Lysosomal Enzyme Changes in Growing and Regressing Mammary Tumours

By RAYMOND J. SHAMBERGER

Roswell Park Memorial Institute, Buffalo, N.Y. 14203, U.S.A.

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Rat mammary tumours induced by 7,12-dimethylbenz[*a*]anthracene can undergo repeated growth and regression during successive pregnancies. In a 10-day period after birth about half of the tumours regressed 50% or more. The concentrations of the lysosomal enzymes increased in regressing mammary tumours to the following multiples of the initial values: β -glucuronidase, 7.7; β -galactosidase, 3.9; cathepsin, 2.9; acid ribonuclease, 2.1; arylsulphatase A, 1.5; acid phosphatase, 1.4. In contrast, several non-lysosomal enzymes failed to increase. Activities in the post-partum uterus increased to the following multiples of the initial values: β -glucuronidase, 5.8; cathepsin, 5.5; acid ribonuclease, 4.3; β -galactosidase, 2.2; acid phosphatase, 1.8. Arylsulphatase A in the post-partum uterus decreased significantly, suggesting a non-lysosomal distribution or a special function related to pregnancy. No other significant changes were observed in the lysosomal or non-lysosomal enzymes in the hormone-independent liver or hormone-dependent normal mammary gland. The ratio of free to bound arylsulphatase A and acid ribonuclease decreased slightly 1–3 days after birth because of problems in homogenizing the tumours. At days 4–8, however, there was a dramatic increase in the ratio of the free to bound activities. The results can be explained in terms of the lysosomal theory of intracellular digestion.

Huggins and Dao have shown that rat mammary carcinoma induced by 3-methylcholanthrene or 7,12-dimethylbenz[*a*]anthracene is hormone-dependent, undergoing repeated growth and regression during successive pregnancies (Huggins, 1962; Dao & Sunderland, 1959). Pregnancy (Dao & Sunderland, 1959; Talalay, Fishman & Huggins, 1946; Huggins, Moon & Morii, 1962), prolactin (McCormick & Moon, 1967*a*), high doses of progesterone given to castrated animals (Dao & Sunderland, 1959; Huggins, Briziarelli & Sutton, 1959) and the suckling stimulus (McCormick & Moon, 1967*b*) enhance tumour growth. On the other hand, birth (Dao & Sunderland, 1959; McCormick & Moon, 1965), ovariectomy (Dao & Sunderland, 1959; Huggins *et al.* 1959), hypophysectomy (Sterental, Dominguez, Weisman & Pearson, 1963) and administration of oestrogen and progesterone mixtures (Huggins, 1962; Huggins *et al.* 1962) result in a decrease in tumour size and nearly complete regression of the tumour in many cases.

The growth and regression of hormone-dependent rat mammary carcinoma suggest a parallel with the behaviour of the mammary gland and the uterus, which are normal mammalian hormone-dependent tissues that grow and regress in connexion with

reversible physiological processes. The mechanism of regression of mammary carcinoma is largely unknown. Nevertheless, since both the hormone-dependent normal mammary glands and the uterus are known to undergo growth and lysosomal-enzyme-linked regression, increased lysosomal enzyme activity may also be associated with mammary carcinoma regression. Woessner & Brewer (1960) and Woessner (1965) have demonstrated striking increases in uterine lysosomal enzymes accompanying post-partum uterine involution. In contrast, several non-lysosomal enzymes failed to increase significantly. The mammary gland also undergoes similar widespread changes in metabolism. Shortly after removal of the litter from a lactating rat, there are abrupt decreases in the activity of many non-lysosomal enzymes including succinate dehydrogenase, arginase, alkaline phosphatase, 6-phosphogluconate dehydrogenase, hexokinase, phosphoglucose isomerase, glucose 6-phosphate dehydrogenase and glutamate-aspartate transaminase, and in many cofactors such as NAD⁺, NADH and ATP (Slater, Greenbaum & Wang, 1963), but the activity of lysosomal enzymes such as cathepsin and β -galactosidase is markedly increased. The ratio of free to bound lysosomal

enzyme activity is greatly increased, indicating that lysosomal membranes are being broken during involution of normal mammary glands (Greenbaum, Slater & Wang, 1965).

The present study was undertaken to determine whether the regression of 7,12-dimethylbenz[*a*]-anthracene-induced mammary tumours is associated with increased lysosomal enzyme activity. The activities of several enzymes known to be lysosomal in other tissues were determined in hormone-dependent growing and regressing 7,12-dimethylbenz[*a*]anthracene-induced mammary tumours and were compared with the lysosomal enzyme activities of several other tissues in the same animal: the normal hormone-dependent mammary glands, the hormone-dependent pregnant and post-partum uterus and, finally, the hormone-independent liver. The second part of this attempt to link tumour regression directly to changes in the ratio of free to bound activity has yielded equivocal results because of problems in homogenizing these tumours, but striking increases in lysosomal enzyme activity obtained during mammary-tumour regression are consistent with the hypothesis that these enzymes are involved in the regression process.

MATERIALS AND METHODS

Conduct of animal experiments

Female Sprague-Dawley rats from this Institute inbred colony were maintained on Purina laboratory chow and water *ad libitum*. At 50–55 days of age each rat was given one dose of 15 mg. of 7,12-dimethylbenz[*a*]anthracene in 0.4 ml. of sesame oil into the stomach by using a 1 ml. syringe fitted with a Teflon catheter. The solution of 7,12-dimethylbenz[*a*]anthracene in sesame oil was prepared by shaking the constituents and heating the mixture to a maximum temperature of 60° for 30 min. on a water bath. After the 7,12-dimethylbenz[*a*]anthracene had dissolved the solution was cooled to room temperature. At 20 days after completion of 7,12-dimethylbenz[*a*]anthracene administration male and female rats were kept together for 1 day. That day was ordinarily considered day 1 of pregnancy. All animals that did not appear to be pregnant at the end of 2 weeks were mated again in the same way. Mammary adenocarcinoma appeared in most of the animals during the ensuing pregnancy. The tumours were palpable and were measured with calipers. The long and the short diameters of non-symmetrical tumours were averaged.

In these experiments tumours were considered hormone-dependent if they decreased in diameter after parturition. Hormone-dependency of growing tumours could not be determined with certainty, because many such tumours continued to grow after birth (Fig. 1). Accordingly, a more stringent criterion was adopted for growing tumours. These tumours were followed after birth until they regressed, the site of each lesion being carefully noted. The animal was mated again. If a tumour reappeared in the same area during the ensuing pregnancy, this tumour was considered a hormone-dependent growing tumour.

Fig. 1. Comparisons of the volumes of 29 7,12-dimethylbenz[*a*]anthracene-induced mammary tumours in eight rats at parturition with their volumes at day 10 after parturition. The numbers of tumours and their respective percentage volume changes are indicated. Tumour growth is indicated by '+'; '-' indicates tumour regression; 'NC' indicates no change; 'New' indicates new tumours.

Tumours generally began growing at about day 16 of pregnancy, and continued to grow rapidly until term. Enzyme studies were carried out on animals killed on day 20 of pregnancy. Other groups of rats were killed on day 4 of lactation, when the tumours had regressed to about one-third to two-thirds of their original diameter.

Tissue preparations

At death, the liver, normal mammary tissue, growing or regressing mammary tumours and the uterus were excised and weighed. Each tissue was placed in a VirTis flask, and 10 ml. of 0.9% NaCl/g. of tissue was added. The tissues were homogenized with a VirTis model 45 homogenizer at high speed for 3 min. The homogenates were centrifuged in an International HR-1 high-speed centrifuge at 15000g for 30 min. The clear supernatant was decanted and was saved for protein and enzyme analysis. The completeness of the lysosomal enzyme release was checked by resuspending the precipitate in 0.9% NaCl. After the addition of a few drops of Triton X-100 this suspension was homogenized again with the VirTis homogenizer at high speed for 10 min. The resulting homogenate was centrifuged again at 15000g for 30 min. Less than 0.5% of the lysosomal enzyme activities were present in the second supernatant. All homogenizations, centrifugations and routine handling of the supernatant were carried out at 0–2°.

Enzyme assays and analytical procedures

The following lysosomal enzymes were assayed: β -galactosidase, arylsulphatase A, β -glucuronidase, cathepsin, ribonuclease and acid phosphatase. For comparison, the following non-lysosomal enzymes were also assayed: succinate dehydrogenase, alkaline phosphatase, cytochrome *c* oxidase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

All enzyme measurements were completed within 12 hr. to avoid freezing the homogenates, since cytochrome *c* oxidase lost activity on freezing. All measurements were made with a Cary recording spectrophotometer. All measurements were linear with concentration and time.

Assay of β -galactosidase (EC 3.2.1.23, β -D-galactoside galactohydrolase). This was carried out by a modification of the method of Sellinger, Beaufay, Jacques, Doyen & de Duve (1960). To 2.7 ml. of 39 mM-sodium acetate buffer, pH 5, containing 16.3 μ moles of *p*-nitrophenyl β -D-galactoside, was added 0.5 ml. of freshly prepared supernatant. The mixture was shaken for 1 hr. at 37° in a test tube on a Dubnoff metabolic shaker. The reaction was stopped with 0.1 ml. of 30% (w/v) trichloroacetic acid. Blanks contained all of the reactants except the enzyme. The enzyme was added after addition of trichloroacetic acid. The nitrophenol was colorized by addition of 1.5 ml. of 0.5 M-NaOH. Liberated nitrophenol was calculated from the extinction at 410 m μ less the blank values and was compared with values obtained with standard amounts of nitrophenol. Results are expressed as m-moles of nitrophenol liberated/hr./mg. of protein.

Assay of arylsulphatase A (EC 3.1.6.1, aryl sulphate sulphohydrolase). This was carried out by a modification of the method of Roy (1953). To 0.4 ml. of 125 mM-sodium acetate buffer, pH 4.7, containing 4.78 μ moles of nitrocatechol sulphate, was added 0.4 ml. of the freshly prepared enzyme solution. Blanks contained all of the reactants except the test enzyme. After 1 hr. in a bath at 37° with shaking at 15 min. intervals the reaction was stopped by the addition of 3 ml. of 2% (w/v) phosphotungstic acid. The enzyme was then added to the blank. The precipitated protein was removed by centrifuging at 10000g for 20 min. and the supernatants were decanted into test tubes. Colour was developed by adding 5 ml. of quinol reagent. After measurement of the extinction at 510 m μ and subtraction of the blank, comparisons were made with standard amounts of nitrocatechol. Results are expressed as m-moles of nitrocatechol liberated/hr./mg. of protein.

Assay of β -glucuronidase (EC 3.2.1.31, β -D-glucuronide glucuronohydrolase). This was carried out by a modification of the method of Talalay *et al.* (1946). To 0.9 ml. of 80 mM-sodium acetate buffer, pH 4.5, containing 0.51 μ mole of phenolphthalein glucuronide, was added 0.1 ml. of the enzyme solution. One blank contained all of the reactants except the enzyme. The mixture was incubated at 37° for 30 min. with shaking at 10 min. intervals. The reaction was stopped and colour was developed with 5 ml. of 0.2 M-glycine-NaOH buffer, pH 10.4. Enzyme was added to the first blank. The extinction was measured at 540 m μ , and then for internal blanks the solutions were decolorized with conc. HNO₃ and the extinction was determined again. After subtraction of both blank values the resultant extinction was compared with those of standard amounts of phenolphthalein. Results are expressed as μ moles of phenolphthalein liberated/hr./mg. of protein.

Assay of cathepsin (EC 3.4.4.-). This was carried out by a modification of the method of Anson (1938). To 4.8 ml. of 42 mM-sodium acetate buffer, pH 3.2, containing 1.6% (w/v) of acid-denatured haemoglobin, was added 0.2 ml. of supernatant from the homogenized tissue. (The haemoglobin was denatured by adding 1 ml. of conc. HCl to 200 ml. of 2% haemoglobin and stirring for 30 min. with a mechanical stirrer.) Blanks contained all of the reactants except the enzyme. The enzyme reaction mixture was incubated at 37° for 30 min. The reaction was stopped with 5 ml. of 5% (w/v) trichloroacetic acid and the enzyme was then added to the blank. The precipitates were filtered with Whatman no. 1 filter paper. Samples were read at 280 m μ

and blank values were subtracted. Comparisons were made with standard amounts of tyrosine. Results are expressed as μ moles of tyrosine liberated/hr./mg. of protein.

Assay of ribonuclease [EC 2.7.7.17, ribonuclease nucleotid-2-transferase (cyclizing)]. This was carried out by a modification of the method of Kalnitsky, Hummel & Dierks (1959). To 0.5 ml. of 50 mM-sodium acetate buffer, pH 5, containing 5 mg. of yeast sodium ribonuclease, was added 0.2 ml. of supernatant from freshly homogenized tissue. Blanks contained all of the reactants except the enzymes. After 15 min. of incubation at 37° the reaction was stopped with 5 ml. of 5% (w/v) HClO₄ in ethanol. Enzyme was added to the blank solutions. The solutions were centrifuged at 15000g for 15 min., and the clear supernatants and the blank were read at 260 m μ . Blank values are subtracted. Results are expressed as increase in *E*₂₆₀ units/hr./mg. of protein.

Assay of acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase). This was carried out by a modification of the method of Lowry, Roberts, Wu, Hixon & Crawford (1954). To 0.55 ml. of 30 mM-sodium acetate buffer, pH 5.2, containing 35.1 μ moles of nitrophenyl phosphate, was added 0.1 ml. of enzyme preparation. Blanks contained all of the reactants except the enzyme. After 30 min. at 37° 0.2 ml. of 10% (w/v) trichloroacetic acid was added to stop the reaction. After addition of the enzyme to the blank 4 ml. of 0.15 M-NaOH was added to develop the colour. Precipitated protein was removed by centrifugation. The extinction of the liberated nitrophenol was measured at 410 m μ . Blank values were subtracted and the resultant extinctions compared with those of standard amounts of nitrophenol. Results are expressed as m-moles of nitrophenol liberated/hr./mg. of protein.

Assay of succinate dehydrogenase [EC 1.3.99.1, succinate-(acceptor) oxidoreductase]. This was carried out by a modification of the method of Slater & Bonner (1952). To 3.1 ml. of a 0.1 M-sodium phosphate buffer, pH 7.4, containing 30 μ moles of KCN, 3 μ moles of K₃Fe(CN)₆ and 40 μ moles of sodium succinate, was added 0.2 ml. of enzyme preparation. Blanks contained all of the reactants except the enzyme. After mixing for 1 min. the initial decrease in extinction at 400 m μ at 22° was observed for 1 min. and after subtraction of blank values results are expressed as decrease in *E*₄₀₀ units/hr./mg. of protein.

Assay of alkaline phosphatase (EC 3.1.3.1, orthophosphoric monoester phosphohydrolase). This was carried out by a modification of the method of Garen & Levinthal (1960). To 0.55 ml. of 0.15 M-tris-HCl buffer, pH 8.0, containing 35.1 μ moles of nitrophenyl phosphate, was added 0.1 ml. of enzyme preparation. After 20 min. at 22° 0.2 ml. of 10% (w/v) trichloroacetic acid was added to stop the reaction and 4 ml. of 0.15 M-NaOH was added to develop the colour. The precipitable protein was cleared by centrifugation. The extinction of the liberated nitrophenol was measured at 410 m μ and after subtraction of the blank was compared with those of standard amounts of nitrophenol. Results are expressed as μ moles of nitrophenol liberated/hr./mg. of protein.

Assay of cytochrome c oxidase (EC 1.9.3.1, ferrocytochrome c-oxygen oxidoreductase). This was carried out by a modification of the method of Smith (1954). To 3 ml. of 30 mM-sodium phosphate buffer, pH 7.4, containing 0.05 μ mole of reduced cytochrome c, was added 0.1 ml. of enzyme preparation. (The cytochrome solution was

reduced by adding 0.1 ml. of freshly prepared 1.2 M- $\text{Na}_2\text{S}_2\text{O}_4$ to 30 ml. of 30 mM-sodium phosphate buffer, pH 7.4, containing 0.5 μmole of reduced cytochrome *c*; the solution was shaken vigorously to oxidize the excess of dithionite.) The reactants were mixed by inverting the cuvette and readings were taken at 22° every 30 sec. at 550 μm . Blanks contained all of the reactants except the enzyme. Cyanide inhibition was tested by adding 1 drop of 0.1 M-KCN adjusted to pH 7.4. After subtraction of blank values results are expressed as change in E_{550} units/hr./mg. of protein.

Assay of glucose 6-phosphate dehydrogenase [EC 1.1.1.49, D-glucose 6-phosphate-NAD(P) oxidoreductase]. This was carried out by a Worthington Biochemical Corp. (1965) enzyme method.

Assay of 6-phosphogluconate dehydrogenase [EC 1.1.1.43, 6-phospho-D-gluconate-NAD(P) oxidoreductase]. This was carried out similarly to the glucose 6-phosphate dehydrogenase assay, but 6-phosphogluconic acid was used at the same substrate concentration as glucose 6-phosphate. After addition of the test enzyme to the appropriate substrates the rate of reaction was measured in terms of the increase in extinction at 340 μm and 22°. Blanks contained all of the reactants except the enzyme. After subtraction of blank values results for both assays are expressed as m-moles of NADP⁺ reduced/hr./mg. of protein.

Determination of thiobarbituric acid reductants. This was carried out by a modification of the method of Kohn & Liversedge (1944). In a 50 ml. Erlenmeyer flask 3.5 ml. of mammary enzyme preparation was shaken on a Dubnoff metabolic shaker at 30° for 3 hr. The reaction was stopped with 3.5 ml. of 10% (w/v) trichloroacetic acid. Blanks were prepared in the same manner except that the reaction was stopped at zero time. All of the samples were filtered through Whatman no. 1 filter paper. To 4 ml. of filtrate was added 1 ml. of 1% (w/v) thiobarbituric acid. The mixture was boiled for 15 min. and the resulting red colour was measured at 535 μm . Blank readings were subtracted. Results are expressed as change in E_{535} units/hr./g. of tissue.

Determination of protein. The protein content of every sample was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine serum albumin was used as the protein standard.

Latency of enzymes

Fractionation of mammary tumour homogenate. Ratios of free to bound activities of arylsulphatase A and acid ribonuclease were determined in six tumours growing at day 20 of pregnancy and in 18 tumours regressing after birth, including six at day 1, six over days 2 and 3 and six between days 4 and 8. Each tumour was washed with ice-cold water and then blotted on Whatman no. 1 filter paper. Tumours weighing 0.60–5.8 g. were used for homogenization. Each tumour was thoroughly minced with surgical scissors in a 20 ml. beaker and the tissue was transferred in ice-cold sucrose to an ice-encased VirTis flask. The volume of 0.25 M-sucrose solution was adjusted to 10 ml. of sucrose/g. of tissue. The tumours were homogenized with a VirTis model 45 homogenizer at high speed in a 30 ml. VirTis flask for 8–10 sec. at 0°. This homogenization procedure with 0.25 M-sucrose and the VirTis homogenizer for a few seconds yielded over 90% of the cytochrome *c* oxidase in the mitochondrial fraction for both the liver and tumours. The homogenates were transferred to stainless-

steel tubes and were centrifuged for 20 min. at 800g in an International HR-1 refrigerated centrifuge. 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 (10 ml./g. initial wt. of tissue) and stirred with a motor-driven pestle. The suspension was centrifuged again at 800g to give a pellet containing primarily nuclei, cell debris and some undisturbed tissue. The supernatant was added to the second 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. Water to make a final concentration equivalent to 20 ml./g. initial wt. of tissue and 1 drop of Triton X-100 were added to each 800g and 15000g pellet. The pellets were rehomogenized. Therefore each of the three fractions (P-1, P-2 and supernatant) consisted of approx. 20 ml. of preparation/g. of starting tissue. Arylsulphatase A and acid ribonuclease activities were determined on all three fractions. Less than 3% of these enzymes were contained in fraction P-1 and therefore this fraction was not considered in the calculations of free to bound activities. The ratios of free to bound activities are summarized in Tables 2 and 3.

Chemicals

Haemoglobin was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Reduced cytochrome *c* and yeast sodium ribonuclease were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. All other enzyme substrates and cofactors were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Striking increases were observed in all of the lysosomal enzymes of the regressing hormone-dependent mammary tumours and in most of the lysosomal enzymes of the hormone-dependent post-partum uteri. The results of the assays are shown in Figs. 2 and 3, and the numerical values and the probabilities of significant differences between growing and regressing tumours are summarized in Table 1. Ratios of lysosomal enzyme activities in regressing tumours to those in growing tumours were as follows: β -glucuronidase, 7.7; β -galactosidase, 3.9; cathepsin, 2.9; ribonuclease, 2.1; arylsulphatase A, 1.5; acid phosphatase, 1.4. Ratios of such activities in post-partum uteri to those in pregnant uteri were as follows: β -glucuronidase, 5.8; cathepsin, 5.5; ribonuclease, 4.3; β -galactosidase, 2.2; acid phosphatase, 1.8. The corresponding ratio for uterine arylsulphatase A, however, was only 0.55. All of the ratios are significant except that for uterine ribonuclease.

No significant changes were observed in the lysosomal enzymes of the hormone-dependent normal mammary gland or the hormone-

Fig. 2. Lysosomal enzyme activities of homogenates of whole tissues when mammary tumours were growing (hatched areas) and regressing (plain areas): (a) acid phosphatase; (b) β -glucuronidase; (c) cathepsin; (d) acid ribonuclease; (e) arylsulphatase A; (f) β -galactosidase. Activity units are defined in the Materials and Methods section. Standard errors and numbers of animals are indicated.

independent liver (Figs. 2 and 3). No significant differences in alkaline phosphatase, succinate dehydrogenase, 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase were observed in the normal mammary glands, mammary tumours, livers or the uteri when the mammary tumours were growing or regressing. Cytochrome *c* oxidase increased significantly in the normal mammary glands, but remained similar in the other tissues. Lysosomal enzyme activities were greater in the normal mammary glands than in mammary tumours for β -galactosidase, ribonuclease, β -glucuronidase and arylsulphatase, but cathepsin and acid phosphatase activities were greater in the growing tumours. When the tumours were regress-

Fig. 3. Non-lysosomal enzyme activities of homogenates of whole tissue when mammary tumours were growing (hatched areas) and regressing (plain areas): (a) succinate dehydrogenase; (b) 6-phosphogluconate dehydrogenase; (c) glucose 6-phosphate dehydrogenase; (d) alkaline phosphatase; (e) cytochrome *c* oxidase. Activity units are defined in the Materials and Methods section. Standard errors and numbers of animals are indicated.

ing all lysosomal enzyme activities were greater in mammary tumours than in the mammary glands.

Lysosomal enzyme content was greater in the normal mammary glands than in the liver for β -glucuronidase, cathepsin, acid ribonuclease, arylsulphatase A and β -galactosidase, but the reverse was true for acid phosphatase. The lysosomal enzyme patterns of the pregnant uterus and the liver were similar, except that both acid ribonuclease and β -glucuronidase as well as acid phosphatase activities were greater in the liver. The arylsulphatase A content was several times as great in the uterus as in the liver. The activities of all of the lysosomal enzymes except ribonuclease were greater in malignant growing mammary tumours than in the liver.

Of the non-lysosomal enzymes, 6-phosphogluconate dehydrogenase and glucose 6-phosphate

Table 1. *Lysosomal enzyme activities in homogenates of growing and regressing mammary tumours and pregnant and post-partum uterus*

Results are expressed as means $\pm$ S.E.M. The numbers of individual determinations are given in parentheses. Values of *P* were calculated by Student's *t* test.

Enzyme	Mean activity (<i>E</i> units/hr./mg. of protein)		<i>P</i>	Mean activity (<i>E</i> units/hr./mg. of protein)		<i>P</i>
	Growing tumour	Regressing tumour		Pregnant uterus	Post-partum uterus	
β -Glucuronidase	0.36 $\pm$ 0.05 (15)	2.76 $\pm$ 0.64 (14)	< 0.001	0.45 $\pm$ 0.02 (15)	2.59 $\pm$ 0.71 (13)	< 0.005
β -Galactosidase	0.76 $\pm$ 0.12 (6)	2.96 $\pm$ 0.58 (7)	< 0.01	1.16 $\pm$ 0.16 (6)	2.54 $\pm$ 0.35 (6)	< 0.001
Cathepsin	0.18 $\pm$ 0.04 (15)	0.59 $\pm$ 0.11 (13)	< 0.001	0.08 $\pm$ 0.02 (15)	0.44 $\pm$ 0.14 (11)	< 0.001
Ribonuclease	1.89 $\pm$ 0.31 (6)	3.99 $\pm$ 0.34 (7)	< 0.001	0.27 $\pm$ 0.09 (6)	0.61 $\pm$ 0.15 (6)	< 0.100
Arylsulphatase A	0.30 $\pm$ 0.02 (6)	0.44 $\pm$ 0.05 (7)	< 0.025	0.96 $\pm$ 0.07 (6)	0.52 $\pm$ 0.16 (6)	< 0.025
Acid phosphatase	6.92 $\pm$ 0.5 (15)	9.35 $\pm$ 0.59 (13)	< 0.01	3.50 $\pm$ 0.24 (15)	6.32 $\pm$ 0.55 (13)	< 0.01

Table 2. *Changes in the arylsulphatase A activity of rat mammary tumours at various stages of tumour growth and regression*

The animals were primiparous females whose litters were restricted to eight pups. For the 'late pregnancy' group the animals were killed on day 20 or 21 of pregnancy. The other groups were killed at various times shortly after birth, when the tumours were regressing. The units of arylsulphatase A are expressed as μ moles of nitro-catechol liberated/hr./mg. of protein. The results are given as means $\pm$ S.E.M. The total activity and the free activity are given/g. wet wt. of tumour.

Status	No. of rats	Tumour wt. (g.)	Total activity (units/g. of tumour)	Free activity (units/g. of tumour)	Free activity Bound activity
Late pregnancy	6	4.45 $\pm$ 0.89	32.3 $\pm$ 2.6	12.3 $\pm$ 0.81	0.62 $\pm$ 0.02
1 day after birth	6	1.22 $\pm$ 0.30	43.6 $\pm$ 10.5	15.7 $\pm$ 2.6	0.56 $\pm$ 0.01
2-3 days after birth	6	1.68 $\pm$ 0.35	42.3 $\pm$ 4.5	13.1 $\pm$ 1.4	0.49 $\pm$ 0.12
4-8 days after birth	6	1.65 $\pm$ 0.21	25.4 $\pm$ 3.1	13.6 $\pm$ 1.4	1.15 $\pm$ 0.03

Table 3. *Changes in the acid ribonuclease activity of rat mammary tumours at various stages of tumour growth and regression*

The animals were primiparous females whose litters were restricted to eight pups. For the 'late pregnancy' group the animals were killed on day 20 or 21 of pregnancy. The other groups were killed at various times shortly after birth, when the tumours were regressing. The units of ribonuclease are expressed as change in E_{260} units/hr./mg. of protein. The results are expressed as means $\pm$ S.E.M. The total activity and the free activity are given/g. wet wt. of tumour.

Status	No. of rats	Tumour wt. (g.)	Total activity (units/g. of tumour)	Free activity (units/g. of tumour)	Free activity Bound activity
Late pregnancy	6	4.45 $\pm$ 0.89	208.0 $\pm$ 37.6	92.0 $\pm$ 17.0	0.73 $\pm$ 0.06
1 day after birth	6	1.22 $\pm$ 0.30	511.0 $\pm$ 99.0	220.0 $\pm$ 11.0	0.77 $\pm$ 0.05
2-3 days after birth	6	1.68 $\pm$ 0.35	329.1 $\pm$ 41.0	107.1 $\pm$ 10.0	0.53 $\pm$ 0.11
4-8 days after birth	6	1.65 $\pm$ 0.21	347.0 $\pm$ 118.0	207.0 $\pm$ 50.0	1.71 $\pm$ 0.38

dehydrogenase were present in greater concentrations in the liver than in mammary tumours, the normal mammary glands or the uterus. The liver contained about as much cytochrome *c* oxidase as did mammary tumours, but less than normal mammary glands or pregnant uteri. Succinate dehydrogenase activities were greater in mammary tumours than in the liver. The alkaline phosphatase

activity in the liver was greater than that in the uterus, equal to that in mammary tumours and less than that in normal mammary glands.

The ratio of free to bound enzyme for arylsulphatase A decreased 1-3 days after birth (Table 2). The ratio of free to bound acid ribonuclease increased slightly at day 1, but decreased 2-3 days after birth (Table 3). By days 4-8,

Table 4. *Changes in the weights and protein contents of rat mammary tumours at various stages of tumour growth and regression*

The animals were primiparous females whose litters were restricted to eight pups. For the 'late pregnancy' group the animals were killed on day 20 or 21 of pregnancy. The other groups were killed at various times shortly after birth, when the tumours were regressing. Protein content is expressed as mg. of protein/g. wet wt. of tumour. Results are expressed as means $\pm$ S.E.M.

Status	No. of rats	Tumour wt. (g.)	Total protein	
			(mg./tumour)	(mg./g. of tumour)
Late pregnancy	6	4.45 $\pm$ 0.89	405.0 $\pm$ 79.7	93.8 $\pm$ 4.3
1 day after birth	6	1.22 $\pm$ 0.30	108.3 $\pm$ 4.8	88.8 $\pm$ 21.6
2-3 days after birth	6	1.68 $\pm$ 0.35	156.9 $\pm$ 19.8	93.6 $\pm$ 12.7
4-8 days after birth	6	1.65 $\pm$ 0.63	101.5 $\pm$ 12.8	61.5 $\pm$ 20.5

however, there was a dramatic increase in the ratios of free to bound activities for the two enzymes. The ratio for arylsulphatase A, 0.62 ± 0.02 in late pregnancy, increased to 1.15 ± 0.03 by 4-8 days after birth (Table 2). The ratio for acid ribonuclease, 0.73 ± 0.06 in late pregnancy, increased to 1.71 ± 0.38 by 4-8 days after birth (Table 3). Both differences are significant, with $P < 0.01$ and $P < 0.025$ respectively.

A slight but insignificant decrease in thio-barbiturate reductants was observed in growing tumours as compared with regressing tumours. Eight growing tumours had an increase of 0.16 ± 0.08 E_{535} unit/hr./g. wet wt. of tissue, whereas nine regressing tumours had an increase of 0.12 ± 0.04 E_{535} unit/hr./g. wet wt. of tissue.

The protein content as well as the average weight of the mammary tumours decreased sharply after birth (Table 4). At days 4-8 after birth there was a decrease in the protein content/g. wet wt. of tumour.

The pattern of tumour growth varied after parturition. Observations were made on 29 tumours during 10 days after birth (Fig. 1). Three tumours regressed completely, three regressed about 80%, four regressed nearly 50%, four regressed 10-20%, four did not change in size, seven continued to grow and four new tumours appeared during the 10-day period.

DISCUSSION

Cathepsin, β -glucuronidase, β -galactosidase, acid phosphatase and ribonuclease activities were similarly increased in the uterus. Surprisingly, there was a significant decrease in arylsulphatase A. This decrease may be related to an extralysosomal function of the enzyme during the rapid growth of the uterus during pregnancy. Woessner (1965) reported three- to four-fold increases in acid cathepsin and β -glucuronidase and two- to three-

fold increases in β -galactosidase and acid phosphatase. These results, although not identical with those obtained in the present study, are nevertheless closely comparable.

The failure of several non-lysosomal enzymes (6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase, succinate dehydrogenase, cytochrome *c* oxidase and alkaline phosphatase) to increase significantly in the uterus is similar to the finding of Woessner (1965) that glutamate-oxalate transaminase did not increase significantly in the involuting uterus. In his studies cytochrome *c* oxidase exhibited a twofold maximum increase. Similar increases in uterine cytochrome *c* oxidase, although not statistically significant, were found in the present study. The increases in both the present study and that of Woessner (1965) may reflect a slower loss of mitochondria than of protein in general.

Several non-lysosomal enzymes (6-phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase, succinate dehydrogenase, alkaline phosphatase and cytochrome *c* oxidase) failed to increase significantly in regressing mammary-tumour tissues.

Hershey, Johnstone, Murphy & Schmitt (1966) studied several non-lysosomal nicotinamide nucleotide-linked dehydrogenases of 3-methylcholanthrene-induced growing and regressing breast cancer. They found that glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase and malate dehydrogenase also did not increase in regressing mammary tumours as compared with growing mammary tumours.

During mammary-tumour regression and uterine involution acid hydrolases increase in concentration and non-lysosomal enzymes fail to increase. These results from the present study may possibly be explained by findings in other tissue-regression studies, which suggest that the increased concentration of acid hydrolases may be attributed to a

mechanism such that the lysosomal enzyme complement is conserved rather than that enzymes are suddenly synthesized.

Woessner (1965) found that the total activity of β -glucuronidase remained constant during late pregnancy and several days after parturition. Weber (1957) reported a nearly 30-fold increase in lysosomal acid cathepsin concentration with little net gain in total units of enzymes. The conservation of lysosomal enzymes may be related to the postulate of de Duve (1964) that lysosomes may function repeatedly, digesting many 'meals' without altering their hydrolase composition. At the end of this process considerable lysosomal enzyme activity should remain, even though much tissue material has been digested and removed.

There is a dramatic increase in the ratio of free to bound activities of acid ribonuclease and arylsulphatase A in mammary tumours at days 4–8 (Tables 2 and 3). Similar increases in the ratios of free to bound activity have been observed for both acid phosphatase and acid protease in the sheep corpus luteum at days 15 and 16, when the corpus luteum is regressing (Dingle, Hayes & Moor, 1968). Striking increases in the ratios of free to bound activity for acid ribonuclease and β -glucuronidase have been observed in regressing mammary glands (Greenbaum *et al.* 1965). The increase in lysosomal fragility and the release of free enzymes facilitates rapid tissue resorption, since these enzymes are in direct contact with their substrates. Another factor that may facilitate rapid tissue resorption by the free enzymes is cellular pH. The pH of the cells at cell death is believed to be lowered to a range where the free enzymes have their maximal activity.

Surprisingly, there is a slight decrease in the ratio of free to bound activity up to days 2 and 3 after birth for both arylsulphatase A and acid ribonuclease. Even so, there are some unique properties of this tumour system that may elucidate the decrease in the ratio of free to bound activity. Immediately after birth the mammary tumours began to soften. The softening was easily observed by palpating tumours before and after birth. The soft tissues in general, and the liver in particular, gave a greater yield of intact particles because less vigorous homogenization was required and fewer particles were broken. Consequently a large increase in yield due to a softer tissue could easily mask the expected small increase in the ratio of free to bound activity. By days 4–8 this ratio for mammary-tumour arylsulphatase A and ribonuclease had increased to the point where the effect of tumour softening might no longer be important. The early softening of these tumours was probably due to the early action of cathepsin, or unknown components such as collagenase, on the fibrous elements of the tumours. Woessner (1965) also observed similar

greater yields of intact subcellular particles from the involuting uterus when the uterine tissue softens and becomes easier to homogenize. Careful homogenization of the growing and regressing mammary tumours with a TenBroeck homogenizer failed to increase the yield of intact particles significantly.

Another important factor in the relationship of free to bound activity may involve the macrophages. In the uterus they increase in number during involution and reach a maximum concentration at 4 days after birth (Lobel & Deane, 1962). In view of the known content of acid hydrolases in macrophages (Cohn & Wiener, 1963) some of the increase in hydrolase concentration may be due to the presence of macrophages. In the rat corpus luteum both lysosomes and macrophages have been shown histochemically to be present in the vicinity of regressing lutein cells and may play a part in luteal regression (Novikoff, 1960; Lobel, Rosenbaum & Deane, 1961). In the rat much of the lysosomal enzyme activity is localized in the invading macrophages, rather than in the lutein cells themselves (Banon, Brandes & Frost, 1964). In the regressing sheep lutein cells, however, little or no macrophage activity is associated with the lutein cells (Dingle *et al.* 1968).

Macrophages appear at days 4–8, and it is possible that their membranes are more fragile at that time, contributing to the increased ratio of free to bound activity of arylsulphatase A and acid ribonuclease. In certain tissues synthesis of lysosomal enzymes by activated macrophages may also contribute significantly to the apparent concentration of lysosomal enzymes. With involuting tadpole tails histochemical methods reveal a progressive accumulation of acid hydrolases by macrophages, probably in response to the initiation of phagocytosis in the regressing tail tissue (Salzman & Weber, 1963; Weber, 1964).

In the present study differences in the lysosomal enzyme pattern and cytochrome *c* oxidase pattern existed between hormone-dependent normal mammary gland and hormone-dependent malignant mammary tumours. Because the pups were not removed from their mothers, involution of the mammary tissue was not expected. In contrast with the lysosomal enzyme pattern observed in the normal mammary tissue, large increases in lysosomal enzymes were observed in mammary-tumour regression as well as in uterine regression, even though the pups were not removed from their mothers. This difference in response may reflect the difference between normal and neoplastic mammary tissue.

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