Function of Arginase in Lactating Mammary Gland

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The potential for a considerable formation of ornithine exists in lactating mammary gland because of its arginase content. Late in lactation arginase reaches an activity in the gland higher than that present in any rat tissue except liver. Occurrence of the urea cycle can be excluded since two enzymes for the further reaction of ornithine in the cycle, carbamoyl phosphate synthetase I and ornithine carbamoyltransferase, are both absent from this tissue. Instead, carbamoyl phosphate synthetase II appears early in lactation, associated with accumulation of aspartate carbamoyltransferase and DNA, consistent with the proposed role of these enzymes in pyrimidine synthesis. The facts require another physiological role for arginase apart from its known function in the urea cycle. Significant activity of ornithine aminotransferase develops in mammary gland in close parallel with the arginase. By this reaction, ornithine can be converted into glutamic semialdehyde and subsequently into proline. The enzymic composition of the lactating mammary gland is therefore appropriate for the major conversion of arginine into proline that is known to occur in the intact gland.

An orderly accumulation of particular enzymes occurs in mammary gland with the onset of and during the increase in lactation (Munford, 1964; Baldwin & Milligan, 1966). One of these enzymes is arginase (EC 3.5.3.1) (Folley & Greenbaum, 1947; Greengard *et al.*, 1970). The function of the high arginase activity in lactating mammary gland, as part of the urea cycle or in another function still unidentified, might be apparent from other enzymes that accumulate along with it during lactation. A major conversion of arginine into proline occurs in the intact gland without formation of labelled citrulline from labelled arginine (Mepham & Linzell, 1966, 1967). This suggests that the urea cycle is inoperative and that arginine can be diverted through the reactions of arginase and the transamination of ornithine to proline. The reaction of ornithine in the urea cycle occurs via ornithine carbamoyltransferase (EC 2.1.3.3), with carbamoyl phosphate formed by the special carbamoyl phosphate synthetase I (EC 2.7.2.5) (Cohen, 1962). Carbamoyl phosphate can also be made by carbamoyl phosphate synthetase II for utilization via aspartate carbamoyltransferase (EC 2.1.3.2) in the synthesis of pyrimidines (Hager & Jones, 1967; Yip & Knox, 1970). The major alternative reaction of ornithine is conversion into glutamic

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semialdehyde by ornithine aminotransferase (EC 2.6.1.13), a step leading to proline synthesis. These several enzymes were determined in rat mammary gland during lactation to decide which of these metabolic pathways were open for the ornithine formed by the arginase present in the gland. It appears that the arginase in this tissue is not part of the urea cycle. It is associated, however, with equally high activity of the next enzyme that leads to proline formation.

Materials and Methods

$NaH^{14}CO_3$ (18.5 mCi/mmol), $K^{14}CNO$ (3.87 mCi/mmol) and L-[*ureido*-^{14}C]citrulline (5 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. 2,5-Diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were obtained from Packard Instrument Co., La Grange, Ill., U.S.A. L-Arginine, L-ornithine, L-glutamine and L-citrulline were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. Carbamoyl phosphate (lithium salt) and ATP were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Radioactive carbamoyl phosphate was prepared from $K^{14}CNO$ as the lithium salt by the method of Jones *et al.* (1955). Ornithine carbamoyltransferase from *Streptococcus faecalis* 8043, with the specific activity of 287 units/mg of protein, was prepared by the procedure of Jones (1962).

Rats used were the inbred NEDH strain. The pregnancies were dated. Ten pups were retained in each litter and weaned by removal from the dam at 22 days of age. Dams were decapitated at the appropriate times and the inguinal mammary glands dissected out for immediate analysis.

Assay of carbamoyl phosphate synthetase I and II

Tissue homogenates (20%, w/v) were prepared in a cold medium containing sucrose, ATP, $MgCl_2$, glycerol, 2-mercaptoethanol and potassium phosphate, pH7.0, in the concentrations previously described (Yip & Knox, 1970). Either the homogenate or the soluble fraction after 30min centrifugation at 100000g was analysed. In the experiments in Table 2 the soluble fraction was first dialysed at 4°C for 2h against 100vol. of the homogenizing medium with ATP omitted.

Both carbamoyl phosphate synthetase I and II were assayed by formation of $[^{14}C]$citrulline from $NaH^{14}CO_3$ (3×10^6c.p.m./μmol) in the presence of the citrulline-generating system. The synthetase I was assayed in the above homogenate with additions including N-acetylglutamate as previously described (Yip & Knox, 1970). Blank values with the complete reaction mixture deproteinized at zero time, or the incubated mixture with N-acetylglutamate omitted, were equally low and were subtracted. The synthetase II reaction mixture was the optimum one previously described containing 5mM-glutamine and no added NH_4Cl (Yip & Knox, 1970), and blanks were minus enzyme.

$[^{14}C]$Citrulline produced in the enzyme assays was separated on columns for counting. The reaction mixtures were stopped by addition of trichloroacetic acid to 10% final concentration. Tubes were gassed with CO_2 for 1h, being kept in boiling water for the first 10min, and 100mg of citrulline was added as carrier. The protein-free supernatants were run through narrow columns containing 1ml bed vol. of Dowex 50W (X8), followed by 20ml of water. Citrulline was eluted into a counting vial with 3ml of 40% (v/v) pyridine and the contents of the vials were evaporated to dryness at 45°C. To the dried vials 1ml of water was added followed by the addition of 10ml of modified Bray's (1960) solution [30g of naphthalene, 2g of 2,5-diphenyloxazole, 0.2g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 10ml of ethylene glycol, 50ml of methanol and 500ml of dioxan]. The recovery from the Dowex 50W (X8) column was 96–98% and the counting efficiency was 76%.

The eluted radioactive product of the carbamoyl phosphate synthetase II reaction in breast extracts was identified as citrulline by ascending t.l.c. on silica-gel G plates (Yip & Knox, 1970). The radio-active material migrated as a single peak in two solvent systems, with R_F values similar to that of the commercial $[^{14}C]$citrulline. It was labelled in the carbamoyl carbon atom of citrulline, as shown by enzymic arsenolysis with purified bacterial ornithine carbamoyltransferase by the method of Hager & Jones (1967). Of the labelled product 90% was lost, compared with the controls, which contained the same reaction mixture minus ornithine carbamoyltransferase. Disappearance of citrulline as measured by the colorimetric method of Archibald (1944) paralleled the decrease in radioactivity. Similar results were obtained when commercial L-[ureido-^{14}C]citrulline was used.

Other determinations

The arginase assay was based on that of Schimke (1962). Tissues homogenized in 4vol. of water were incubated with 0.05M-$MnSO_4$ for 5min at 55°C. Samples (0.05–0.5ml) were then incubated for 15min at 37°C with L-arginine (0.25M) and glycine buffer (0.05M, pH9.5) in a final volume of 2ml. To stop the reaction 1ml of 20% $HClO_4$ was added. Urea was measured by the method of Archibald (1944).

Conditions for the colorimetric assay of ornithine carbamoyltransferase were those described by Schimke (1962). The enzyme was also assayed more sensitively under the same conditions by using $[^{14}C]$carbamoyl phosphate, with the mixture proportionately decreased in volume to 0.5ml. The $[^{14}C]$citrulline was separated as described above. Both assays gave the same activities in adult liver preparations.

Ornithine aminotransferase was measured as described by Herzfeld & Knox (1968).

All of the enzymes were measured in standard rat livers by the same methods used on mammary glands, so that the activities could be expressed as relative concentrations (Knox, 1972). Adult male rats provided the liver reference standards for all but synthetase II, for which foetal liver was the standard. All assays were proportional to the amounts of standard tissue used. All activities were expressed in units or munits (μmol or nmol of product formed/min at 37°C) per g of milk-free tissue. The lactose contents of rat milk (30.0±1.5mg/ml, mean±S.D. of six determinations) and of the mammary gland homogenates were determined by the method of Slater (1957). The lactose present was used to correct the weights of tissue for milk content.

DNA was determined by the diphenylamine method (Burton, 1956) after the extraction of the nucleic acids from a 10% (w/v) water homogenate as described by Greenbaum & Slater (1957). The results are expressed as mg of DNA/g of tissue corrected for milk content.

Results

Arginase was measured quantitatively in rat mammary gland at intervals during pregnancy, lactation and involution. It increased and then fell (Fig. 1), approximately in parallel with milk production as reported by Folley & Greenbaum (1947). The latter authors did not compare the activity with that in other tissues by the same assay. By our assay the peak activity in mammary gland was at least twice that recorded for any tissue except liver (Greengard *et al.*, 1970) and about one-quarter of that in adult liver (Table 1). It is to be noted (Fig. 1) that ornithine aminotransferase appeared in the mammary gland during lactation in parallel with arginase and reached nearly half the activity found in control livers (Table 1).

No significant activity of ornithine carbamoyltransferase, the enzyme following arginase in the
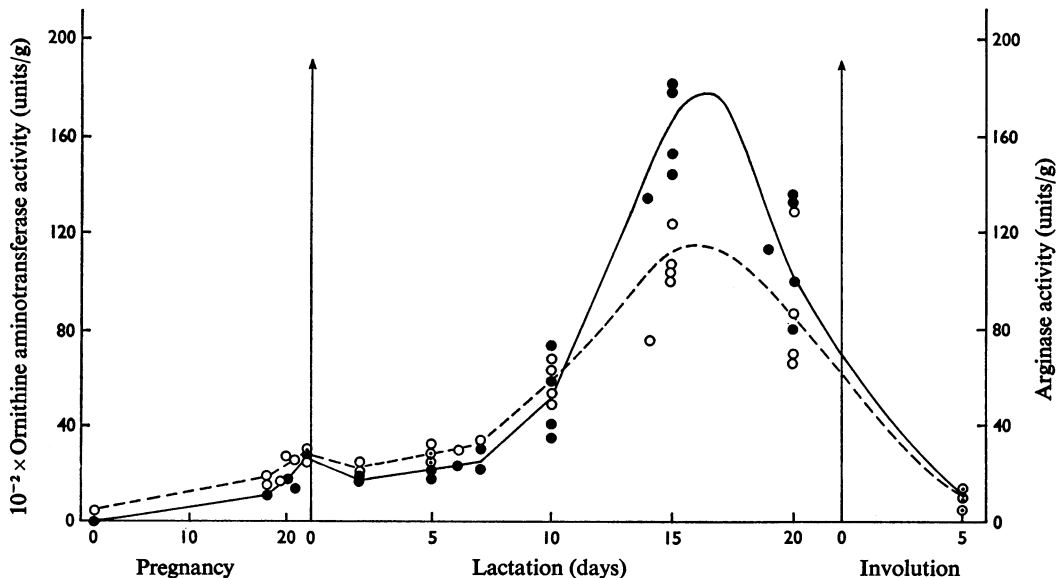

Fig. 1. *Parallel development of arginase and ornithine aminotransferase activities in rat mammary gland during late lactation*

Both arginase (●) and ornithine aminotransferase (○) activities are expressed in units (μmol/min) per g of milk-free gland from individual animals.

Table 1. *Enzyme activities in rat liver and mammary gland*

Means of the maximum values reached in lactating mammary gland and the mean values in livers of standard rats are given, each representing four or more determinations. Means of activities of adult male rat livers are given as the reference standards for the methods used in μmol/min per g at 37°C, except for carbamoyl phosphate synthetase II. Activities of carbamoyl phosphate synthetase II are nmol/min per g, and foetal rat liver at 16 days gestation served as the reference standard.

	Lactating mammary gland (units/g)	Standard liver (units/g)	Activity in mammary gland (% of that in liver)
Arginase	180	669	27
Carbamoyl phosphate synthetase II	2.4 munits	6.0 munits	40
Aspartate carbamoyltransferase	0.83	0.59	140
Carbamoyl phosphate synthetase I	<0.0001	10	<0.001
Ornithine carbamoyltransferase	<0.005	214	<0.002
Ornithine aminotransferase	1.3	3	43

urea cycle, was detected in breast tissue at any stage of lactation. Activities were zero with the colorimetric assay and less than $0.005\,\mu$mol/min per g of tissue above the control without enzyme in the more sensitive radioactive assay. The activity of $214\,\mu$mol/min per g of tissue was easily detected in adult male liver by both assays (Table 1).

Carbamoyl phosphate synthesis occurred in the gland homogenates, but at the low tissue activity (measured in munits) that is typical of the carbamoyl phosphate synthetase II, which utilizes glutamine, and not at the high tissue activity (measured in units) of the carbamoyl phosphate synthetase I, which utilizes only NH_3 (Yip & Knox, 1970). All of this activity was present in the soluble fraction after centrifugation (Table 2), and it was unstable. In these properties it was like carbamoyl phosphate

Table 2. *Activity of carbamoyl phosphate synthetase II in the rat mammary gland*

Incorporation of $NaH^{14}CO_3$ into citrulline was measured under the conditions of the assay for carbamoyl phosphate synthetase II activity. Each value is the mean from two or three animals.

Status of rat mammary gland	[^{14}C]Citrulline produced (nmol/min per g)	
	Homogenate	100000g supernatant
Virgin	0.34	0.24
Pregnant, 20 days	0.48	0.52
Lactating, 2 days	1.30	1.50
Lactating, 6 days	1.50	1.74
Lactating, 22 days	1.46	1.53
Involuting, 6 days	0.19	0.23

synthetase II and not like the stable particulate carbamoyl phosphate synthetase I. Specific assay of the particulate fraction for the latter activity that was dependent on addition of N-acetylglutamate was repeatedly negative at all stages of the lactation cycle. The assay was made equally as sensitive as that for carbamoyl phosphate synthetase II by use of bicarbonate with the same specific radioactivity.

Short dialysis of the soluble tissue fractions preserved the carbamoyl phosphate synthetase activity while decreasing the amounts of endogenous substances sufficiently to show characteristic increases in activity with the appropriate additions. Characteristics of the soluble dialysed system prepared from glands at two stages of lactation are given in Table 3. Incorporation of $NaH^{14}CO_3$ into citrulline was dependent on the addition of the soluble enzyme preparation from the mammary gland and the citrulline-generating system. The dialysed supernatant required the addition of glutamine for full enzymic activity. Ammonia also served as the nitrogen donor, giving activities higher than with glutamine, as previously observed (Yip & Knox, 1970). The activity with NH_4Cl and glutamine together was not higher than with NH_4Cl alone, indicating that a single enzyme was involved in the synthesis of carbamoyl phosphate. The glutamine analogue 6-diazo-5-oxo-L-norleucine inhibited the reaction. Addition of N-acetylglutamate, the cofactor for the particulate synthetase I reaction, did not stimulate the incorporation when NH_4Cl was used as substrate. The above characteristics of carbamoyl phosphate synthesis in rat mammary gland were different from carbamoyl phosphate synthetase I but were similar in detail to those of the synthetase II in tumours and certain tissues of the rat (Yip & Knox, 1970), Ehrlich ascites cells (Hager & Jones, 1967) and hemopoietic mouse spleen (Tatibana & Ito, 1967).

Table 3. *Characteristics of the soluble carbamoyl phosphate synthetase from lactating rat mammary gland*

The dialysed supernatant fraction of breast tissue prepared as described in the Materials and Methods section was used as the enzyme source.

System	Lactating gland ...	Activity (nmol of citrulline/min per g)	
		5 days	10 days
Complete		1.58	2.16
Minus enzyme source		0.10	0.10
Minus ornithine and ornithine carbamoyl-transferase		0.13	0.07
Minus glutamine		0.90	1.44
Minus glutamine, plus 50mM-NH_4Cl		1.98	2.67
Minus glutamine, plus 50mM-NH_4Cl and 5mM-N-acetylglutamate		1.98	2.76
Complete, plus 10mM-6-diazo-5-oxo-L-norleucine		0.50	0.87
Complete, plus 5mM-N-acetylglutamate		1.53	2.12

Carbamoyl phosphate synthetase II activity was just detectable in the virgin gland, rose sharply immediately after parturition and remained at about the same value until the gland involuted after removal of the pups (Table 2). It developed earlier in lactation than did arginase. Development of synthetase II approximately paralleled the early increases in DNA concentration in the gland that are shown in Fig. 2. Fig. 2 also shows how closely the development of carbamoyl phosphate synthetase II paralleled the early development of aspartate carbamoyltransferase in the same tissues, as reported previously from this laboratory (Herzfeld & Knox, 1972). The association in time of the three curves in Fig. 2 is consistent with the participation of these two enzymes in the pyrimidine synthesis necessary for the cell proliferation that is known to occur early in lactation (Munford, 1964).

The maximum activities during lactation of all enzymes measured in mammary gland are given in Table 1, in comparison with the standard liver values for these enzymes determined by the same methods. The quantitative patterns in these two tissues are generally similar, with values in the mammary gland roughly one-third of those in liver, except for the

virtual absence from mammary gland of two of the three urea-cycle enzymes studied.

Discussion

The enzymes that accumulate in breast tissue during lactation clearly do so at different stages, depending on the timing of the functions they subserve (Baldwin & Milligan, 1966). In the present study two examples were found of closely parallel development of enzyme pairs that are sequentially related in function. In addition to the development of arginase and ornithine aminotransferase late in lactation was the early and parallel development of carbamoyl phosphate synthetase II and aspartate carbamoyltransferase. An elevation of the latter two activities to form pyrimidines may be a prerequisite for the accelerated cell proliferation occurring at and shortly after parturition, which was indicated by the coincident increase in DNA concentration in the gland. Other instances have been recorded of both these enzymes increasing with cell proliferation. In transplanted rat tumours they are elevated in proportion to the measured growth rates (Yip & Knox, 1970; Herzfeld & Knox, 1972).
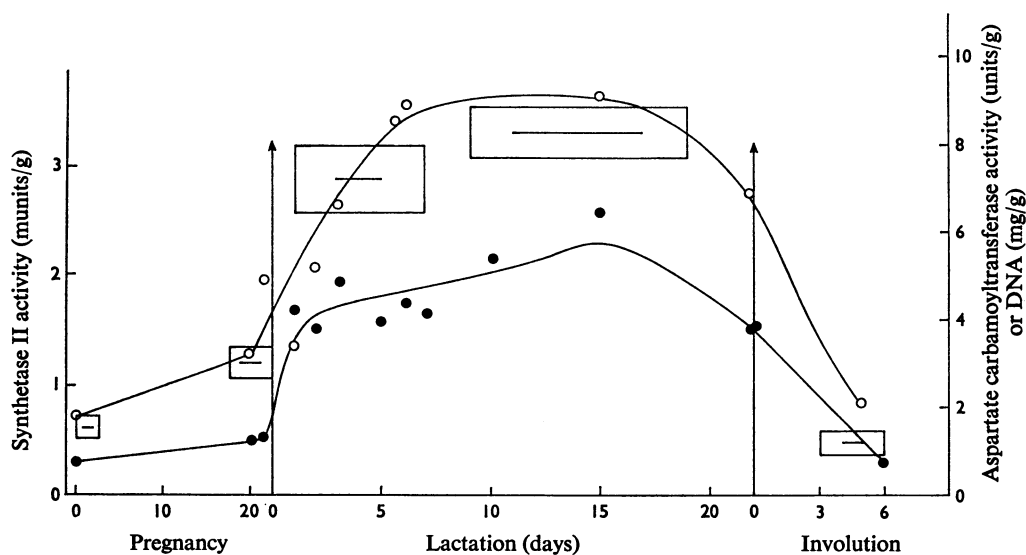

Fig. 2. *Parallel development of carbamoyl phosphate synthetase II, aspartate carbamoyltransferase and DNA in rat mammary gland*

Mean values of aspartate carbamoyltransferase over periods of several days are represented in boxes showing 2 s.e.m. vertically and have been recalculated from those previously reported (Herzfeld & Knox, 1972). Values are expressed in units or munits (μmol or nmol/min) per g of milk-free gland from individual animals, except for aspartate carbamoyltransferase, which represents the means from six or more animals. ●, Carbamoyl phosphate synthetase II activity; ○, DNA.

One or more of the urea-cycle enzymes have been found in a number of tissues, but the distribution of the whole functional urea cycle in tissues other than liver is uncertain (Knox, 1972; Knox & Greengard, 1965). Mammary gland offers a prime example of this. The development of arginase in rat breast during lactation was discovered by Folley & Greenbaum (1947), unfortunately with an assay that could not be compared directly with the assay of liver. The elevation was recently confirmed by methods that allowed direct comparison with the activity in liver (Greengard et al., 1970). The present measurements duplicate the curve rising to a maximum late in lactation, as described by Folley & Greenbaum (1947), and confirm the stated belief of these authors that the activity reached is relatively high. It is the second highest found in animal tissues. The maximum is about one-quarter of the activity in liver (Table 1)' and several times greater than in small intestine and kidney, the next richest tissues (Greengard et al., 1970).

If the relatively high arginase activity in mammary gland does not function in the urea cycle, it might participate in the substantial synthesis of proline that occurs in the intact gland. Mammary gland puts out in milk only about one-third of the arginine but considerably more proline than it extracts from the blood. It also extracts ornithine from the blood (Mepham & Linzell, 1966). The uptake of labelled arginine from blood and its incorporation as labelled proline into casein was demonstrated in perfused lactating cow and goat udders (Mepham & Linzell, 1967). In the latter experiments, the action of arginase and inactivity of the urea cycle could be inferred from the appearance of labelled ornithine, but not of labelled citrulline, in udders perfused with labelled arginine. The reverse conversion of labelled proline into ornithine could be forced in homogenates of several animal tissues that did not include mammary gland, but tests in the direction of proline were not reported (Smith et al., 1967). However, the urea cycle is absent from the adult silkmoth, and there arginine is converted into proline by the fat-body tissue through the expected enzyme reactions (Reddy & Campbell, 1969).

It is clear that the arginase in mammary gland does not function as part of the urea cycle, because the cycle is incomplete. Carbamoyl phosphate synthetase I and ornithine carbamoyltransferase, both required in the urea cycle for the step after arginase, could not be detected at any stage in the mammary gland by assays adequate to detect very small fractions of these activities that are present in liver. On the other hand, arginase was accompanied in the gland by ornithine aminotransferase. It increased in exact parallel with arginase to a maximum that was nearly half that in liver. The product of the ornithine aminotransferase reaction, glutamate γ-semialdehyde (or Δ^1-pyrroline-5-carboxylate), is the immediate precursor of proline (Peisach & Strecker, 1962). The relatively high and associated activities of these two enzymes provide appropriate means for the conversion of arginine into proline that increases in parallel with the synthesis of milk proteins. The δ-transamination of ornithine would also conserve nitrogen in the form of glutamate for use in the synthesis of certain other amino acids that the gland needs (Mepham & Linzell, 1967).

Roles for arginase outside the urea cycle have been considered for other animals (Folley & Greenbaum, 1947; Knox & Greengard, 1965) and insects (Reddy & Campbell, 1969). Close association with cell proliferation in animals is now unlikely because of the very low activity of arginase found in rapidly growing transplanted rat tumours (Greengard et al., 1970), and because arginase activity rose only after the stage of cell proliferation in the mammary gland (Figs. 1 and 2). However, an indirect relation to protein synthesis, at least to the massive synthesis of milk during lactation, is inherent in the role of forming proline. The arginase activity in liver also doubles during lactation (Folley & Greenbaum, 1947), and it is possible that some fraction of this arginase in liver and other tissues also functions in proline formation.

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