ENZYME DISTRIBUTION IN THE RAT
FETUS AND PLACENTA FOLLOWING
THE ADMINISTRATION OF ETHIONINE

RICHARD L. SCHULTZ, Ph.D., and PHYLLIS W. SCHULTZ, Ph.D.

From the Department of Anatomy, University of Colorado Medical Center, and the University of Colorado, Denver Center

ABSTRACT

Enzyme changes which accompany ethionine-induced resorption of the rat conceptus have been studied by both histochemical and biochemical techniques. Pregnant rats were injected with ethionine over a 3-day period prior to autopsy on day 12 of pregnancy. Sections of the whole conceptus were studied for acid phosphatase with both the Burstone and Gomori methods and for succinoxidase activity with nitro-BT. Biochemical determinations of cathepsins, acid phosphatase, and succinoxidase were performed on homogenates of the fetuses, placentae, and deciduas basalis from ethionine-treated and saline-treated rats. The histochemical study has shown that resorption is accompanied by an increase in the size and number of acid phosphatase granules in the decidual tissues and a concurrent loss of acid phosphatase granules in the fetal tissues. Biochemical methodology indicated that there was no increase in total cathepsin or acid phosphatase activities in the resorbing tissues. No change in succinoxidase activity was found with either histochemical or biochemical techniques. The significance of these results was discussed with reference to the lysosome hypothesis.

Enzyme changes which accompany cell death and tissue degeneration have been reported for a variety of tissues and have been summarized in a recent review (19). Correlation of histochemical and biochemical data on enzymatic patterns associated with tissue destruction has led to a functional concept of cell death and the lysosome theory (7, 8).

As reported previously (24), the administration of ethionine to the pregnant rat leads to resorption of the conceptus which is characterized by large scale degeneration of embryonic and/or maternal tissues. The present study was undertaken to determine whether this cell destruction is characterized by an increase in hydrolytic enzymatic activity either preceding or concurrent with the resorption process. The presence of lysosomes and lysosomal or proteolytic activity was established histochemically by acid phosphatase techniques and the state of metabolic activity by a succinoxidase technique.

Metabolic and proteolytic activities were also determined chemically by acid phosphatase, succinoxidase, and cathepsin techniques.

MATERIALS AND METHODS

Animal Treatment

Pregnant Sprague-Dawley rats were injected intraperitoneally with a total dose of 0.8 mg DL-ethionine (Nutritional Biochemicals Co., Cleveland, Ohio)/gm body weight. The amount was given in six injections, starting the evening of day 7 of pregnancy, and administered at 12-hour intervals. Control animals were injected over the same period of time with
FIGURE 1
Sections of conceptus from 12-day pregnant saline-treated control. Reacted with Burstone's method for acid phosphatase.

la Yolk sac: arrow indicates concentration of acid phosphatase granules in distal portion of yolk sac epithelium. × 700.

lb Placental labyrinth: note dark areas lining maternal blood spaces. × 700.
Decidua basalis: arrows indicate leucocytes with acid phosphatase granules in the maternal blood vessel. \( \times 700 \).

Decidua capsularis: note dark reaction in lining of blood vessels. \( \times 700 \).
Figure 2
Sections of conceptus from 12-day pregnant saline-treated control. Reacted with a modified Gomori method for acid phosphatase.

2a Yolk sac. X 700.

2b Placental labyrinth: arrows indicate acid phosphatase granules in tissues. X 700.
2c Decidua basalis: arrows indicate leukocytes with acid phosphatase granules in maternal blood vessels. X 700.

2d Decidua capsularis. X 700.
0.85 per cent saline, the vehicle for ethionine. All animals were sacrificed on day 12 of pregnancy.

**Histochemical Methods**

Whole conceptuses were removed from the uterus and either frozen and sectioned immediately at 6 μm on the cryostat or fixed in cold acetone. The acetone-fixed tissues were embedded in paraffin (m.p. 48 to 50°C) under vacuum and sectioned at 6 μm.

The acetone-fixed paraffin-embedded tissues were reacted for acid phosphatase according to the method described by Burstone (4). The sections were deparaffinized in cold petroleum-ether, incubated with a substrate of naphthol AS-BI phosphate (Sigma Chemical Co., St. Louis, Missouri) and Fast Bordeaux Salt OL (Carbic-Hoechst Corp., Mountainside, N. J.), for 18 hours at 37°C, and mounted in glycerol gel. Control sections were inhibited with sodium fluoride and with tartaric acid.

Frozen sections for acid phosphatase determinations were reacted with a modified Gomori technique (12) and for succinoxidase activity with nitro-BT (23). Control sections for each technique were reacted with the incubating medium without the substrate.

**Biochemical Methods**

Homogenates were made of the 12-day fetuses, the placental labyrinth, and decidua basalis in cold distilled water with McShan-Erway homogenizers. Aliquots of these homogenates were analyzed for cathepsin activity, acid phosphatase activity, succinoxidase activity, and total protein nitrogen.

Cathepsin activity was determined by incubation of the samples with a casein-urea substrate (14), pH 4.9, for 18 hours at 37°C. Tyrosine, released during the proteolysis of the substrate, was quantified with the Coleman Jr. spectrophotometer at

![Figure 3](image-url)

**Figure 3**

Section of conceptus from 12-day pregnant ethionine-treated rat showing early stages of resorption. Reacted with Burstone's method for acid phosphatase. E, degenerating embryo; YS, yolk sac; L, placental labyrinth; arrow indicates region of accumulation of cells with acid phosphatase granules in decidua basalis. X 125.
700 mm following the addition of phenol reagent and was used as the measure of enzyme activity.

Acid phosphatase content of the homogenates was determined by incubation of the aliquot with phenyl disodium phosphate substrate, pH 5.1, for 1 hour at 37°C (5). Phenol reagent was added to each sample and the amount of free phenol was determined in a Coleman Jr. spectrophotometer at 660 mm. Non-incubated aliquots of homogenate with the phenyl disodium phosphate substrate were used as blanks for each sample.

Succinoxidase activity was determined by incubation of the samples with a neotetrazolium substrate, pH 7.9, for 2 hours at 37°C (22). The enzyme activity was stopped by the addition of 40 per cent trichloroacetic acid. The diformazan produced was extracted in water-saturated n-butanol and quantitated in a Coleman Jr. spectrophotometer at 515 mm.

Total protein nitrogen was determined by nesslerization.

Each type of enzyme activity was expressed as enzyme activity/μg total protein nitrogen. The significance of enzyme activity for each tissue was determined by the t test.

RESULTS

The variation in the degree of ethionine-induced resorption attained at 12 days of pregnancy made possible a reconstruction of the sequential phases of progressive destruction of the conceptuses (24). Histochemical determination for acid phosphatase upon these various stages of resorbing conceptuses showed that the concentration and pattern of distribution of proteolytic enzymes change with the progressive phases of resorption.

FIGURE 4

Section of conceptus from 12-day pregnant ethionine-treated rat showing advanced stage of resorption. Reacted with Burstone's method for acid phosphatase. DC, decidua capsularis; DB, decidua basalis; B, blood and embryonic debris; arrows indicate concentrations of leucocytes with acid phosphatase granules at periphery of embryonic debris and blood. X 125.
Figure 5
Section of decidua capsularis of conceptus from 12-day pregnant ethionine-treated rat showing stage of advanced resorption.

5a Reacted with Burstone's method for acid phosphatase. X 700.
5b Reacted with Gomori method for acid phosphatase. X 700.
Both the Gomori and the Burstone methods (Figs. 1 and 2) showed that control 12-day conceptuses had acid phosphatase granules in the distal portion of the cells of the yolk sac epithelium, and within the leucocytes in the blood vessels of the decidua basalis and capsularis. In the fetus itself, acid phosphatase granules were found in the neural tube, the ventral roots of developing spinal nerves, the sympathetic ganglia, the lens, the gut epithelium, and the developing kidney tubules. With the Gomori method, the granules were more discrete, showing less evidence of diffusion.

Some tissues of the control conceptuses reacted differently to the two methods. With the Gomori method, discrete acid phosphatase granules were found in the cells of the placental labyrinth (Fig. 2b). With the Burstone method, a positive reaction was found in the lining of the blood vessels of the placental labyrinth and the decidua capsularis (Figs. 1b and d). Since this reaction was not inhibited with sodium fluoride or tartaric acid, its significance is not understood.

Study of resorbing 12-day conceptuses showed that the sequential phases of resorption exhibited a definite pattern of acid phosphatase distribution. During the early phases of resorption, characterized by disorganization of the cells of the embryo and reduction in the size of the placental labyrinth, acid phosphatase activity disappeared completely from the cells of the embryo and placental labyrinth. Concurrently, an apparent increase in acid phosphatase activity occurred in the tissues of the decidua basalis and capsularis (Fig. 3); this was due to larger acid phosphatase granules within the decidual cells and within the phagocytes which occur in increasing numbers during this stage of resorption. During later stages of resorption, after the lumen of the uterus had filled with blood and embryonic debris (Fig. 4), the high concentration of acid phosphatase activity of the deciduas persisted (Figs. 5 and 6) and a “ring” of phagocytes containing large acid phosphatase granules appeared on the peripheral portion of the blood and debris (Fig. 7). When resorption of the fetal and placental tissues was...
Figures 7

Section of periphery of embryonic debris of conceptus from 12-day pregnant ethionine-treated rat showing stage of advanced resorption; area is indicated by lower arrow in Fig. 4.

7a Reacted with Burstow's method for acid phosphatase. Dark cells are leucocytes containing large acid phosphatase granules. \( \times 700 \).

7b Reacted with Gomori method for acid phosphatase. Arrows indicate leucocytes with acid phosphatase granules. \( \times 700 \).
complete and only a remnant of the decidua basalis remained, this remnant contained cells with large acid phosphatase granules (Fig. 8).

In resorbing conceptuses found occasionally in the saline-treated controls, the same pattern of loss of specific reacting granules in the fetal tissues and of increase in the size of granules in cells of the deciduas was observed.

Succinoxidase preparations from control rats showed diffuse distribution of small granules containing reduced tetrazolium throughout the tissues of the fetus, placenta, and uterus. There was a marked concentration of these enzyme-positive granules in the region of the junction of the placental labyrinth and the decidua basalis (Fig. 9). There was no change in the distribution or concentration of the granules with resorption. In fact, when only debris remained in the center of the uterine lumen, the cells in the debris contained enzyme-positive granules. The granules were not present in the control sections incubated without the substrate.

Biochemical assays of saline-treated and ethionine-treated conceptuses showed that no significant differences in total cathepsin, acid phosphatase, and succinoxidase activities existed in the fetus, the placenta, or the decidua basalis (Fig. 10). Moreover, no differences in enzyme concentration, i.e. cathepsin, acid phosphatase, and succinoxidase, were found during the successive stages of the destruction of the conceptus in ethionine-treated resorbing animals.

The total protein nitrogen in the fetus and decidua basalis of ethionine-treated rats was

![Figure 8](image.png)

**Figure 8**
Section of conceptus from 12-day pregnant ethionine-treated rat showing complete resorption. Reacted with Burstone’s method for acid phosphatase. DB, remnant of decidua basalis; arrow indicates cells with large acid phosphatase granules. X 125.
significantly lower than that of the controls (Table I). This would be a reflection of the lower weights of these tissues with ethionine treatment since the mg protein nitrogen/gm wet weight ratio did not differ significantly in any of the tissues.

**DISCUSSION**

The detection of early necrosis of a cell or tissue by the enlargement of acid phosphatase granules (enlarged lysosomes or "cytolysomes" (18)) has been described for a series of tissues, e.g., liver, kidney, brain, Müllerian ducts, and amphibian tail (2, 6, 16, 18, 21). In light of these observations it seemed plausible to expect the necrotic changes characteristic of fetal and placental resorption to be accompanied by detectable changes in the lysosome concentration or distribution in the dying cells. However, not all of the data presented above lend themselves easily to interpretation on the basis of the lysosome hypothesis.

The histochemical reactions of the decidual tissues were the most unequivocal. The enlargement of the lysosomes within the decidual cells is probably a good indication of immediate or incipient cell degeneration. Similarly the presence of acid phosphatase-positive granules in the phagocytes found in large numbers in this tissue during resorption (25) indicates proteolytic destruction. The presence of high levels of acid phosphatase in phagocytotic vacuoles has been reported in previous work by Essner (11).

The proteolytic reactions of the deciduas must be interpreted in the light of and correlated with the cell death occurring within the embryonic and extraembryonic tissues. An increase in the size or numbers of acid phosphatase granules in the cells of the embryo during degeneration, which might be interpreted as an increase in proteolytic activity, did not occur at any time. The only detectable change in the small acid phosphatase granules of the fetal tissues was their disappearance just prior to the first signs of pronounced cell disorganization during resorption. This need not imply lack of proteolytic activity but rather a release of enzymes from the...
Figure 10
Enzyme determinations of homogenates of fetuses, placentas, and decidua basalis from ethionine-treated and saline-treated 12-day pregnant rats.

Table 1
Protein Nitrogen Data
Expressed as mg protein nitrogen (PN$_2$)/gm original wet weight of the tissue and as total mg PN$_2$/individual fetus, placenta, or decidua basalis. Mean ± standard error.

<table>
<thead>
<tr>
<th></th>
<th>Fetus</th>
<th>Placenta</th>
<th>Decidua Basalis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg PN$_2$/gm wet wt</td>
<td>total mg PN$_2$/fetus</td>
<td>mg PN$_2$/gm wet wt</td>
</tr>
<tr>
<td>Saline-treated control</td>
<td>5.21 ±0.20</td>
<td>201.8 ±9.3</td>
<td>14.72 ±0.61</td>
</tr>
<tr>
<td>(38)*</td>
<td>(61)</td>
<td></td>
<td>(62)</td>
</tr>
<tr>
<td>Ethionine-treated</td>
<td>5.23 ±0.34</td>
<td>171.5 ±12.1</td>
<td>14.18 ±0.70</td>
</tr>
<tr>
<td>(33)</td>
<td>(47)</td>
<td></td>
<td>(49)</td>
</tr>
</tbody>
</table>

* ( ) indicate number of animals.

R. L. Schultz and P. W. Schultz  Enzyme Distribution after Administration of Ethionine
lysosomes into the soluble portion of the cell where visualization is not possible. Indeed, one idea holds that increase in size and number of acid phosphatase granules immediately precedes enzymatic activity and that subsequently during proteolysis the lysosomes decrease in size or disappear (19).

This idea may not be correct. Fetal cells may not take an active part in self-destruction, but rather may be disorganized and destroyed by the lysosomal activity of the decidual cells and of the phagocytes which invade the area of degenerating fetal cells late in resorption. Biochemical determinations have shown that the fetal cells have a relatively low concentration of cathepsin and acid phosphatase which would indicate little potential for activity during cell death.

Biochemical determinations of the cathepsin and acid phosphatase contents of resorbing fetal and placental tissues show that no significant change occurs in total enzyme concentration during resorption. This was unexpected in view of other observations in which an increase in total lysosomal enzymes was associated with either normal or induced degeneration of tissues (3, 10, 13, 27). However, other studies have shown that lack of increase in total enzyme activity is accompanied by a shift of the enzymes from the particulate to the soluble fractions of the cell (1, 9, 18). We are at present engaged in enzyme determinations of fractions of fetal and placental homogenates to determine whether such a shift of proteolytic enzymes does occur during resorption.

Both the histochemical and biochemical observations indicate a continuation of respiratory enzyme activity after tissues have undergone considerable disorganization and degeneration. This would appear to agree with the report of King et al. (15) who found that no immediate diminution in glucoytic cycle activity occurred when Ehrlich tumor cells were injured and that enzymatic activity was eventually reduced many hours after the initial injury.

The reservations which must be considered in the interpretation of results with the use of tetrazolium salts for the determination of succinoxidase activity have been presented by Novikoff (17, 20). Since one of the major misinterpretations that can be made is due to the solubility of the diformazan in lipid, the distribution of the diformazan was compared to that of fat in sections (26). The only area in which there was a similarity of distribution of the two reactions was in the yolk sac, which has been discussed previously by Padykula (22).

The authors make grateful acknowledgement to Juanita Graves, Barbara D. Fleischaker, and Donald L. Secore for technical assistance, and to Dr. John T. Willson for use of the cryostat. Naphthol AS-Cl phosphate and Fast Red Violet LB were generously donated by Dr. Burstone for the initial histochemical determination of acid phosphatase. The Fast Bordeaux Salt OL was generously donated by Carbica-Hoechst Corp.

This work was supported by United States Public Health Service grant RG-6747. The material was presented at the second meeting of the Teratology Society, March, 1962, Gainesville, Florida.

Received for publication, May 24, 1962.

REFERENCES


17. Novikoff, A. B., Enzyme cytochemistry: pitfalls in the current use of tetrazolium techniques, J. Histochem. and Cytochem., 1959, 7, 301.


