Lysosomal Enzyme Changes in Enforced Mammary-Gland Involution

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1. Changes occurring in the activities and latency of acid ribonuclease and β -glucuronidase have been studied in rat mammary gland during the early phases of mammary involution. 2. It was found that there were rapid increases in the proportions of both enzymes in the 'free' (i.e. unsedimentable) form. The total activities of both enzymes did not show the rapid decrease characteristic of most other enzymes studied during mammary-gland involution. 3. The effects of pre-treating the rats with reserpine or the antihistamine drug Phenergan on these lysosomal changes was studied. Neither treatment affected the enzymic redistribution that occurs during early involution. 4. It was concluded that lysosomal rupture occurs as an early manifestation of mammary-gland involution. Although reserpine treatment preserves the histological appearance of the gland and, apparently, inhibits involution, this drug did not prevent the lysosomal changes normally found in suspensions.

The results of Slater & Greenbaum (1965) indicated that the experimental induction of acute liver necrosis is unaccompanied, in the early stages at least, by biochemically detectable damage to the lysosomes. It was concluded that lysosomal changes have but little significance in the early mechanisms of such forms of experimental toxic liver injury (Slater & Greenbaum, 1965). However, this conclusion cannot be generalized to cover all cases of tissue injury, for it has been clearly shown that lysosomal damage occurs as an early feature of several regressive situations. For example, de Duve & Beaufay (1959) have shown that the proportions of lysosomal enzymes appearing in unsedimentable forms in the left hepatic lobe increase shortly after ligation of the vasculature supplying that lobe. Weber (1963) has provided evidence to support the view that lysosomal rupture occurs as an early manifestation of metamorphosis in the tadpole, and Schrieb (1963) has reported similar conclusions on the regression of the Mullerian ducts in the chick embryo.

Thus, at present, so far as lysosomal participation is concerned, there appear to be two types of tissue response to injury: one proceeding independently of significant lysosomal damage (Slater & Greenbaum, 1965) and another in which lysosomal rup-

ture occurs early in the course of the injury (de Duve & Beaufay, 1959; Weber, 1963; Schrieb, 1963).

In attempts to understand more clearly the differences in the mechanisms that result in this division, we have studied lysosomal changes occurring in mammary-gland involution. The problems posed by involution are of interest in several ways. For instance, the tissue regression that occurs is on a large scale, commences rapidly after removal of the litter and is under tight physiological control; further, the destruction that occurs is spatially confined and, at least in the early stages, is reversible by reintroduction of the pups. Mammary-gland involution therefore provides a good system for studying the role of lysosomes in a physiologically induced tissue regression, a type of injury worthy of careful comparison with the previously investigated experimentally produced toxic liver injuries (Slater & Greenbaum, 1965). Further, by using a treatment to inhibit involution after withdrawal of the litter (Benson, 1958, 1960), or by reintroducing the pups to re-establish lactation, there seemed the possibilities of studying the hormonal control of lysosomal stability and the reversibility of lysosomal changes that occur in such situations. The present paper deals with work towards these aims.

METHODS

Animals and treatment. The rats used were primiparous albino females, body wt. approx. 200 g.; they were fed on a standard diet and water *ad libitum*. Involution was induced by removing litters from rats on the sixteenth day of lactation. The rats were killed by cervical dislocation and the abdominal and inguinal glands were quickly removed, weighed and cooled in ice-cold 0.25 M-sucrose. The glands were normally homogenized by passing through an Emanuel & Chaikoff (1957) homogenizer, twice with a piston clearance of 64 μ , and once at 25 μ . Intractable connective tissue was removed by straining through cheese-cloth after the second 64 μ stage. The tissue suspension was then diluted with cold 0.25 M-sucrose to a volume equal to five times the original wet wt. of the gland. In experiments aimed at inhibiting involution by prior treatment with drugs, rats were injected intraperitoneally 18 hr. before weaning and then twice a day for the duration of the experiment. The drugs used (and doses/injection) were N^{10} -(2-dimethylamino-*n*-propyl)phenothiazine hydrochloride (Phenergan; 1.25 mg./100 g. body wt.) and reserpine (50 μ g./rat).

Mammary-gland estimations. Acid ribonuclease was measured by the procedure of Slater (1961); β -glucuronidase was measured essentially by the procedure of Gianetto & de Duve (1955). However, in the presence of high contents of milk in involuting mammary gland, there was considerable variation in the normal β -glucuronidase blanks. This was overcome by using as blanks the test solutions themselves after decolorization of the phenolphthalein with acid. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Serum estimations. Acid ribonuclease and β -glucuronidase were measured in serum samples by the procedures given by Slater & Greenbaum (1965).

RESULTS

Table 1 shows the results obtained for acid ribonuclease in rat mammary gland during late lactation and early enforced involution. Although the total activity/gland fluctuates during the first few days of involution, there is a considerable and rapid rise in the free activity/gland over this period

(Table 1, column 5). By the second day of involution the free activity/gland is almost three times the value obtained during late lactation.

The results in Table 1 (columns 4-6) were obtained after homogenizing the glands as described in the Methods section. In several cases, however, the final passage through the Emanuel-Chaikoff homogenizer was made with a 17 μ aperture rather than the routine 25 μ aperture. This had the effect of increasing the proportion of acid ribonuclease in the unsedimentable (i.e. free) form. Table 1 (column 7) gives the results obtained with the 17 μ aperture. A pattern of change in the bound/free ratio similar to that with homogenates prepared with the 25 μ aperture was found, although the bound/free ratios were numerically lower.

Table 2 gives the corresponding results for β -glucuronidase. Unlike acid ribonuclease, this enzyme showed an increased total activity/gland during involution. Even so, the bound/free activity ratio showed a decrease during involution (Table 2, column 6).

Table 3 gives the results obtained for acid ribonuclease and β -glucuronidase in mammary-gland suspensions after 2 days of involution with concomitant treatment with either Phenergan or reserpine. Neither drug had any effect in preventing the changes in the bound/free activity ratios that are associated with the development of the involutionary process. The activities of acid ribonuclease and β -glucuronidase in sera obtained from rats on the first day of involution are also included in Table 3; normal values are given for comparison.

DISCUSSION

The presence in mammary-gland tissue obtained from lactating rats of particles having properties similar to the lysosomes of rat liver was first demonstrated by Greenbaum, Slater & Wang (1960). It

Table 1. *Changes in the acid-ribonuclease activity of rat mammary gland at different stages of mammary involution*

Status	No. of rats	Gland wt. (g.)	Total activity (units/gland)	Free activity (units/gland)	Bound activity	
					Free activity	Free activity (17 μ orifice)
Late lactation	5	6.8 $\pm$ 0.70	834 $\pm$ 132	141 $\pm$ 30	5.25 $\pm$ 0.89	1.25 $\pm$ 0.16 (4)
12 hr. of involution	4	11.2 $\pm$ 0.84	648 $\pm$ 96	289 $\pm$ 74	1.92 $\pm$ 0.59	—
1 day of involution	5	12.0 $\pm$ 0.83	576 $\pm$ 6	202 $\pm$ 15	2.01 $\pm$ 0.27	0.67 $\pm$ 0.12 (4)
2 days of involution	5	9.8 $\pm$ 0.52	728 $\pm$ 84	373 $\pm$ 43	0.88 $\pm$ 0.11	0.49 $\pm$ 0.07 (4)
3 days of involution	5	4.2 $\pm$ 0.93	493 $\pm$ 114	303 $\pm$ 76	0.55 $\pm$ 0.09	—

The animals were primiparous females and the litters were restricted to eight pups. For the 'late lactation' group animals were killed on the sixteenth and seventeenth days of lactation. For the involution groups the pups were weaned on sixteenth day of lactation. The number of rats used in experiments with the 17 μ orifice (column 7) are shown in parentheses. The units of acid-ribonuclease activity are expressed as change in $E_{260\text{ m}\mu}$ /hr. under the conditions of assay (see the Methods section). The results are given as means $\pm$ S.E.M.

Table 2. *Changes in the β -glucuronidase activity of rat mammary gland at different stages of mammary involution*

The animals were primiparous females and the litters were restricted to eight pups. For the 'late lactation' group animals were killed on the sixteenth or seventeenth day of lactation. For the involution groups the pups were weaned on the sixteenth day of lactation. The units of β -glucuronidase activity are expressed as μ g. of phenolphthalein liberated/hr. under the conditions of assay (see the Methods section). The results are given as means $\pm$ S.E.M.

Status	No. of rats	Gland wt. (g.)	Total activity (units/gland)	Free activity (units/gland)	Bound activity Free activity
Late lactation	13	6.66 $\pm$ 0.57	1274 $\pm$ 217	781 $\pm$ 157	0.82 $\pm$ 0.16
1 day of involution	3	11.9 $\pm$ 0.80	3080 $\pm$ 510	1940 $\pm$ 333	0.64 $\pm$ 0.30
2 days of involution	8	7.5 $\pm$ 1.11	2140 $\pm$ 244	1642 $\pm$ 171	0.31 $\pm$ 0.09
3 days of involution	9	4.1 $\pm$ 0.52	3260 $\pm$ 280	2960 $\pm$ 311	0.13 $\pm$ 0.03

Table 3. *Effect of Phenergan and of reserpine on the bound/free activity ratios of β -glucuronidase and acid ribonuclease in involuting rat mammary gland*

The units of β -glucuronidase activity and acid-ribonuclease activity are expressed as μ g. of phenolphthalein liberated/hr. and increase in $E_{260\text{ m}\mu}$ /hr. respectively under the conditions of assay (see the Methods section). The results are given as means $\pm$ S.E.M., with the numbers of rats in parentheses; where only two estimations were performed the individual values are quoted.

Status	Treatment	Mammary-gland		Serum activity (units/ml.)	
		$\frac{\text{bound activity}}{\text{free activity}}$			
		β -Glucuronidase	Acid ribonuclease	β -Glucuronidase	Acid ribonuclease
Normal female	None	—	—	34 $\pm$ 7 (5)	13.02 $\pm$ 1.54 (7)
1 day of involution	None	0.64 $\pm$ 0.30 (3)	2.01 $\pm$ 0.27 (5)	50 $\pm$ 9 (4)	16.92 $\pm$ 3.33 (4)
	Phenergan	0.60, 0.30	2.0, 1.3	100 $\pm$ 12 (4)	14.40 $\pm$ 2.28 (4)
2 days of involution	None	0.31 $\pm$ 0.09 (8)	0.88 $\pm$ 0.11 (5)	—	—
	Phenergan	0.00, 0.05	0.85, 0.45	—	—
	Reserpine	0.19 $\pm$ 0.06 (4)	0.83 $\pm$ 0.10 (4)	—	—

was shown that, provided that the homogenization step was carefully controlled, β -glucuronidase and acid ribonuclease (and cathepsin and β -galactosidase; T. F. Slater & A. L. Greenbaum, unpublished work) were largely associated with particles that exhibited the property of latency (see de Duve, 1959). A recent publication has amplified the previous findings (Slater, Greenbaum & Wang, 1963).

Shortly after removing the litter from a rat in full lactation, extensive changes occur in the metabolism of the mammary gland. There are changes over 4 days in the activities of many important enzymes [e.g. glutamate-aspartate transaminase (Greenbaum & Greenwood, 1954), succinate oxidase (Greenbaum & Slater, 1957), glucose 6-phosphate dehydrogenase (McLean, 1958)] and in the concentrations of many cofactors [e.g. NAD and NADP (Glock & McLean, 1959), acid-soluble ribonucleotides (Greenbaum & Wang, 1960)]. In fact, of all the enzymes and other components studied during early involution, few fail to decrease in amount or activity. Three enzymes that are exceptions to this

generalization are the lysosomal acid hydrolases, β -glucuronidase and cathepsin (Greenbaum & Greenwood, 1954) and acid ribonuclease (Slater, 1961). This fact, that lysosomal enzyme activity does not appreciably decrease (and might even increase) during the first few days of involution, suggested the hypothesis that the lysosomes were closely concerned in the processes of mammary-gland involution (see Slater *et al.* 1963). The increased activity of lysosomal hydrolases is probably the result of several factors. First, there can be an increase in the amount of enzyme present owing to an induced synthesis or a decreased catabolism. The latter possibility seems the less likely, since lysosomal enzymes apparently 'turn over' relatively slowly in normal tissue (Von Lancker, 1964). Secondly, milk is known to contain several acid hydrolases and milk retention increases markedly during the first 24 hr. of involution. Thirdly, there is probably a contribution arising from an increased number of tissue macrophages in early involution (Okada, 1957). However, this cannot be large since histological examination of the tissue shows the

macrophage invasion to be only moderate. This contribution is particularly difficult to assess quantitatively (Slater, 1962). A similar condition has been reported in dystrophic rabbits where a large increase in acid-hydrolase activity occurs concomitantly with macrophage invasion (Zalkin *et al.* 1962).

The results given in Tables 1 and 2 show that there are rapid changes in the bound/free activity ratios obtained for acid ribonuclease and β -glucuronidase. The bound/free activity ratio (see de Duve, 1959) is a measure of the integrity of the lysosomal membrane. A decrease in this ratio indicates an increased availability of enzyme to substrate, often as the result of liberation of enzyme into a soluble form that is not sedimented by, for example, centrifuging at 3×10^6 g-min. Thus it could appear that lysosomal disturbance is an early feature of mammary-gland involution. It is possible, however, to explain the results given in Tables 1 and 2 in an alternative way that would depend on the lysosomes present in mammary-gland tissue becoming increasingly fragile to homogenization as involution proceeds.

Such a possibility is difficult to eliminate, but some results bearing on this point are presented in Table 1 (column 7). In these experiments, the mammary-gland tissue was subjected to a rather more vigorous homogenization procedure than that normally used, by passing the suspension through the Emanuel-Chaikoff homogenizer with a 17μ clearance. This produced a substantial breakage of lysosomal particles compared with the routine 25μ procedure. Further, the greatest decrease in the bound/free activity ratio that occurred as a result of the smaller clearance was in the late lactating group. These results therefore provide some evidence against the possibility that there is an increased lysosomal fragility during early involution.

The bound/free activity ratio obtained for β -glucuronidase at all of the stages studied of lactation and involution was far smaller than the corresponding ratio for acid ribonuclease (Tables 1 and 2). There are two main possible contributory factors that could result in this difference. First, β -glucuronidase is not wholly associated with the lysosomes in rat liver but also occurs in the microsomal fraction (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955); the latter activity does not exhibit latency. Thus, if a considerable proportion of the total activity of β -glucuronidase in mammary gland were associated with the microsomes, this would lead to a low bound/free activity ratio. Secondly, damage to the lysosomes of mammary gland could result in a preferential release of β -glucuronidase compared with acid ribonuclease. A situation has been reported for this type of pre-

ferential release by Tappel, Sawant & Shibko (1963), studying the effects of free-radical systems on lysosomal integrity. It is impossible with present data to decide the relative contributions of these mechanisms that result in a low bound/free activity ratio for β -glucuronidase.

A variety of treatments (see Folley, 1961) has been shown to slow (i.e. inhibit) the processes of mammary-gland involution after removal of the litter; for example, oxytocin (Benson & Folley, 1956) and reserpine administration (Benson, 1960) both have this effect. With reserpine, it was shown (Benson, 1960) that administration of 50μ g. daily for 9 days after removing the litter from lactating rats significantly maintained the proportion of alveolar parenchymal cells when compared with control animals. It was decided in the present investigation to determine whether reserpine exerted this 'protective action' by decreasing the degree of lysosomal damage that has been shown to be an early feature of mammary-gland involution. We have also studied the effects of Phenergan treatment on lysosomal changes in involution in view of the protective action of this substance on experimental liver injury (Rees, Sinha & Spector, 1961).

The results of Table 3, however, show that neither reserpine nor Phenergan had any noticeable effect on the lysosomal changes that occur in early involution. Reserpine treatment has previously been shown (Darby, Wang & Greenbaum, 1964; Greenbaum & Darby, 1964) to result in only small modifications of the changes that occur in other biochemical parameters measured in suspensions of mammary-gland tissue in early involution. Thus the results obtained by biochemical measurements on suspensions give no indication of a protective action for reserpine on the lysosomal component in cells, in contrast with the established histological results (Benson, 1958, 1960).

From these results it is possible to suggest that considerable intracellular changes can occur in mammary-gland tissue despite apparent maintenance of the histological profile and appearance. Such a view has, of course, precedence in other types of tissue injury (e.g. carbon tetrachloride poisoning) where large-scale biochemical changes have been reported at times when no histological disturbance is apparent (e.g., see Slater, Sträuli & Sawyer, 1964; Smuckler & Benditt, 1962; Reynolds, 1963).

Finally, sera obtained from animals in early involution (Table 3) did not exhibit increased lysosomal enzyme activities despite the large-scale increases in free activities present in the gland. This contrasts with tissue injury produced by carbon tetrachloride where there are rapid increases in the serum activities of β -glucuronidase, acid phosphatase and acid ribonuclease (Slater & Greenbaum,

1965). Further, milk is known to contain significant amounts of β -glucuronidase, acid phosphatase, cathepsin and ribonuclease (Bingham & Zittle, 1962, 1963; T. F. Slater, unpublished work), so that early involuting mammary-gland tissue contains appreciable quantities of these enzymes in the free form. The absence of any rise in serum activities points to a selective permeability barrier being present between the gland and the blood, since other substances (e.g. lactose) are known to increase rapidly in the serum in involution.

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