Acid Hydrolases of the Rat Uterus in Relation to Pregnancy, Post-Partum Involution and Collagen Breakdown

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1. The rat uterus contains acid cathepsin, β -glucuronidase, β -galactosidase, acid phosphatase and deoxyribonuclease II at concentrations comparable with those found in liver. Two non-hydrolytic uterine enzymes, cytochrome c oxidase and aspartate aminotransferase, display only 2–6% of the activity found in liver.
2. The concentrations of acid cathepsin and β -glucuronidase are significantly decreased in pregnancy and increase 3–4-fold during post-partum involution.
3. The concentrations of β -galactosidase and acid phosphatase are not decreased in pregnancy and increase only 2–3-fold during involution.
4. The concentrations of these four acid hydrolases increase linearly during the first 4 days *post partum* and reach their peak values at the same time that wet weight and collagen content fall to their lowest point.
5. The concentration of deoxyribonuclease is depressed in pregnancy but does not rise above normal in the post-partum period.
6. Only a small proportion of each hydrolytic activity can be isolated in the mitochondrial-lysosomal fraction of sucrose homogenates of the rat uterus. This proportion increases during involution. However, the extensive mitochondrial rupture occurring during homogenization indicates that the technique is probably too harsh to obtain a true measure of the proportion of lysosomes present in the intact tissue.
7. There are no significant changes in either the concentration or subcellular distribution of the five acid hydrolases in the livers of the experimental rats during pregnancy or involution. In each case the largest proportion of the activity is found in the mitochondrial-lysosomal fraction of liver homogenates.
8. The results are interpreted in terms of the lysosomal theory of intracellular digestion.

One of the most striking examples of a rapid and massive breakdown of tissue occurring under physiological conditions is to be found in the post-partum involution of the mammalian uterus. This involution involves the resorption of many different tissue components, including muscle protein, cellular material and the connective-tissue framework. Of particular interest is the rapid breakdown of collagen, a protein that normally displays minimal metabolic activity. A previous paper (Woessner, 1962) reported some of the events occurring during collagen breakdown in the involuting rat uterus. The evidence points to a hydrolytic destruction of collagen culminating in the appearance of free hydroxyproline.

Considerable attention has been given to the detection of hydrolytic processes in the involuting uterus that might be responsible for the digestion of collagen, muscle protein and other components of the uterus. A marked proteolytic activity at pH 3.5 and a weak activity at pH 8.5 have been

shown in the involuting rat and human uterus (Woessner & Brewer, 1960, 1963; Woessner, 1962), with haemoglobin or uterine protein as substrate. But collagen digestion could be demonstrated only at pH 3.5. Similar results have been obtained by Morrión & Seifter (1962) and by Schaub (1963).

If the acid-cathepsin activity of the uterus plays a role in the involutionary processes, it must either function at a pH far removed from its optimum or else there must be a mechanism for lowering the pH in certain restricted parts of the tissue. One approach to the elucidation of the role of the cathepsin is offered by the lysosome theory, as expounded by its chief proponent de Duve (1964). In brief, it is found that certain cathepsins and a wide variety of hydrolytic enzymes, all possessing acid pH optima, tend to be segregated from the cytoplasm of the cells of liver, kidney and other tissues by an enclosing membrane. The resultant small particle is named the 'lysosome'. These lysosomes function in normal intracellular digestion either by fusion

with endocytotic vacuoles (phagosomes or pinosomes) or by forming digestive organelles themselves. An acid pH is presumed to obtain within these digestive vacuoles. These lysosomal particles have been implicated in a number of processes of tissue regression or resorption, and in each case there is a marked rise in the concentration of the acid hydrolases during the process. Examples include the regression of the chick Müllerian duct (Brachet, Decroly-Briers & Hoyer, 1958) and the resorption of the tadpole tail (Weber, 1957), both of which involve an elevated cathepsin activity.

It is reasonable to expect that a similar phenomenon of intracellular digestion involving lysosomal enzymes may be a prominent mechanism in uterine involution, and some evidence suggesting this has been accumulating recently. Maibenco (1960) has shown that involution does not involve necrosis or degenerative changes, but rather that the tissue substances such as muscle protein seem to be resorbed by controlled intracellular processes. Macrophages are a prominent cell type in the involuting uterus and may help to remove the extracellular material. Lobel & Deane (1962) showed that macrophages increase rapidly in the post-partum rat uterus and that they contain high concentrations of acid phosphatase localized in particles believed to be lysosomes. Electron-microscope evidence presented by Luse & Hutton (1964) reveals large numbers of intracellular digestive vacuoles filled with collagen fragments. This indicates that even the extracellular elements are probably ultimately digested within cells. However, the quantitative relationships of the various hydrolytic enzymes to the involutionary processes have not been investigated to date.

The present study was undertaken to investigate the pertinency of the lysosomal theory to the process of uterine involution. The behaviour of a variety of enzymes that are characteristically found in the lysosomes in other tissues was examined in the rat uterus during pregnancy, post-partum involution and in the non-gravid state. The enzyme activities were measured quantitatively on tissue homogenates and attempts were made to characterize the intracellular distribution of the enzymes. The second part of this approach has yielded equivocal results owing to problems of homogenizing the fibrous tissue, but changes in the acid hydrolases in the various functional states of the uterus are consistent with the hypothesis that these enzymes may be involved in the processes of involution.

METHODS

Tissue preparation. Pregnant and non-pregnant albino rats were purchased from Sprague-Dawley Inc., Madison, Wis., U.S.A. All rats were of the same general age and

weight; the average weight, exclusive of the uterus and its contents, was 230g. All animals were maintained on Purina chow and water *ad lib.* until used; this was generally within 2 weeks after purchase. In animals used before parturition, the stage of pregnancy was estimated from the supplier's information and from the size of the foetuses. In studying the post-partum uteri, it was usually possible to ascertain the time of parturition within the limits ± 2 hr.

The rats were killed by decapitation during ether anaesthesia and the uteri were rapidly dissected out. The uterine contents were removed, and the mesenteries and small fatty nodules corresponding to the placental attachment sites were trimmed off. The cervix was cut off immediately below the junction of the horns. The number of foetuses, offspring or placental sites was recorded. Uteri containing less than ten or more than 12 foetuses were discarded because of the influence of this number on the uterine size and collagen content (Harkness & Moralee, 1956). The uteri were rinsed in 0.9% NaCl and then placed in cold 0.25M-sucrose solution containing EDTA (1mM).

Each uterus was blotted on paper towelling and weighed on an analytical balance. Portions weighing approx. 0.8g. were used for homogenization. With larger uteri, representative portions of lengthwise-sectioned horns were used. Smaller uteri were pooled from two rats to provide sufficient tissue. The tissue portion was thoroughly minced in a 10ml. beaker with iridectomy scissors and the fragments were transferred with ice-cold sucrose to a chilled all-glass homogenizer of the TenBroeck type (A. H. Thomas Co., Philadelphia, Pa, U.S.A.). The volume of sucrose-EDTA solution was adjusted to 12ml. for 0.8g. of tissue. The tissue was first ground by hand until the glass pestle reached the bottom of the homogenizer. The pestle was then attached with rubber tubing to a motor and five rapid passes of the pestle were made at a speed of 1500 rev./min. It proved impossible to homogenize the uterine tissue satisfactorily with the Teflon-pestle homogenizers usually used for subcellular fractionations.

Fractionation of homogenates. All homogenates were subjected to differential centrifugation in attempts to prepare lysosomal particles. The homogenates were transferred to celluloid tubes and centrifuged at 600g for 15 min. in the Spinco model L preparative ultracentrifuge (rotor no. 40). The pellet (P-1) was saved and the supernatant was centrifuged for 20 min. at 15000g to obtain a combined mitochondrial-lysosomal pellet (P-2). No further attempt was made to separate the lysosomes from the mitochondria.

The first pellet (P-1) was resuspended in sucrose solution (10ml./g. initial wt. of tissue) and homogenized with the motor-driven pestle. This suspension was centrifuged again at 600g to give the final low-speed pellet containing nuclei, cell debris and undisrupted tissue. The supernatant was poured on top of the mitochondrial pellet (P-2) and centrifuged for 20 min. at 15000g to give the final mitochondrial-lysosomal pellet. The remaining supernatant was combined with the original 15000g supernatant. Distilled water (25ml./g. initial wt. of tissue) and 1 drop of Triton X-100 were added to each 600g and 15000g pellet. The pellets were rehomogenized to give uniform suspensions. Thus each of the three fractions (P-1, P-2 and supernatant) consisted of approx. 25ml. of preparation/g. of starting tissue.

In most of the experiments described below, parallel comparative studies were made of the rat livers. Approx.

1g. of liver was homogenized in 10ml. of sucrose-EDTA. The further procedure was the same as for uterus tissue except that the final pellets (P-1 and P-2) were resuspended in 10ml. of water.

Each of the three fractions of each tissue sample was divided into several portions, which were frozen separately for subsequent enzyme assay. Enzyme assays were all completed within 4 days after killing the rats. Freezing and storage did not affect the activities appreciably except for cytochrome *c* oxidase, which was assayed immediately on preparing the homogenates.

Enzyme assays. A preliminary series of experiments was performed to find the optimum pH for the assay of each enzyme activity in the uterine homogenates. In most cases these optima were similar to the values published for liver enzymes. Each activity was assayed with two different concentrations of homogenate fraction as a check on the linearity of the assay and to improve accuracy. Cytochrome *c* oxidase (EC 1.9.3.1, cytochrome *c*-oxygen oxidoreductase) was assayed by the method of Smith (1955). Aspartate aminotransferase (EC 2.6.1.1, L-aspartate-2-oxoglutarate aminotransferase, glutamate-oxaloacetate transaminase) was assayed by the method of Karmen, Wróblewski & LaDue (1955). Acid deoxyribonuclease (EC 3.1.4.6, deoxyribonuclease 3'-nucleotidohydrolase, deoxyribonuclease II) was measured at pH 4.6 by the procedure of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The technique of Sellinger, Beaufay, Jacques, Doyen & de Duve (1960) was employed for β -galactosidase (EC 3.2.1.23, β -D-galactoside galactohydrolase) measurement at pH 4.0. β -Glucuronidase (EC 3.2.1.31, β -D-glucuronide glucuronohydrolase) was assayed at pH 4.8 by the method of Fishman, Springer & Brunetti (1948). Acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase) was measured in citrate buffer, pH 4.9, with *p*-nitrophenyl phosphate as substrate (*Sigma Technical Bulletin*, no. 104; Sigma Chemical Co., St Louis, Mo., U.S.A.). The assay for acid cathepsin (EC 3.4.4.-) with haemoglobin at pH 3.5 was as described by Woessner & Brewer (1963). All assay results are expressed as μ moles of substrate hydrolysed/hr./g. wet wt. of tissue. Two exceptions are aspartate aminotransferase (μ moles/min./g. wet wt. of tissue) and cytochrome *c* oxidase. The reaction of the latter enzyme follows a first-order kinetic expression, and so the results are expressed as a first-order rate constant/g. wet wt. of tissue. The rate constant is calculated in units of minutes. Cathepsin activity was expressed in terms of tyrosine released from haemoglobin and was further corrected for departures from linearity by an empirical correction curve. The total activity of each enzyme in the entire uterus was calculated by multiplying the sum of the three separate subcellular fraction activities/g. wet wt. of tissue by the weight of the uterus.

Collagen assay. Samples of each of the three subcellular fractions were hydrolysed and analysed for hydroxyproline by the methods previously outlined (Woessner, 1961) to determine the amount of collagen/uterus.

RESULTS

Changes in wet weight and collagen. Fig. 1 illustrates the weight changes of the rat uterus during pregnancy and involution. The base-line weight for 30 nulliparous non-gravid uteri is 0.41 g.

Fig. 1. Wet weight of the rat uterus during pregnancy and post-partum involution. The vertical line indicates the time when parturition occurred. The horizontal line represents the mean weight of 30 non-gravid uteri (0.412 ± 0.107 s.d.).

The weight increases during the second half of pregnancy to a maximum of approx. 3g. at term (an 8-fold increase). Involution begins immediately after delivery and the weight falls rapidly to the base line within 4 days. This process obeys first-order reaction kinetics. The results from 0 to 100 hr. *post partum* are described by the expression:

$$\text{Weight (g.)} = \exp(1.089 - 0.02158t)$$

where *t* is the time (hr.) elapsed after delivery. This equation was obtained by fitting a least-squares linear regression line to a plot of $\log(\text{wet wt.})$ against time (coefficient of correlation = 0.96). The involution loss of weight does not stop at the base line, but continues to a lower value and remains there for many days.

The pattern of collagen (hydroxyproline) change is different (Fig. 2). Again the increase occurs in the second half of pregnancy, but the peak value reached at term (about 8mg. of hydroxyproline/uterus) is only 5 times the base-line value of 1.6mg. found for nulliparous non-gravid uteri. Thus the increase in collagen fails to keep pace with the increase in wet weight. Further, the process of collagen loss during involution does not follow first-order kinetics.

Fig. 2. Total hydroxyproline of the rat uterus during pregnancy and post-partum involution. These values may be converted into an approximate collagen equivalent by multiplying by 7.46. The horizontal line represents the mean hydroxyproline content of 19 non-gravid uteri (1.59 ± 0.60 s.d.).

A more complex kinetic expression has been developed (Woessner, 1962) relating the square root of the hydroxyproline content to the time *post partum*. The present results from 0 to 90hr. *post partum* can be described by this expression:

$$\text{mg. of hydroxyproline/uterus} = (-0.663t + 90.5)^2 \times 10^{-3}$$

where t is time (hr.) *post partum*. Again the involutionary processes with respect to collagen are essentially complete by 4 days *post partum*, and the collagen value falls below the normal level for a considerable time. The initial lag period reported previously (Woessner, 1962) is still found in the immediate post-partum period. The average hydroxyproline value for 6–12hr. *post partum* (7.46mg.) is not significantly lower than the value at parturition (8.07mg.).

The difference in the kinetics of the loss of weight and of collagen leads to a changing concentration of collagen/g. wet wt. of tissue during involution. There is a rise in concentration, reaching a peak about 60hr. *post partum* and then returning to a lower value at 4 days. However, even at the peak, the concentration does not reach the concentration found in the non-gravid uterus.

Table 1. Comparison of enzyme activities in homogenates of non-gravid uterus and liver

The enzyme units, with two exceptions, are expressed as $\mu\text{moles/hr.}$ Aspartate aminotransferase is given as $\mu\text{moles/min.}$ Cytochrome *c* oxidase is given as the first-order rate constant, k , in units of common logarithms and minutes. Protein refers to mg. of solubilized protein as determined by the method of Warburg & Christian (1941). The results are expressed as means $\pm$ s.d., with the numbers of animals in parentheses.

Enzyme	Activity (units/g. wet wt. of tissue)	
	Uterus	Liver
Deoxyribonuclease	47.6 ± 23.3 (19)	28.0 ± 10.4 (8)
Acid cathepsin	59.5 ± 6.3 (25)	76.8 ± 9.1 (9)
β -Galactosidase	5.6 ± 1.9 (14)	5.9 ± 0.2 (9)
Acid phosphatase	178 ± 48 (25)	576 ± 57 (9)
β -Glucuronidase	22.5 ± 5.4 (18)	132 ± 49 (9)
Cytochrome <i>c</i> oxidase	49.1 ± 28.9 (14)	1760 ± 802 (9)
Aspartate aminotransferase	521 ± 90 (14)	8450 ± 1360 (9)
Protein	40.1 ± 6.2 (14)	116 ± 10 (9)

Enzymic activity of the non-gravid uterus. Table 1 presents a comparison of some enzyme activities in the non-gravid uterus with activities found in liver tissue from the same animals. Deoxyribonuclease II, acid cathepsin, β -galactosidase, acid phosphatase and β -glucuronidase were selected as examples of acid hydrolases normally associated with lysosomes. It is clear that the uterus contains high activities of these hydrolases. In fact, there is more deoxyribonuclease in the uterus than in liver. Cathepsin and β -galactosidase concentrations are similar in the two tissues, whereas uterine acid-phosphatase and β -glucuronidase activities are about one-quarter those of the liver. These high activities in the uterus are further magnified if they are expressed on the basis of soluble protein, since much of the uterus weight is due to water and insoluble protein. On the other hand, the mitochondrial enzyme cytochrome *c* oxidase and the predominantly soluble enzyme aspartate aminotransferase are found in uterus to the extent of only one-fifteenth to one-thirtieth the activities found in liver. This indicates that the hydrolytic function of the uterus is highly developed in comparison with some of its other metabolic functions. This hydrolytic function is further increased in the post-partum period.

Changes in enzyme concentrations in pregnancy and post partum. The results for the acid hydrolases fall into three general patterns. Cathepsin (Fig. 3) and β -glucuronidase (Fig. 4) are noticeably depressed in concentration during pregnancy. The growth of enzyme fails to keep up with the growth of

Fig. 3. Cathepsin concentration in the rat uterus. The horizontal line indicates the mean activity of the non-gravid uterus (see Table 1 for details and standard deviation). The average value during pregnancy is significantly different from the value for the non-gravid uterus ($P < 0.001$).

Fig. 5. β -Galactosidase concentration in the rat uterus. The horizontal line indicates the mean activity of the non-gravid uterus.

Fig. 4. β -Glucuronidase concentration in the rat uterus. The horizontal line indicates the mean activity of the non-gravid uterus (see Table 1). The average value during pregnancy is significantly lower than the value for the non-gravid uterus ($P < 0.01$).

Fig. 6. Acid phosphatase concentration in the rat uterus. The horizontal line indicates the mean activity of the non-gravid uterus. The average value during pregnancy is significantly higher than the value for the non-gravid uterus ($P < 0.02$).

weight. But, starting at term, there is a rapid rise in the concentration of these enzymes and a peak is reached about 4 days (100hr.) *post partum*. The increase results in maximum concentrations about 3-3.5 times the normal level or 4-5 times that found at the end of pregnancy: the return to normal concentrations is prolonged and is not yet complete by 12 days *post partum*. β -Galactosidase (Fig. 5) and acid phosphatase (Fig. 6) follow a somewhat different pattern in that they are not appreciably decreased during pregnancy and may even be slightly elevated towards the end of pregnancy. However, they resemble the first group in showing a rapid rise in concentration immediately *post partum*. β -Galactosidase increases to about 3 times its normal concentration, but acid phosphatase shows only a little more than 2-fold increase. The return to normal values is prolonged, as in the first group, but acid phosphatase appears to return more rapidly than the others. The final hydrolase, deoxyribonuclease, behaves differently (Fig. 7). Its concentration is considerably decreased during pregnancy, but the increase in the post-partum period does little more than restore the concentration of the enzyme to normal. There is also a considerable scatter in the results for this enzyme.

Fig. 7. Deoxyribonuclease II concentration in the rat uterus. The horizontal line indicates the mean activity of the non-gravid uterus. The average value during pregnancy is significantly lower than the value for the non-gravid uterus ($P < 0.01$).

Still other patterns of change are found for the non-hydrolytic reference enzymes. Aspartate aminotransferase (Fig. 8) displays a wide spread of values, but there is no significant change in concentration during pregnancy or *post partum* (except for a possible decrease in early pregnancy). This enzyme seems to be directly proportional to the wet weight of tissue. Cytochrome *c* oxidase (Fig. 9) shows no change during pregnancy, but does increase during involution. About a 2-fold maximum increase is found, indicating a slower loss of mitochondria than of wet weight.

Immediate post-partum period. The most striking changes in enzyme concentration occur within 100hr. after delivery. To show these changes more clearly, this region of Fig. 3 has been enlarged to give Fig. 10. Clearly the results can be fitted by a simple linear regression line of the form:

$$y = ax + b$$

The same approach can be applied to acid cathepsin, acid phosphatase and β -galactosidase. Table 2 summarizes these results.

Up to this point enzyme activities have been treated in terms of concentration, i.e. enzyme units/wet wt. of tissue. To illustrate the behaviour of these enzymes on the basis of total units/uterus, the results for β -glucuronidase are given in Fig. 11.

Fig. 8. Aspartate aminotransferase (glutamate-oxaloacetate transaminase) concentration in the rat uterus. The horizontal line indicates the mean activity of the non-gravid uterus.

The general pattern resembles wet weight (Fig. 1) at first glance. There is a rise during pregnancy to a value 7-8 times normal, followed by a fall in the post-partum period. However, the two curves do not coincide. The enzyme increase lags behind that of wet weight during pregnancy; this results in a depressed concentration in this period compared with normal. And in the post-partum period the decline in enzyme/uterus is greatly protracted,

requiring about 12 days to reach the base line. It is clear, then, that the increased β -glucuronidase concentration during involution is not due to a net increase in the total units of enzyme/uterus, but rather to an increase in enzyme relative to the wet weight. This statement also applies to the cathepsin, acid phosphatase and β -galactosidase increases. The increases in enzyme concentration may be due simply to a conservation of certain

Fig. 9. Cytochrome *c* oxidase concentration in the rat uterus. The activity is expressed as a first-order rate constant, *k*. The horizontal line indicates the mean activity of the non-gravid uterus.

Fig. 10. Cathepsin concentration in the period immediately after parturition (0-100hr.). The straight line has been fitted to the points as described in Table 2.

Table 2. *Change in uterine enzyme concentrations during the first 100hr. post partum*

The activity of each enzyme was plotted against the time (hr.) *post partum* (e.g. Fig. 10). A straight line was fitted statistically to the data by the method of least squares, assuming errors in activity but not in time. The resulting equations were of the form: activity = *at* + *b*, where *a* is the slope, *t* is time (hr.) and *b* is the intercept at 0hr. The 100hr. activity was calculated from the equation and corresponds approximately to the time when maximal activity is reached during involution. The zero-time and 100hr. activities are expressed as μ moles/hr./g. wet wt. of tissue.

Enzyme.....	β -Glucuronidase	Acid cathepsin	β -Galactosidase	Acid phosphatase	Deoxyribonuclease II
Zero-time activity	19.10	47.21	6.40	157.3	55.96
100hr. activity	79.20	175.91	19.01	380.7	67.75
Factor of increase $\frac{(100hr.)}{(0hr.)}$	4.15	3.73	2.97	2.42	1.21
Slope of straight line	0.6010	1.287	0.1261	2.234	0.1181
Coefficient of correlation	0.92	0.89	0.86	0.85	0.18
Number of points	72	76	53	73	45

Fig. 11. Total activity of β -glucuronidase/rat uterus. The horizontal line indicates the mean total activity/non-gravid uterus.

enzymes while other tissue components are lost. Alternatively, there may be increased synthesis of certain enzymes to offset the losses due to tissue breakdown.

Subcellular distribution of enzymes. The homogenization procedure outlined for uterus preparation (see the Methods section) was first applied to rat livers to test whether subcellular distributions obtained by this method were comparable with results published in the literature. The average distribution for the five acid hydrolases was about 18% in the nuclear and debris fraction, 54% in the mitochondrial-lysosomal fraction and 28% in the supernatant-microsomal fraction. The activities in the debris and supernatant point to incomplete disintegration of the tissue and rupture of parts of the lysosomes. Thus the homogenization is not ideal, but it is not too dissimilar to classical results for liver. A typical result for a uterine hydrolase is shown in Fig. 12. Only about 14% of the uterine cathepsin is recovered in the mitochondrial-lysosomal fraction of homogenates of the non-gravid uterus. A maximum of about 27% is found in this fraction by the end of involution (4 days *post partum*). Before it is concluded that the bulk of the acid hydrolases of the uterus is not in the lysosomes, the results for cytochrome *c* oxidase should also be taken into account.

When liver is homogenized by the procedures used in the present work, less than 1% of the total

Fig. 12. Subcellular distribution of rat-uterus cathepsin. The experimental details of fractionation are given in the Methods section. The bars are divided into three segments corresponding to 600g pellet (bottom), 15000g pellet (middle) and supernatant (upper). The percentage of the total activity that is found in the 15000g fraction (mitochondria plus lysosomes) appears on the bar. Each bar represents the average of 12-20 experimental points. N, Non-gravid; P, pregnancy; 0-1 includes all points between 0 and 24hr. *post partum*, and similarly for the other groups.

cytochrome *c*-oxidase activity is found in the supernatant-microsomal fraction, indicating that minimal disruption of mitochondria is occurring. However, when the non-gravid uterus is homogenized, about 40% of this enzyme appears in the supernatant fraction (Fig. 13), indicating considerable rupture of mitochondria. During involution the tissue becomes somewhat easier to homogenize, and a much larger proportion of the cytochrome *c* oxidase appears where it belongs, in the mitochondrial fraction. But at least 15% of the activity always remains in the supernatant fraction. In view of the extensive damage to mitochondria, considerable caution must be used in interpreting the results with respect to lysosomes.

Liver acid hydrolases. The livers of the experimental animals from the post-partum period were fractionated in a manner similar to the uteri. In contradistinction to the uterus, the liver showed no significant changes in the concentrations of cathepsin, β -glucuronidase and acid phosphatase. Deoxyribonuclease and aspartate aminotransferase also remained unchanged. β -Galactosidase increased to about 25% above normal and cytochrome *c* oxidase was 50% higher (2650 units/g. wet wt. of

Fig. 13. Subcellular distribution of cytochrome c oxidase. For details see the legend of Fig. 12.

tissue). With regard to the subcellular distribution of these seven enzymes, there were no significant differences between normal livers and livers from rats in the post-partum period. A possible exception is deoxyribonuclease, which may occur in a somewhat higher proportion in the lysosomal-mitochondrial fraction during the post-partum period.

DISCUSSION

Lysosome theory and uterine involution. The lysosomal theory of intracellular digestion and its possible relationship to uterine involution are discussed in the introduction. The hypothesis that the processes of uterine involution involve lysosomal hydrolases has been tested by measuring quantitatively the behaviour of certain hydrolases characteristically found in lysosomes and by attempting to isolate lysosomal particles from the rat uterus.

The quantitative studies of the acid hydrolases (cathepsin, β -glucuronidase, β -galactosidase, acid phosphatase and deoxyribonuclease II) have given results that are compatible with the hypothesis that the acid hydrolases are active in catabolic processes within the uterus. This is seen most strikingly during post-partum involution, when all of the acid hydrolases, except deoxyribonuclease, show a 2-4-fold increase in concentration. The peak concentrations are reached about the same time that the wet weight and collagen have fallen

to their lowest values. The failure of deoxyribonuclease to show much change may be related to the observation that there is little change in cell numbers (and hence of DNA) during pregnancy and involution (Maibenco, 1960).

On the other hand, in pregnancy there is a tendency for several of the hydrolases, especially acid cathepsin and β -glucuronidase, to lag behind the growth of other uterine constituents. This results in a decreased enzyme concentration. Since this period is one of rapid synthesis of uterine components, there may be less call for hydrolytic processes, although they would scarcely be excluded altogether.

If it is true that the acid hydrolases are involved chiefly in catabolism, then one would expect that catabolic processes would be important even in the non-gravid uterus. This follows from the observation that the five acid hydrolases all had high activity in the non-gravid uterus and compared favourably with a tissue as metabolically active as the liver. Kao, Hilker & McGavack (1961) found that collagen, which accounts for 15-20% of the protein of the uterus, shows a high rate of turnover in the non-gravid uterus in comparison with the collagen of other tissues of the rat.

The first part of the study has shown that acid hydrolases increase in concentration during involution; the second part of the study has yielded equivocal results. The attempt to show that these acid hydrolases, reported as having a lysosomal distribution in the liver and other tissues, are localized predominantly in lysosomes in the uterus has not met with great success. It is certain from the results that at least part of the activity of uterine cathepsin, β -glucuronidase, β -galactosidase, acid phosphatase and acid deoxyribonuclease resides in lysosomal particles. However, the homogenization procedure required to disrupt the tough fibrous tissue is so vigorous that it probably disrupts most of the lysosomes. Certainly the 40% rupture of mitochondria in the non-gravid uterus is a good indicator of the harshness of the homogenization conditions. That the fault lies with the tissue, and not with the homogenizing apparatus, is shown by the relatively good recoveries of mitochondria and lysosomes from rat liver homogenized in the same apparatus. Wakid (1960) and Wakid & Needham (1960) have also drawn attention to the difficulties involved in preparing subcellular fractions of rat-uterus tissue. In their experience, only 60% of the mitochondrial enzyme succinate dehydrogenase was recovered in the mitochondrial fraction prepared from uteri at the end of pregnancy; 31% was recovered in the microsomal fraction and 9% in the supernatant.

On the basis of the present results, not more than 25% of the acid-hydrolase activities are found in the

lysosomes. However, Lobel & Deane (1962) have presented histochemical evidence that most of the acid phosphatase of the uterus is in the form of granules resembling lysosomes in their size and distribution, and β -glucuronidase also has a granular distribution in the rat-uterus cells (Hayashi, 1964). O'Dell (1962) presented evidence that the cathepsin of involuting pig uterus is lysosomal. When one considers the five hydrolases studied in the present work with respect to their general subcellular distribution in a large variety of other tissues (de Duve, Wattiaux & Baudhuin, 1962) it is seen that almost without exception they are localized chiefly in the lysosomal fraction. It is to be expected that, with the development of more successful fractionating methods for the uterus, this tissue will follow the general pattern, with the acid hydrolases largely in the lysosomal fraction.

Though the present results on lysosomes have little quantitative significance, they show qualitatively that, if any change is occurring in involution, it is probably in the direction of an increasing proportion of enzyme activity within the lysosomes. Both the total enzyme activity in the lysosomal fraction and the proportion of the total enzyme activity of the tissue recovered in the lysosomal fraction increase during involution (Fig. 12). These results may be partly due to artifacts, as a result of changes in resistance of the tissue to homogenization, but in any event the condition at the end of involution cannot be typified as a state of complete discharge of the lysosomal contents. Further evidence for an increase in lysosomes during uterine involution is presented by Lobel & Deane (1962), who found a marked increase in acid-phosphatase-staining granules. An increasing concentration of lysosomes as involution proceeds would correspond to the situation observed in many other cases of tissue resorption, e.g. tadpole tail resorption (Eeckhout, 1964).

Given that acid hydrolases increase in concentration during involution and that this increase is probably due to an increase in lysosomes, how can we relate this to tissue breakdown? Why is the maximum activity attained at the end of the process rather than at the beginning? What causes the increase in lysosomes? The present results show that the increased concentration of acid hydrolases need not be attributed to a sudden synthesis of enzyme. Fig. 11 shows that there is never more total hydrolase activity present than at the time of parturition, so that any mechanism which merely conserves enzyme (lysosomes) during involution could account for the increased concentration. The ultimate expression of this is found in the resorbing tadpole tail, where there is a 30-fold increase in lysosomal acid-cathepsin con-

centration with little net gain in total units of enzymes (Weber, 1957). However, if the enzyme is conserved, how does it play a role? Lysosome theory would require a discharge of enzyme into digestive vacuoles of uterine cells. de Duve (1964) proposes that the lysosomes may function repeatedly, digesting many 'meals' without losing their enzyme complement. At the end of this process considerable enzyme activity may still persist, though much tissue material has been digested and removed. However, it is also possible that the cells continue to synthesize hydrolases during the involution period. One of the key experiments still to be performed is the determination of the turnover rates of some of the acid hydrolases in situations of rapid tissue breakdown.

There are probably two main cell types playing the major role in uterine involution. The smooth-muscle cells undergo hypertrophy during pregnancy, and during involution they appear to lose cytoplasm without evidence of cell death or degeneration (Maibenco, 1960). Presumably, autophagic digestion of cell components occurs completely within the muscle cell. The other prominent cell type is the macrophage, which increases in number during involution and reaches a maximum concentration at 4 days *post partum* (Lobel & Deane, 1962). It no doubt plays an important role in removing extracellular materials by heterophagy. In view of the macrophage's known content of acid hydrolases (Cohn & Wiener, 1963a) and the observed increase in macrophages during involution, it is likely that much of the increase in enzyme concentration of the involuting uterus is due to the macrophages. The presence of several cell types in the uterus explains, in part, why three different behaviour patterns were displayed by five presumably lysosomal enzymes. But even within one cell type the lysosomes are probably heterogeneous as to enzyme content.

Nature and role of the acid cathepsin. It is, of course, impossible to show from the present type of study a direct cause-and-effect relationship between any one of the hydrolases and the loss of a specific chemical component of the uterus. Nonetheless, the possible role of the acid cathepsin in the breakdown of the uterine proteins, especially of collagen, can be considered. The uterine cathepsin is not a collagenase, i.e. it does not digest native collagen at neutral pH. Rather, it is a general proteolytic enzyme that will also digest collagen if the pH is lowered to the range pH 3-4. The rat-uterus cathepsin has not been extensively characterized, but it is probably similar to the cow-uterus cathepsin. The latter enzyme has been extensively purified and has been shown (Woessner, 1965) to be identical in specificity with cathepsin D of bovine spleen (Press, Porter & Cebra, 1960). It is

not related to the lysosomal collagenase of liver reported by Frankland & Wynn (1962), since this latter enzyme is apparently not a proteolytic enzyme (Wynn, 1963). For the uterine cathepsin to be implicated in the breakdown of native collagen it would be necessary to introduce an acid environment. This could conceivably occur at a cell-fibre interface or in a digestive vacuole, although there is no strong evidence to support this idea.

Gross, Lapiere & Tanzer (1963) have proposed a mechanism for collagen breakdown in the resorption of the tadpole tail in metamorphosis. The tadpole tail does not seem to contain any detectable collagenase, but, like the uterus, it contains a considerable concentration of acid-cathepsin activity (Weber, 1957). However, if slices of tadpole tail are cultured on gels of reconstituted collagen, a collagenase is secreted extracellularly and diffuses away from the tissue. This enzyme attacks fibrous collagen at physiological pH values. Cultured slices of rat uterus appear to elaborate a similar enzyme (Gross *et al.* 1963), but details of these experiments have not been published. Such an enzyme could account for the extracellular digestion of collagen in uterine involution.

In view of this report, considerable effort has been expended searching for such a collagenase in homogenates of involuting uterus. Since the results to date have been entirely negative, they are not detailed in the present paper. Tissues have been homogenized by a variety of methods, in a variety of buffers and with the addition of possible stabilizers or activators such as Ca^{2+} , EDTA, thiols, proteases etc. The homogenates have been incubated with collagen that was prepared from the uterus or reconstituted from rat-tail tendon. An amount of tissue that *in vivo* would cause the breakdown of collagen and release of 80 μg . of hydroxyproline/hr., when homogenized and incubated at neutral pH for 24 hr. with collagen containing 1500 μg . of hydroxyproline, will not release any detectable hydroxyproline (limit of detection 3 μg .).

Gross & Lapiere (1962) failed to find a collagenase in homogenates of tadpole tail, and they attributed such failure to the presence of a labilizing, or inhibitory, principle in the tissue. Thus the enzyme can be demonstrated only when it is in a situation where it can leave the cell and diffuse away from the tissue. Such a hypothesis might also account for the failure to find a collagenase in uterine homogenates. It may well be that such a collagenase is responsible for the initial disruption of the collagen fibres in involution.

However, it has been shown by Luse & Hutton (1964) that in the involuting rat uterus collagen is taken up into phagocytic cells in the form of large

fragments identifiable as collagen by their characteristic cross-striations. Even in the tadpole tail, Gross *et al.* (1965) have found collagen in vacuoles within macrophage-type cells of the tail. Though it is possible that the vacuoles may contain newly synthesized collagen, this picture of collagen fragments inside cells is reminiscent of the usual intracellular digestive processes seen in a variety of phagocytic cells. In the macrophages (Cohn & Wiener, 1963b), and in most of the other cases investigated to date, such intracellular digestion clearly involves the acid hydrolases of the lysosomes and presumably involves an acid pH within the vacuoles. Therefore it would be premature to dismiss the acid cathepsin of the uterus from a consideration of the processes of collagen breakdown, even if it should prove to be of no importance in the earliest stage of attack on the intact extracellular fibre.

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