulations of reaction product and regions of little or no activity along bile canaliculi at one-half and 1 hour might reflect the complex reorganizations in microvilli in the canaliculi seen with the electron microscope.[1] Although the electron microscope shows marked alterations in the sinusoidal margin, consisting of bleb formation and extrusion of cytoplasmic contents,[1] dimunition and change in the pattern of the ATPase and 5' nucleotidase activity in this area was more subtle and could not be definitely seen until 2 hours. These findings might reflect the difference in reaction of the plasma membrane at these poles of the cell.

In studying the effect of a necrogenic diet, thioacetamide, and 3' methyl-4-dimethyl-amino-azo benzene on rat liver, Wachstein, Meisel and Falcon[43] noted a reduction in staining of bile canaliculi with the ATPase and 5' nucleotidase reactions as an early manifestation (5 hours) of liver cell damage. In their material (rat), sinusoidal margins showed only weak activity of ATPase in controls, whereas in the mouse we observed fairly strong activity. They also noted diminution in activity of acid phosphatase at 5 hours, but no significant change in DPN diaphorase, succinic dehydrogenase, or mitochondrial ATPase was seen during the first 8 hours of their experiments.[43] Membranous blebbing (potocytosis) has been observed to occur in living cells as a consequence of injury.[44]

The finding of only very gradual disappearance of the reaction for the demonstration of glucose-6-phosphatase is somewhat difficult to correlate with the biochemical[2] and electron microscopic findings.[8] Biochemical determinations of glucose-6-phosphatase activity in tissues undergoing autolysis revealed decreases in activity of 8, 23 and 45 per cent after 1, 2 and 4 hours respectively. Only a negligible amount of phosphatase activity was measurable at 8 and 24 hours.[2] In a similar study, Van Lancker and Holtzer[45] noted a 20 per cent diminution in glucose-6-phosphatase activity after 2 hours of autolysis. Glucose-6-phosphatase activity is associated biochemically with the microsomal fraction,[19] and this fraction has been found to consist predominantly of smooth- and rough-surfaced endoplasmic reticulum.[20] The glucose-6-phosphatase activity is said to be associated predominantly with the rough-surfaced reticulum.[17] In our system the endoplasmic reticulum became dilated, and at 1 hour there were numerous vesicular images instead of the usual tubular system seen in controls. Until 4 hours, however, some of these were still studded with ribonucleoprotein particles. At 4 hours, smooth-surfaced reticulum was not recognizable, and there was apparent degranulation of some cisternae of rough-surfaced reticulum. Further dilatation and fragmentation took place, and at 8 hours wide areas of the hepatic cell cytoplasm were devoid of recognizable reticulum. Up to 4 hours, however, some tubular images of rough-surfaced endoplasmic reticulum have been seen to persist.[8] In spite of these rather marked changes, no appreciable change in the intensity or distribution of the reaction was seen up to 8 hours, and only the central zones were reduced in activity at 12 hours. Control sections incubated in the reaction mixture without substrate showed no reaction in the ATPase, 5'-nucleotidase and acid phosphatase, whereas in the glucose-6-phosphatase reaction mixture without substrate, slight diffuse precipitates of lead were seen. These were also present in somewhat increased amount in histochemical controls of experimental tissues incubated in the reaction mixture without

substrate at each time interval. This might reflect a release of inorganic phosphate in the fresh-frozen sections, whereas in the fixed tissue it was washed out in the fixative. Acid-soluble phosphate has been shown to be released during cell disintegration due to ischemia.[45]

Taft, in an examination of rat liver undergoing autolysis at 25° C found slight decreases in ATPase and acid phosphatase activity at 5 and 6 hours, but there seemed to be an increase in alkaline phosphatase, 5' nucleotidase, and glucose-6-phosphatase activity after 6 hours, as assayed by quantitative microchemical techniques.[46] He did not see any morphologic alterations up to 6 hours in histologic preparations. It is difficult to compare these data with our own because of the difference in incubation temperature (25° C to 37° C).

The two oxidative enzymes were selected because one (succinic dehydrogenase) is known to be associated with the mitochondrial membrane,[12,18] while the other is evidently localized in the mitochondrial matrix[21] or is loosely attached to the membranes. Biochemically, these 2 enzymes reacted somewhat differently in our system. Succinic dehydrogenase showed little or no diminution in activity at 4 hours, and, compared to controls, still had over 60 per cent of its activity after 8 hours of incubation. Glutamic dehydrogenase activity, however, declined markedly, and after only 4 hours of incubation the total activity was reduced to 40 per cent of that of controls.[5,7] In view of this, one might expect that succinic dehydrogenase activity would still be demonstrable histochemically after 8 hours, but it is interesting that glutamic dehydrogenase activity as well should apparently be so little altered. Sections incubated in the reaction mixture without substrate were always negative, even after prolonged incubation, and it thus appeared that deposition of the formazan was actually due to enzymatic reduction. It is curious too, that the deposits remained rather discrete when the electron microscope showed marked alterations in the structure of the mitochondria.[5]

In a histochemical study of autolysis of dog liver, kidney and heart at 37° and 4°C, Kent found appreciable reduction in liver succinic dehydrogenase reaction between 6 and 12 hours in liver maintained at 37° C[47]; activity disappeared from this organ by 18 hours, while some was still present in the heart and kidney. At 4° C, the histochemical reaction was little affected at these times. He also found histologic changes consisting of eosinophilia and hyalinization of the cytoplasm of liver cells at 4 to 6 hours and decreased basophilia of the nuclei beginning at 6 hours. Glycogen disappeared at 4 hours, while alkaline phosphatase activity was unchanged after 15 hours at 37° C. He did find microscopic bacterial contamination in his specimens at 6 hours which was not present in our material.

Several authors[48-50] have found little or no diminution in succinic de-

hydrogenase activity until a few hours after the onset of ischemia in human and experimental myocardial infarction. Kent and Diseker [48] could not demonstrate changes in succinic dehydrogenase activity until more than 4 hours after ligation of the coronary arteries of dogs. Wachstein and Meisel,[49] however, were able to show definite diminution in activity in sites of infarction in human necropsy material. It is interesting that in their series, activity was demonstrable in the tissues of individuals who had been dead for up to 14 hours prior to necropsy. Nachlas and Shnitka [50] as well as Wachstein's group,[49] have used this reaction for gross identification of early myocardial infarction.

Discrete droplets of acid phosphatase activity were readily apparent at 4 hours, and their size, number, and distribution were similar to those of controls. Biochemical assay has shown a decrease in total activity of this enzyme and an increase in unsedimentable activity. Chang, Stowell, Betz and Berenbom [4] found an increase in acid phosphatase activity at 24 hours in acetone-fixed but not in frozen-dried material. It is probable that our finding of disappearance of activity by 8 hours is a reflection of the difference of the technique. Electron microscopic histochemistry [7] has confirmed these light microscopic observations and extended them to show that the sites of activity are the single membrane-limited bodies termed cytosomes.[38] These have previously been shown to contain acid phosphatase activity in the rat liver.[13] Furthermore, there is no localized increase in cytoplasmic dissolution surrounding the bodies containing acid phosphatase. Therefore, if these are the morphologic equivalent of De Duve's lysosomes,[11] our findings do not support the concept that release of acid hydrolases initiates the changes seen in this system. The lack of increased cytoplasmic damage surrounding these bodies further suggests that the release of enzymes found biochemically [2,45] is not reflected in the morphologic appearance of the cells. This may suggest that there is an increased fragility of these bodies, which leads to their rupture during preparation for the biochemical analysis, rather than to actual intracellular release initiating the process of cellular disintegration or occurring during this process.

Summary

Some of the histochemical changes associated with cell death and necrosis were investigated in a simple *in vitro* system utilizing slices of mouse liver incubated at 37° C in a sterile, moist environment. These findings were correlated with electron microscopic and biochemical studies on the same system.

Of the enzymes examined, ATPase showed the earliest alteration consisting of fragmentation and granular deposition of reaction product along bile canaliculi at one-half hour and virtual disappearance of stain-

ing along bile canaliculi by 2 hours. These findings correlate with the previous demonstration by Chang, Stowell, Betz and Berenbom[4] of similar changes in non-specific alkaline phosphatase activity in the same system, and with electron microscopic observations of early changes in the plasma membrane of both the sinusoidal and canalicular margins of hepatic parenchymal cells.

5′ Nucleotidase (AMPase) showed changes in sinusoids and endothelium of blood vessels, similar in time and extent to those of ATPase. Its activity was greatly diminished in sinusoids by 4 hours. Activity was virtually absent after 8 hours of incubation *in vitro* at 37° C.

The glucose-6-phosphatase activity declined slowly, but was little changed by 8 hours. By 12 hours there was some diminution in the reaction of the centrilobular region. These findings were difficult to correlate with rather marked alterations in the endoplasmic reticulum seen with the electron microscope; and rapid diminution in enzyme activity found by biochemical assay.

The oxidative enzymes, succinic dehydrogenase and glutamic dehydrogenase were apparently little altered in histochemical activity after 8 hours and there was demonstrable activity of both by 12 hours, despite marked alterations in mitochondrial morphology seen by electron microscopy, and progressive loss of biochemical activity.

Discrete droplets of acid phosphatase activity were demonstrable in hepatic parenchymal cells up to 4 hours after sacrifice of the animal. This observation, coupled with the electron microscopic histochemical finding of reaction product in cytosomes by 4 hours and the lack of increased cytoplasmic destruction surrounding these bodies, suggested that in this system the "lysosomes" do not initiate the changes of cellular disintegration and that early release of acid phosphatase shown biochemically may reflect increased fragility of these structures and rupture during biochemical processing rather than actual intracellular release.

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LEGENDS FOR FIGURES

FIGS. 1–4.   Morphologic changes in mouse liver after 4 (Fig. 2), 12 (Fig. 3), and 24 (Fig. 4) hours necrosis *in vitro* as compared with normal control (Fig. 1). Formalin fixation, hematoxylin and eosin stain. × 530. (AFIP Neg. 64-4665-1)

FIG. 1.   Control. Hepatic cords are made up of polygonal cells with granular cytoplasm and round nuclei having a distinct nuclear membrane and somewhat clumped chromatin. Sinusoids (S) contain an occasional erythrocyte (E) and nuclei of sinusoidal lining cells (SL) are scattered at angulations of sinusoids. Occasionally a bile canaliculus (BC) may be seen.

FIG. 2.   Four hours. The sinusoids (S) appear somewhat dilated but the hepatic cells are similar to the controls in appearance. There is rounding of nuclei of sinusoidal lining cells (SL).

FIG. 3.   Twelve hours. Most nuclei have a dense homogeneous appearance. Occasional nuclei appear to have lost definition and chromatin is dispersed in the cytoplasm (arrows). There is some dilatation of sinusoids (S), but nuclei of sinusoidal lining cells (SL) are still present. The cytoplasm of hepatic cells is coarsely granular.

FIG. 4.   Twenty-four hours. Nuclei are barely discernible (N). Hepatic parenchymal cells appear rounded, sinusoids (S) are dilated containing eosinophilic debris, and sinusoidal lining cells are not seen.
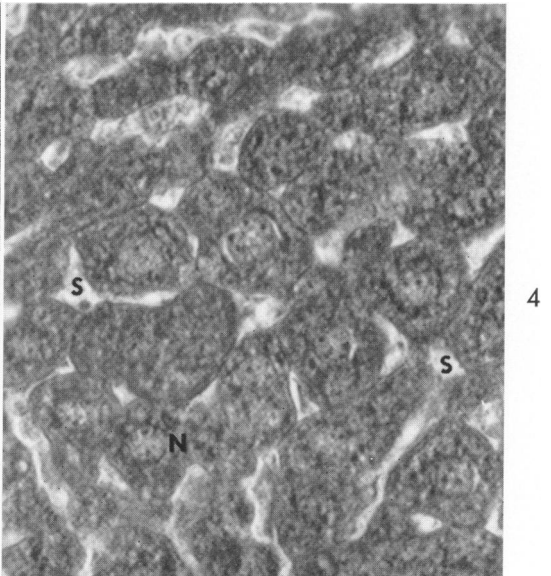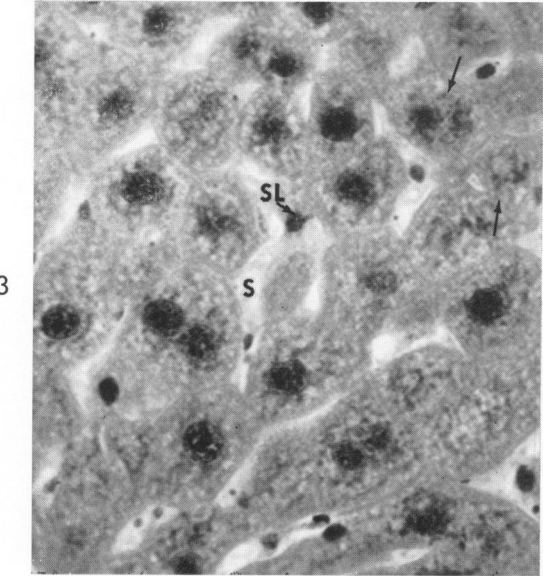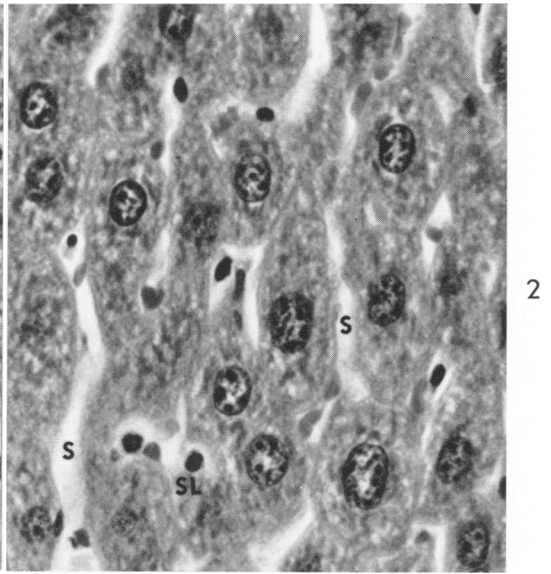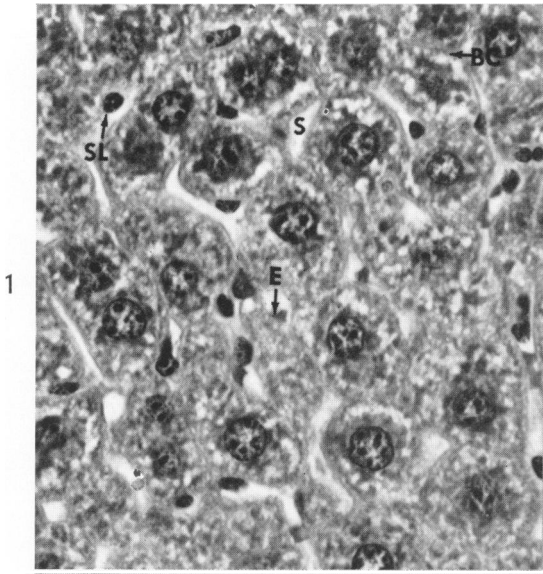
FIGS. 5–10.   Adenosine triphosphatase (ATPase) reaction in mouse liver after incubation *in vitro* at intervals up to 8 hours. × 700. (AFIP Neg. 64-4665-2)

FIG. 5.   Control. Dense deposits of reaction product (lead sulphide) are present along sinusoids (S) and in the bile duct (B), artery (A) and vein (V) of a portal area. Bile canaliculi have a straight tubular appearance (BC).

FIG. 6.   Thirty minutes. The sinusoids (S), bile duct (B), artery (A) and vein (V) appear similar to controls. Delicate bile canaliculi (BC) are still present, but some have a granular appearance (unlabeled arrows) due to uneven deposition of reaction product.

FIG. 7.   One hour. In some areas, reaction along sinusoids (S) spreads into adjacent hepatic cell cytoplasm. Many bile canaliculi (BC) are now represented by rather large focal accumulations of reaction product, which frequently give a beaded appearance (arrows).

FIG. 8.   Two hours. The reaction in some sinusoids (S) is diminished, but it is still strong in the portal vein (V). Bile canaliculi are not seen here.

FIG. 9.   Four hours. Only a few sinusoids (S) continue to react, but fairly strong reaction is still present in bile duct (B) and portal vein (V). Occasional nuclei (N) show granular deposits thought to represent nonspecific reaction.

FIG. 10.   Eight hours. Very slight reaction is still present in portal vein (V) but granular deposits in hepatic cell cytoplasm represent nonspecific deposition of lead reaction product (unlabeled arrows).
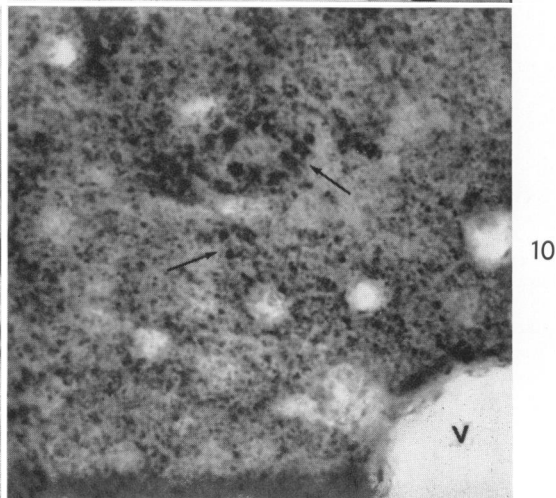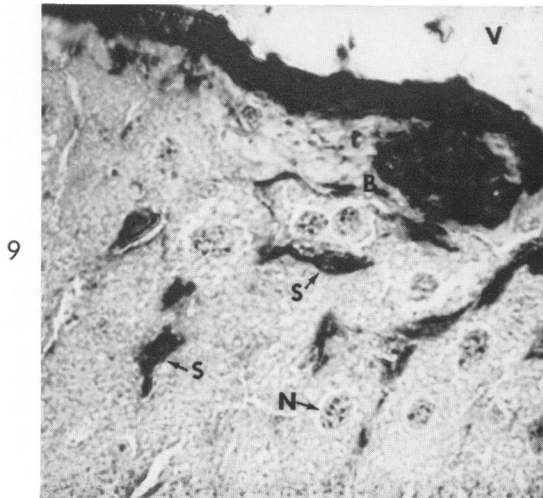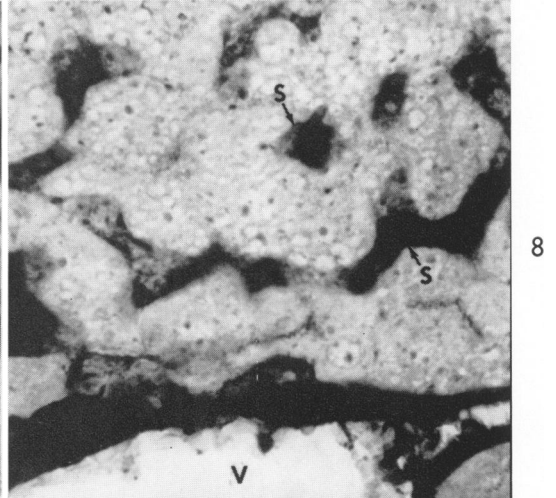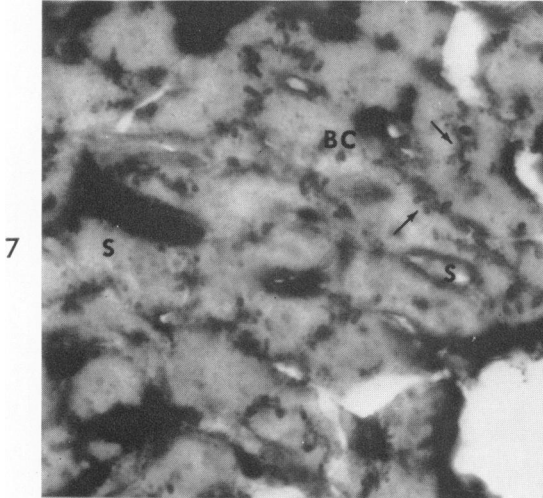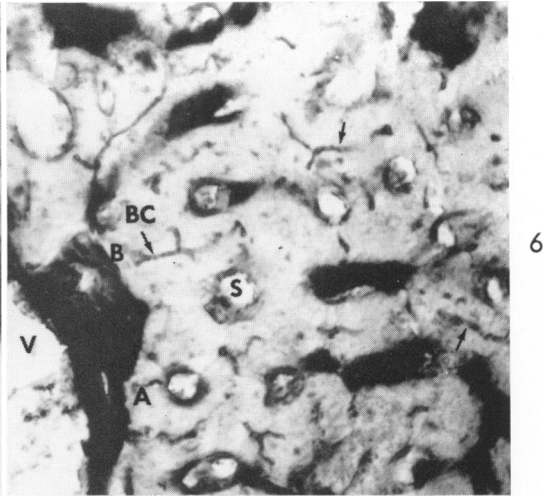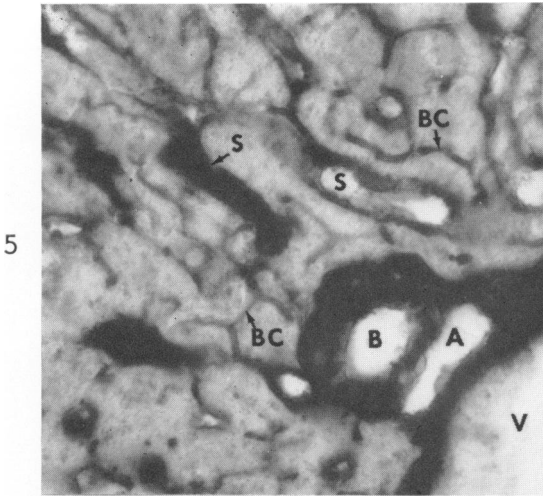
FIGS. 11–16.   Glucose-6-phosphatase reaction in control mouse liver and after up
to 12 hours incubation *in vitro*. The appearances at 1 (Fig. 12), 2 (Fig. 13) and
4 (Fig. 14) hours incubation are essentially unchanged from the control (Fig.
11) and show the same differential between portal (P) and central (C) zones.
× 100. (AFIP Neg. 64-4665-3)

FIG. 11.   Control. There are dense accumulations of reaction product in portal zones
(P), with lighter deposition in central areas (C). The reaction is diffuse in cyto-
plasm of the hepatic cells, and nuclei stand out as clear zones.

FIG. 15.   Eight hours. There is very slight reduction in activity, with a somewhat
narrower zone of intense activity in the portal area (P) as compared to that in
the controls.

FIG. 16.   Twelve hours. The reaction is negative in central zones (C), but there
is still a zone of reaction a few cells in width surrounding portal zones (P).
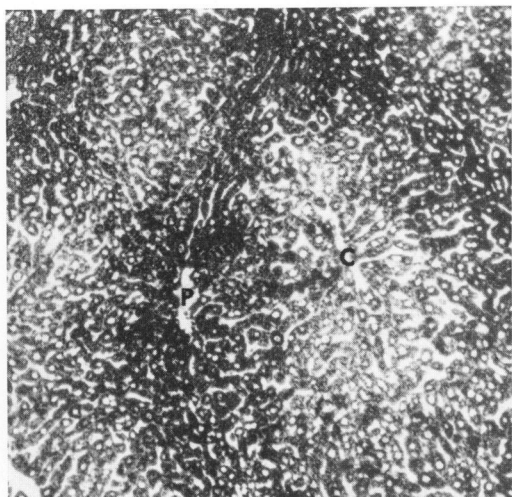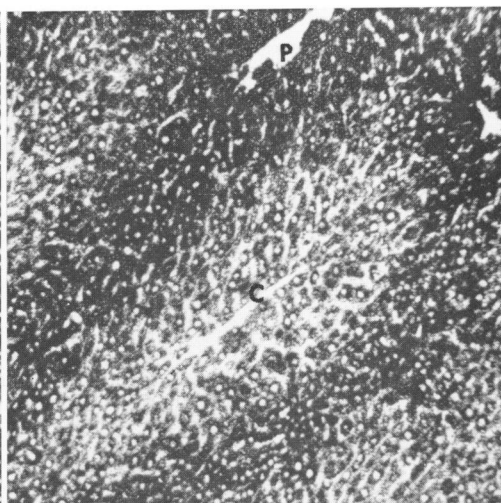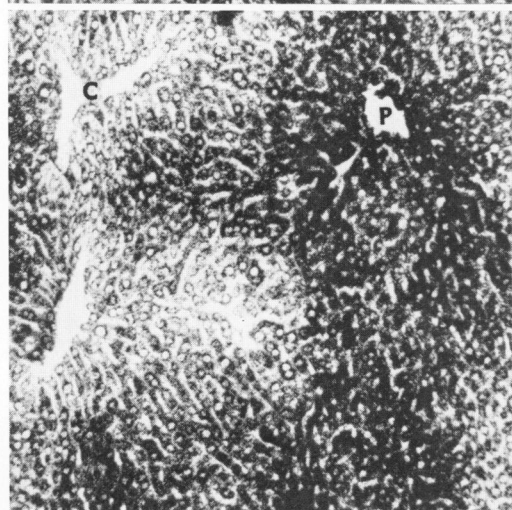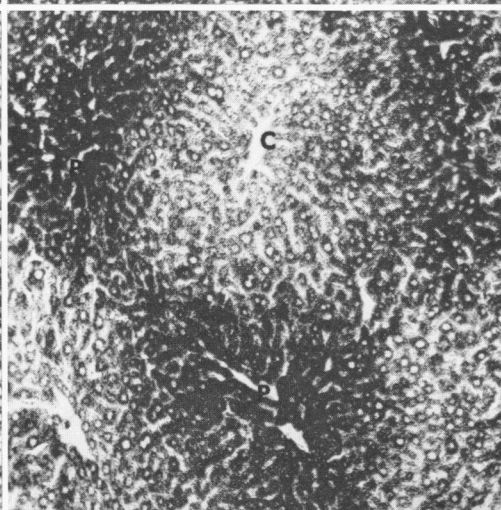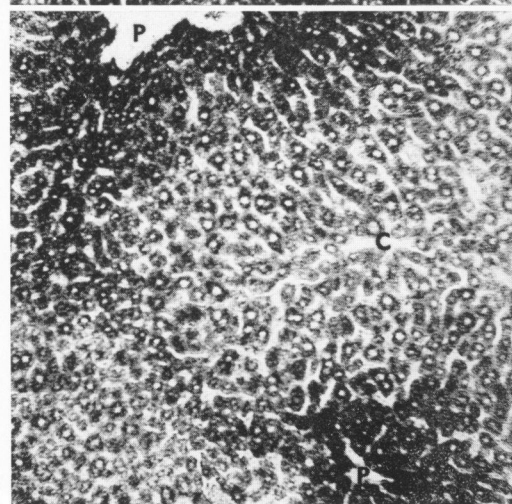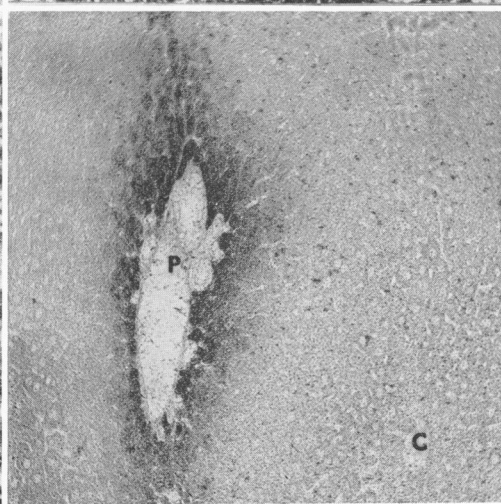
FIGS. 17–22.   Succinic dehydrogenase (NBT) reaction in control and incubated
   mouse liver. × 100. (AFIP Neg. 64-4665-4)

FIG. 17.   Control. The enzyme is localized in the cytoplasm of hepatic parenchymal
   cells, endothelial cells and sinusoidal lining cells, presumably in mitochondria.
   The concentration of reaction product is greater in portal zones (P) than in
   central areas (C).

FIGS. 18 and 19.   One hour and 4 hours, respectively. Note that the reaction is
   apparently slightly heavier than control, but the distribution is the same.

FIG. 20.   Eight hours. Reaction is very slightly diminished in central zones (C),
   with a rim of heavy reaction around portal areas (P).

FIG. 21.   Twelve hours. Reaction is slightly diminished in central areas (C) but is
   still heavy in portal zones (P).

FIG. 22.   Twenty-four hours. Specific reaction is entirely absent. The section re-
   sembles sections of control tissue incubated in the reaction mixture without
   substrate. The granular appearance of the cytoplasm is due to light diffraction
   and not to deposition of formazan.
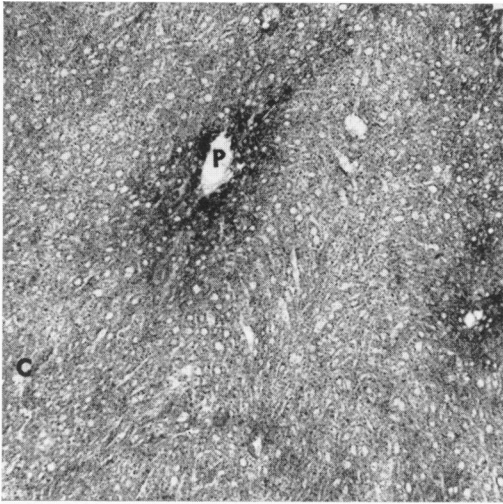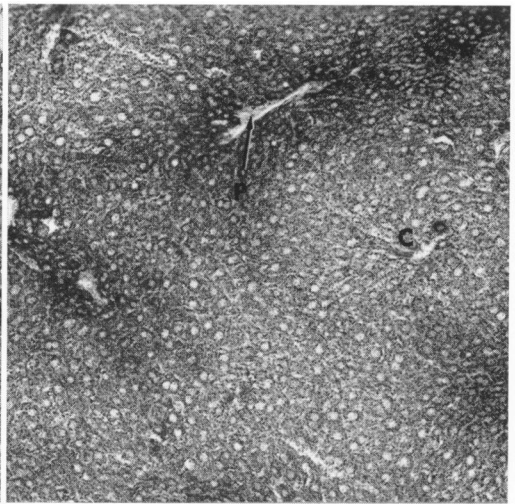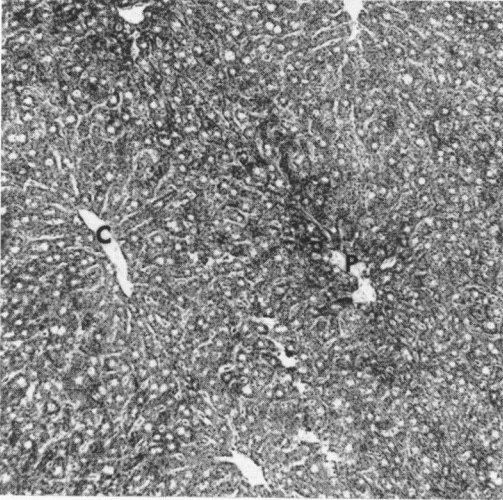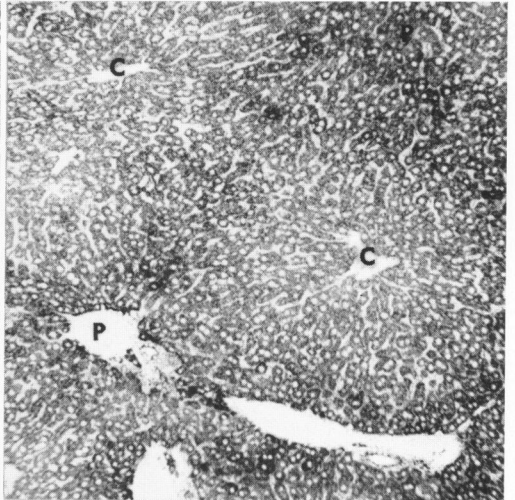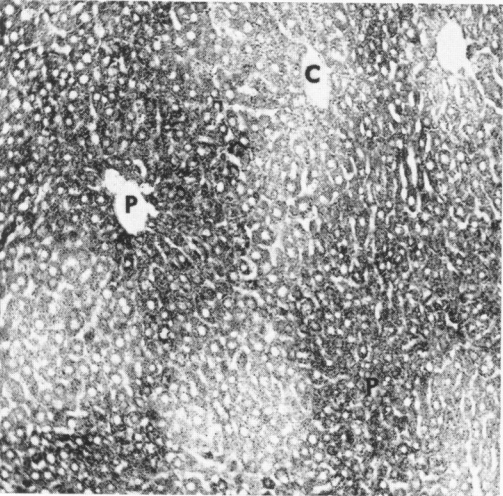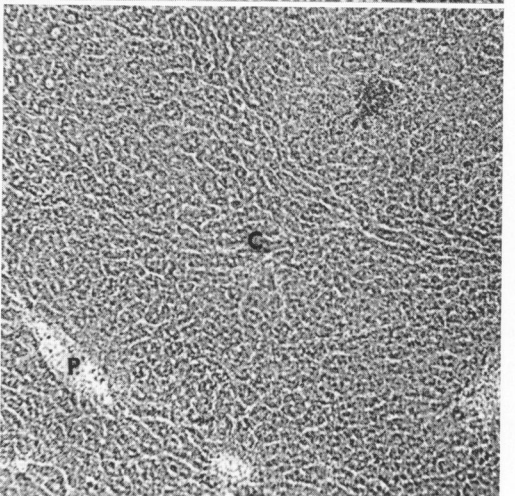
FIGS. 23–28.   Glutamic dehydrogenase (NBT) in normal and incubated mouse liver.
× 100. (AFIP Neg. 64-4665-5)

FIG. 23.   Control. In these sections of mouse liver the portal areas (P) react more
strongly than central zones. The large accumulations of formazan often seen
in control sections are not shown. Nuclei stand out as clear areas.

FIG. 24.   One hour. The more intense portal reaction (P) is more clearly evident,
and the over-all intensity of the reaction is somewhat greater than in controls.

FIG. 25.   Four hours. The portal areas (P) still react more intensely than central
zones (C), and the reaction remains stronger than in controls.

FIG. 26.   Eight hours. The reaction remains stronger than in controls, with a pre-
dominantly portal distribution (P). This is interesting in view of the marked
decline in enzyme activity found biochemically at this time.

FIG. 27.   Twelve hours. There is a marked decline in enzyme activity, though portal
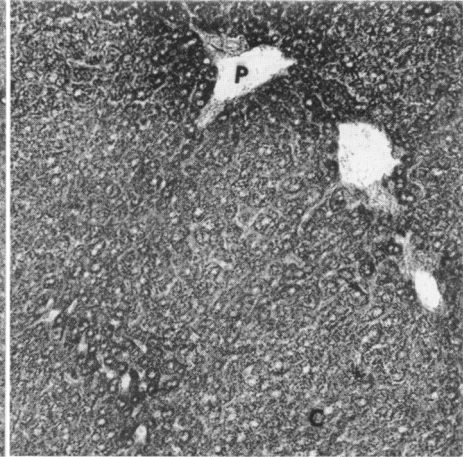zones (P) still react somewhat more strongly than central areas (C).

FIG. 28.   Twenty-four hours. No enzyme activity is seen. Granular appearance of
cytoplasm is due to light diffraction and some slight non-specific deposition of
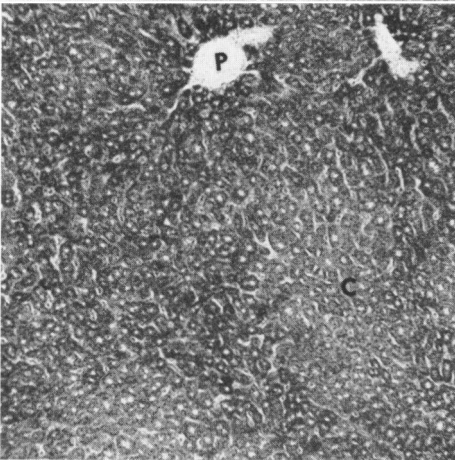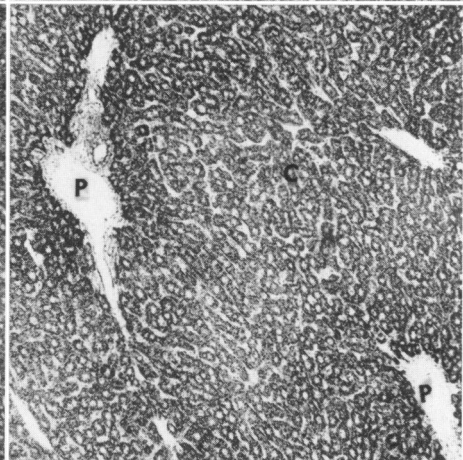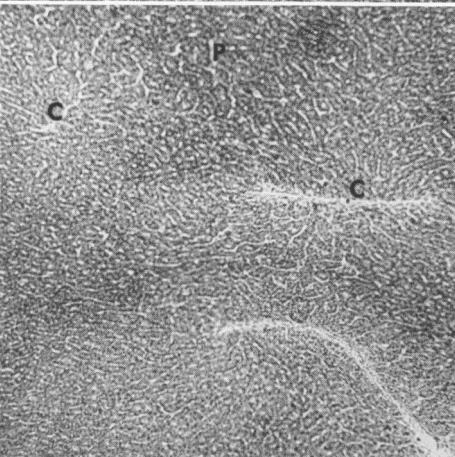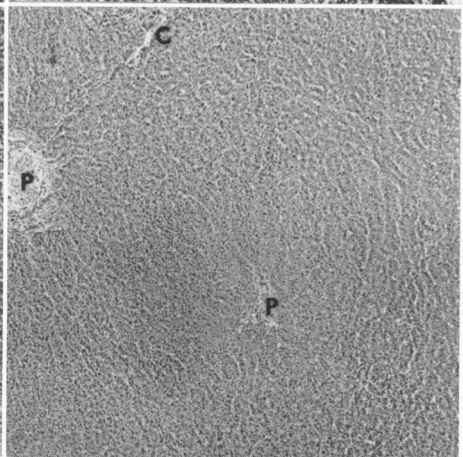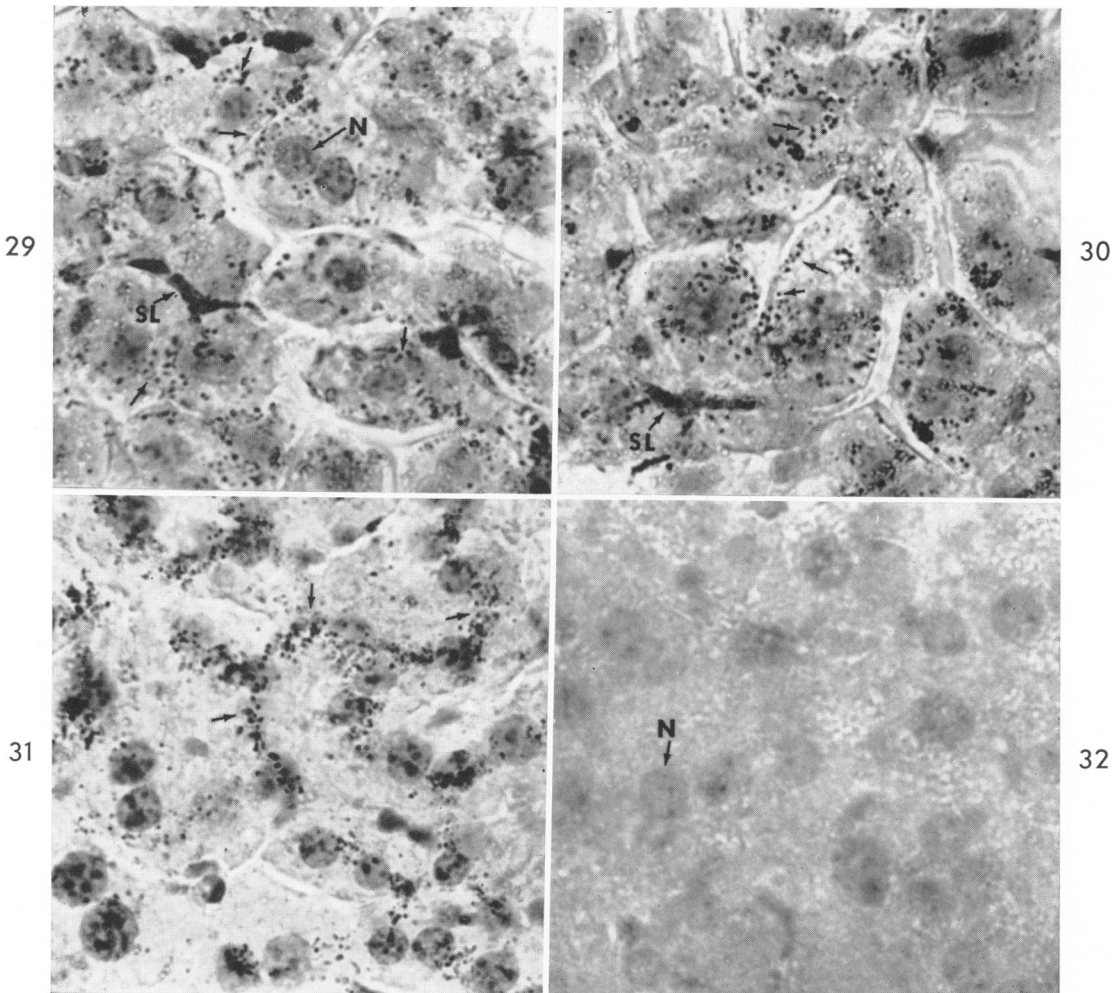formazan.
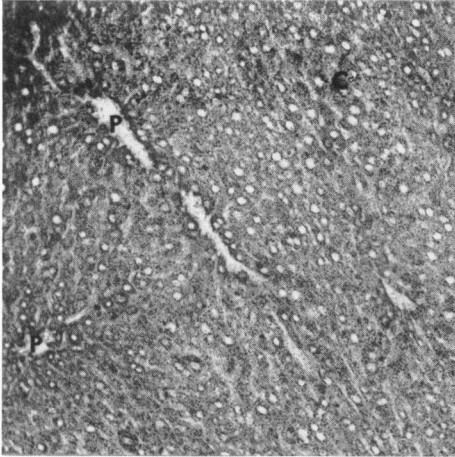
FIGS. 29–32. Acid phosphatase reaction in normal and mouse liver incubated *in
vitro* up to 8 hours. × 820. (AFIP Neg. 64-4665-6)

FIG. 29. Control. Nuclei (N) have been lightly counterstained with methyl green.
The cytoplasm of sinusoidal lining cells (SL) is blackened with reaction product.
Tiny black droplets indicating enzyme activity (unlabeled arrows) are seen in
the cytoplasm of hepatic parenchymal cells, particularly near the bile canaliculi
and the sinusoidal border. In electron micrographs the sites of activity are con-
fined to the cytosomes.

FIG. 30. One hour. The size and distribution of droplets indicating activity (un-
labeled arrows), as well as their number, appear unaltered as compared to the
controls. Sinusoidal lining cells (SL) show great activity.

FIG. 31. Four hours. Discrete droplets (unlabeled arrows) are still numerous, par-
ticularly along the sinusoidal border. The droplets are the same size as those in
the control section.

FIG. 32. Eight hours. Nuclei (N) can be seen, but no acid phosphatase activity is
demonstrated.