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Some Enzymic Changes in the Mammary Gland of Rats during Pregnancy, Lactation and Mammary Involution

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Recent work has emphasized that the lactating mammary gland is a site of intense synthetic activity (see Folley, 1949). During the past five years, fat synthesis in the gland has received considerable attention, but protein synthesis has been relatively neglected, although the mammary gland is known to synthesize large quantities of specific proteins, and, since these are promptly secreted into the milk, an equilibrium condition is never reached and the high rate of protein synthesis is maintained.

The application of isotopic methods to the study of milk-protein precursors by Campbell & Work (1952) is clearly a most promising line of investigation, but it seemed to us that the study of some of the enzymes involved might also yield interesting information. In view of the present state of knowledge of the mechanism of protein synthesis, it seemed more profitable to make a general survey of some of the enzymes which might be implicated in these processes, rather than an intensive study of a particular enzyme system. We have therefore studied glutamic dehydrogenase, glutamic-aspartic transaminase and cathepsin, which have been suggested as playing some part in protein synthesis. In addition, we have measured the activity of β -glucuronidase, first because this enzyme is also found in the particulate fractions (Walker & Levvy, 1953) and is one which, because it is believed not to be concerned with tissue growth in the rat, would differentiate between a specific enzymic change and a general change in the level of mitochondrial enzymes; and secondly because of its postulated role in oestrogen metabolism (Fishman, 1947).

EXPERIMENTAL

Animals. Virgin rats of the hooded Norway strain of the Medical Research Council, aged 3 months, were mated with males of the same strain and age. Nine groups of rats, each

containing five animals, were used; three groups of pregnant animals which were killed on the 10th, 15th and 20th days of pregnancy, four groups which were killed on the 5th, 10th, 15th and 20th days of lactation and two further groups which had their litters removed on the 21st day of lactation and were killed on the 2nd and 4th days of mammary involution. During lactation the litter size was reduced to eight pups, four male and four female, where possible. Rats were killed by dislocation of the cervical vertebrae and the three abdominal mammary glands quickly removed, weighed and disintegrated in water in a Folley & Watson (1948) blender to give a final tissue concentration of 1:5 for glutamic dehydrogenase and transaminase estimations, and 1:10 for cathepsin and β -glucuronidase estimations.

Materials. α -Oxoglutarate was prepared by the method of O'Kane (1949) and was 96% pure by carbonyl-group estimation.

Cytochrome c was prepared by the method of Keilin & Hartree (1945) from horse heart. This had a concentration of $3.26 \times 10^{-3} M$ as estimated by the method of Potter (1941).

Diphosphopyridine nucleotide (DPN) was prepared by the method of Williamson & Green (1940); various preparations gave assays between 55 and 65% pure.

p-Chlorophenyl glucosiduronic acid was prepared by the method of Spencer & Williams (1951). We found that a better yield could be obtained if the dose of *p*-chlorophenol (0.5 g./kg. of rabbit in 50 ml. of water) was divided into two portions and one given at 10 a.m. and the other at 6 p.m.

Haemoglobin was prepared by the method of Anson (1937) and stored in the freeze-dried state. For use, a 5% (w/v) solution of haemoglobin in water was prepared fresh each day.

Chemical methods. Lactose was estimated by the method of Hinton & Macara (1927) as modified by Folley & Greenbaum (1947). The milk content of the mammary gland suspensions was calculated from the lactose content and all enzyme assays were corrected for the 'milk error' as described by Folley & Greenbaum (1947).

Enzymic methods. Glutamic dehydrogenase was measured by the method described by Copenhaver, McShan & Meyer (1950), except that the reaction was carried out in the presence of 0.1 M nicotinamide, in view of the observation of Moore & Nelson (1952) that mammary gland contains an active nucleotidase and that of Turner (1952), who also

found a rapid destruction of DPN, and the protection of DPN by added nicotinamide. The results of this determination are expressed as $\mu\text{l. O}_2$ uptake/hr./g. wet wt. mammary tissue corrected for the presence of milk (Folley & Greenbaum, 1947).

Transaminase was measured at three tissue levels by the method of Greenwood & Greenbaum (1953). Protein precipitation from mammary-gland suspensions leaves a pad of fatty material floating on the surface after centrifugation and the removal of this was best effected by filtration. The results are expressed as $\mu\text{moles oxaloacetic acid produced/hr./g. wet wt. of mammary gland corrected for the presence of milk.}$

Cathepsin. The method described by Anson (1938) was used, except that the scale of operations was reduced by a factor of 4. The concentration of haemoglobin solution was also double that used by Anson (1938) since, at this concentration, a linear relationship between the level of the enzyme

and the tyrosine produced over the range 5–25 mg. of tissue (Fig. 1) was obtained. No activation of catheptic activity by cysteine, as suggested by Anson (1937), could be detected and accordingly no cysteine was added to the reaction mixture. Laskowski & Laskowski (1951) have demonstrated the presence of a powerful anti-trypsin in bovine colostrum. We have tested for the presence of such an inhibitor in suspensions of mammary gland taken from rats on the 20th day of pregnancy by assaying separately the catheptic activity of three mammary-gland suspensions, one at the 20th day of pregnancy, one at the 10th day of lactation and a mixture of equal volumes of these two suspensions. The value for the mixture was exactly the arithmetical mean of the two separate values, so that any inhibitor present had no action on the cathepsin assay used. Throughout these determinations the same batch of haemoglobin was used to ensure uniformity of substrate.

Preliminary experiments using this method have established that the liberation of tyrosine is linear over a period of 1 hr. (Fig. 1) and a 15 min. incubation period was chosen for routine use. Since the presence of a cathepsin in mammary gland had not previously been described in the literature, a pH characterization of the enzyme was carried out (Fig. 2). A sharp optimum was found at pH 3.7 and the estimation was therefore routinely carried out at that pH. The 1:10 tissue suspension was diluted with an equal volume of 0.2M acetate buffer, pH 3.7, immediately before use. Each determination was carried out at three tissue levels, 5, 15 and 25 mg. uncorrected wet wt., and the reaction product measured colorimetrically, using an Ilford Spectrum Red filter no. 608, transmitting from 6200 Å onwards, and a standard tyrosine curve. The results are expressed as mg. tyrosine/hr./g. of wet wt. of mammary tissue corrected for the presence of milk.

β -Glucuronidase was measured by the method of Spencer & Williams (1951). Owing to the high fat content of the mammary gland, a cloudy solution was obtained when the supernatant was diluted according to the above method. By diluting with freshly prepared 0.2N-KOH in 80% (v/v) ethanol instead of the aqueous 0.2N-NaOH recommended,

Fig. 1. (a) Relation between cathepsin concentration and reaction velocity in rat mammary-gland tissue suspensions, with substrate concentrations of 5% (w/v) haemoglobin (●) and 2.5% (w/v) haemoglobin (○). All experiments run for 15 min. at 37° and at pH 3.7. (b) Cathepsin reaction-velocity curves for rat mammary-gland tissue suspensions. All experiments using 10 mg. wet wt. of tissue; substrate concentration 5% (w/v) haemoglobin; temperature 37°; pH 3.7 (●). The open circles represent the incubated controls.

Fig. 2. pH-optimum curve of rat mammary-gland cathepsin (○). '---' Represents the reagent blank and 'x' represents the values for the incubated controls.

the final solution was found to be suitable for spectrophotometric measurements for periods up to 1 hr. For β -glucuronidase measurements, the 1:10 tissue suspension was diluted with an equal volume of 0.2M acetate buffer, pH 5.2. Each determination was performed at three tissue levels, 5, 12.5 and 20 mg. uncorrected wet wt. A straight-line relationship exists between the increase in optical density due to the liberated *p*-chlorophenol and the tissue level up to 40 mg. of tissue. Walker & Levvy (1951, 1953) have suggested that the maximum activity of β -glucuronidase in rat-liver suspensions can only be realized in the presence of a non-ionic detergent which serves either to render the enzyme from the particles soluble or to overcome the inhibition due to some unknown endogenous inhibitor. We have attempted to assay β -glucuronidase in mammary-gland tissue suspensions using the detergent used by Walker & Levvy, Triton-X-100, in the concentrations they suggest, but we have found that the effect of this substance is to produce cloudy filtrates owing to the presence of stabilized fat droplets. Such filtrates were also found after the precipitation of the protein either by the method of Spencer & Williams (1951) or of Talalay, Fishman & Huggins (1946). It was impossible, therefore, to evaluate the effect of Triton-X-100 in the mammary gland.

However, Walker & Levvy (1953) have found that the use of citrate buffer in place of acetate also overcomes the inhibition and, in fact, gives values closely approximating to those obtained using Triton-X-100. We have made several determinations of mammary-gland β -glucuronidase in the

presence of both these buffers and find no difference in the values obtained. It would appear, therefore, that rat mammary gland differs in this respect from rat liver.

RESULTS

Changes in the levels of the various enzymes measured during the lactation cycle are shown in Figs. 3-6.

Glutamic dehydrogenase. No change in the activity of this enzyme is detectable between the 10th and 15th day of pregnancy, but a highly significant increase ($P < 0.001$) occurs between the 15th and 20th days (Fig. 3). This rise probably occurs abruptly later than the 17th day, since we have several results, not included in Fig. 3, at 16 and 17 days of pregnancy, all of which clearly fall in the same grouping as those at the 15th day.

By the 5th day of lactation the level had declined ($P < 0.001$) but thereafter increased again to a maximum at 15 days of lactation, which is more or less maintained until the end of lactation. Very little change is noted in the first 2 days of mammary involution, but between the 2nd day and the 4th day the level drops precipitously.

The curve showing the total glutamic dehydrogenase activity in the three abdominal mammary

Fig. 3. Changes in the glutamic dehydrogenase activity of rat mammary-gland tissue suspensions: (a) activity/g. moist tissue, (b) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

Fig. 4. Changes in the glutamic-aspartic transaminase activity of rat mammary-gland tissue suspensions: (a) activity/g. moist tissue, (b) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

glands follows a very similar course. The level of activity in this tissue is of a low order, being about one-tenth of that in liver.

Transaminase. The level of this enzyme (Fig. 4) increases slowly during pregnancy and rises abruptly at parturition so that at the 5th day of lactation the level is over double that found at the end of pregnancy ($P=0.006$). This high level is maintained, and even increases slightly, throughout the remainder of lactation. There is a slight decline in the first 2 days of mammary involution but in the next 2 days the level falls virtually to zero. The curves showing the level of enzyme/g. of tissue and the total enzyme are of similar form.

The activity of the mammary enzyme is about one-third of that found in liver.

Cathepsin. The changes in the level of cathepsin are shown in Fig. 5. Very little change is observed in the level of cathepsin/g. of tissue over the second half of pregnancy, but after parturition there is a steady increase, so that by the 20th day of lactation the level is over four times that obtaining on the 20th day of pregnancy ($P<0.001$). This rise continues into the period of mammary involution.

Fig. 5. Changes in the cathepsin activity of rat mammary-gland tissue suspensions: (a) activity/g. moist tissue, (b) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no line is given the S.E.M. is too small to show.

The curve for the total cathepsin activity follows essentially the same course, except that it reaches a well-marked peak on the 2nd day after weaning and is still high on the 4th day, indicating that the increase in activity/g. from the 2nd to the 4th days of involution takes place less rapidly than the loss in dry weight of the gland over the same period.

The catheptic activity found in the mammary gland greatly exceeds that of other tissues examined, the value at the 10th day of lactation being over double that for lymph nodes, the most active tissue so far recorded (Rothschild & Junqueira, 1951).

β -Glucuronidase. Changes in the β -glucuronidase activity are shown in Fig. 6. The activity of the enzyme/g. of tissue increases but slightly between the 10th and 15th days of pregnancy ($P=0.50$), but rises to a sharp maximum by the 20th day ($P=0.001$). The level then declines progressively throughout the course of lactation, although it does not fall as low as that found up to the 15th day of pregnancy. There is a dramatic increase in the level

Fig. 6. Changes in the β -glucuronidase activity of rat mammary-gland tissue suspensions: (a) activity/g. moist tissue, (b) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

of the enzyme during involution and indeed the highest value recorded, at the 4th day of mammary involution, is over twice the value found at the end of lactation ($P=0.007$). A similar trend is seen in the total β -glucuronidase activity, although here the peak value is found on the 2nd day of involution, the 4th day value being greatly decreased by the fall in weight of the regressing gland.

The β -glucuronidase activity of mammary gland is approximately one-third of that found in rat liver by Mills, Paul & Smith (1953) and Walker & Levvy (1953) using a different method of assay. For the purpose of this comparison we have recalculated our data into the units used by these authors.

DISCUSSION

The original suggestion of Braunstein & Azarkh (1945) that the coupling of the transaminating systems with glutamic dehydrogenase could play an important part in both the degradation and synthesis of amino acids has gained in significance from the demonstration by Cammarata & Cohen (1950) of the wide scope of the transamination reaction. The mechanism of urea synthesis put forward by Ratner (1949) proposes a scheme for amino acid catabolism which also centres round the interaction of these two systems. Since all the reactions in the Ratner scheme are reversible, the same scheme could be interpreted as a possible route for amino acid synthesis. A great deal of attention has been devoted to establishing a relationship between transaminase activity and protein synthesis. The fall in transaminating activity found by Cohen & Hekhuis (1941) in growing tissues led these authors to postulate an inverse relationship between protein synthesis and transamination activity, a conclusion also reached by Bartlett & Glynn (1950*a*) as a result of their study of muscle transaminase after hypophysectomy. On the other hand, Bartlett & Glynn (1950*b*) have found an increased transaminase activity in the livers of hypophysectomized rats treated with growth hormone and suggest that the increase was due to an increased synthesis of amino acids required for protein deposition.

The contrary nature of these results is not surprising, since a high level of transaminating activity could equally well indicate an increased level of amino-acid synthesis or an increased rate of degradation. According to the scheme indicated earlier, the arbiter of whether an amino group, when once transferred to α -oxoglutarate to form glutamate, is then again transferred to another keto acid to form a new amino acid, or is split off and passed into the ornithine cycle, would be the functional level of glutamic dehydrogenase.

In view of these considerations one might expect to find an inverse relationship between transami-

nase and glutamic dehydrogenase in the protein-synthetic phase of the lactation cycle, while in conditions where amino acid degradation predominates both enzymes should change in the same direction. Lactation in the rat is characterized by a progressive rise in milk yield (Brody & Nisbet, 1938) and hence of protein output. That the inverse relationship of the two enzymes does not hold in lactation can be seen from Figs. 3 and 4. If these results can be interpreted in terms of the Ratner scheme, then the changes of glutamic dehydrogenase and transaminase appear rather to indicate an increased amino acid degradation, a conclusion which it is difficult to reconcile with the known synthetic activity of the gland. However, a consideration of the changes in the morphological structure of the gland could lead to a different interpretation of these results.

Weatherford (1929) has reported a progressive increase in the number of mitochondria in the secretory cells of the mammary gland during pregnancy and lactation and a change in shape from a globular to a filamentous form. Jeffers (1935) has confirmed the change in form, and Dempsey, Bunting & Wislocki (1947) the increased number, of mitochondria through pregnancy and lactation. If such an increase really does occur then it is probable that the enzymes located in the mitochondria would increase at the same rate in a non-specific way. This could account for the rise in glutamic dehydrogenase at the end of pregnancy and for the changes in succinic dehydrogenase and cytochrome oxidase found by Moore & Nelson (1952).

Transaminases occur in both supernatant and particulate fractions, predominantly in the supernatant (Nakada & Weinhouse, 1950; Hird & Rowsell, 1950). Glutamic-aspartic transaminase would, therefore, not be expected to follow the same course as the mitochondrial enzymes. That transaminase activity increases as milk secretion increases seems to indicate that transaminase is connected with milk-protein synthesis, but such a connexion might be indirect. All that the increase in transaminase does indicate is an increased turnover of amino groups. Indeed Copenhaver, Shipley & Meyer (1951) have found a striking increase in liver transaminase in diabetic rats, a condition favouring deamination.

The question arises whether all mitochondrial enzymes increase, or only those concerned with the process of milk formation. Two more mitochondrial enzymes were therefore studied, one, cathepsin, probably connected with protein synthesis, and the other, β -glucuronidase, almost certainly not involved in protein metabolism in the rat.

Of the mammary enzymes so far assayed, the increase of cathepsin most clearly parallels the rate

of milk secretion in that it is both rapid and progressive. It is possible to envisage a role for cathepsin in protein synthesis either in terms of the 'template' hypothesis of Haurowitz (1950) or in the transpeptidation hypothesis of Fruton (1951). More recently, Rothschild & Junqueira (1951) have suggested a direct correlation between cathepsin activity and protein synthesis by a reversible proteolysis.

A further possible function of cathepsin has, so far, not been considered. It is possible that cathepsin plays a purely degradative role in maintaining a supply of free amino acids, which are then available for protein synthesis by another system. This explanation would have the virtue of postulating only one function for cathepsin both during the phase of active protein synthesis and during mammary involution, when degradation of the tissue proteins is proceeding. This conclusion would fit in with the results of Page, Glendening & Parkinson (1951) on the changes of cathepsin in the endometrium during the menstrual cycle and with unpublished results of our own on the growing uterus in pregnancy and in uterine involution where an increasing level of cathepsin during uterine growth was further increased during the involutionary phase.

The cathepsin activity clearly does not follow the same pattern of changes as the other mitochondrial enzymes that have been measured, e.g. cytochrome oxidase and succinic dehydrogenase, and it must be concluded that the enzymic make-up of the mitochondria is not constant. This view is borne out by the results obtained with β -glucuronidase.

Although a rough relationship seems to exist between the level of β -glucuronidase during pregnancy and the oestrogen metabolism of the gland, it is difficult to account for the high level of the enzyme during lactation and the peak during involution when oestrogen stimulation is presumed to be diminished. It is worth noting, however, that a similar rise in β -glucuronidase occurs in the involution of placentomas (Stafford, 1949).

Reviewing the mitochondrial enzymes studied in the mammary gland we thus have three enzymes, succinic dehydrogenase, cytochrome oxidase and glutamic dehydrogenase, which follow one pattern, showing a more or less constant activity during lactation; one enzyme, cathepsin, which increases progressively throughout lactation; and one enzyme, β -glucuronidase, which decreases throughout lactation. It thus appears that mitochondria can have different enzymic constitutions.

It is suggested, therefore, that during the lactation cycle there is a constant turnover of mitochondria and that as these are regenerated at each stage of the cycle they are regenerated complete with those enzymes adapted to the metabolic requirement of the cell at that time.

In view of these considerations it is clearly desirable to extend this study to isolated mitochondria and to attempt to correlate the changes in enzyme levels with the physiological changes of the mammary gland.

SUMMARY

1. Changes in the activity of glutamic dehydrogenase, glutamic-aspartic transaminase, cathepsin and β -glucuronidase have been studied in the mammary gland of the rat at various stages during pregnancy, lactation and mammary involution.

2. The activities of glutamic dehydrogenase and glutamic-aspartic transaminase increase only slightly during pregnancy, but rise sharply after parturition to a high level which is maintained throughout lactation, falling precipitously after weaning.

3. β -Glucuronidase activity increases sharply between the 15th and 20th days of pregnancy but then falls progressively over the period of lactation. There is a dramatic increase in β -glucuronidase after weaning during the period of mammary involution.

4. The catheptic activity of the mammary gland increases progressively from the 10th day of pregnancy up to the 20th day of lactation and increases even further during mammary involution.

5. The results are discussed in relation to possible changes in the mitochondria during the lactation cycle and the possible function of these enzymes in providing precursors for the synthesis of milk protein.

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Multiple Components in Horse-Radish Peroxidase

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The horse-radish (*Cochlearia armoracia* L.) is the source of an active peroxidase which has been the subject of many studies. These studies have comprised both accurate work on the kinetics and spectroscopy of the purified enzyme (Keilin & Hartree, 1951; Chance, 1951), and work on the chemical action of relatively crude enzyme extracts. Theorell (1942) first crystallized horse-radish peroxidase, and subsequent workers have used methods of preparation which are substantially modifications of his methods. Although Theorell showed that a substantial portion of the enzyme ('Peroxidase I') was different in electrophoretic and other properties from the material which he finally crystallized ('Peroxidase II'), this finding has been generally neglected. Subsequent workers have tended to assume that their specimens of crystalline peroxidase are a representative sample of the native enzyme of the horse-radish. Keilin & Hartree (1951) go so far as to deny the existence in nature of any component of horse-radish peroxidase other than the major one.

None the less, horse-radish peroxidase is described in textbooks as a typical example of an enzyme with more than one component (see, for example, Sumner & Somers, 1947). In the course of other work it seemed desirable to compare multi-component enzyme systems from *Aspergillus oryzae* (Gillespie, Jermyn & Woods, 1952) with this

apparently well-authenticated example of the natural coexistence of enzymes of the same specificity. A brief account of an investigation which showed the existence of four components in horse-radish peroxidase was published by Jermyn (1952*a*). Kondo & Morita (1953) have recently isolated from the sweet potato two peroxidases, *a* and *b*, by differential adsorption on alumina gel. Their peroxidase *a* was similar in properties to horse-radish peroxidase II; the properties of peroxidase *b*, although sharply differentiated from those of peroxidase *a*, were also quite distinct from those reported for horse-radish peroxidase I by Theorell.

Filter-paper chromatography as used by Jermyn (1952*a*) demanded both partial purification of the enzyme and a rigid control of the conditions of development of the chromatogram. A certain amount of trial-and-error adjustment of enzyme concentration was necessary to show up the various components. There was thus always room for the suspicion that some form of artifact had been produced. We have therefore used the method of filter-paper electrophoresis (Cremer & Tiselius, 1950) which could be applied to untreated horse-radish juice. The enzyme system has thus been investigated in a state as little removed as possible from that existing *in vivo*, and the application of any methods of purification has been deliberately avoided.