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Metabolism of Arginine in Lactating Rat Mammary Gland

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Significant activities of the four enzymes needed to convert arginine into proline and glutamate (arginase, ornithine aminotransferase, pyrroline-5-carboxylate reductase and pyrroline-5-carboxylate dehydrogenase) develop co-ordinately in lactating rat mammary glands in proportion to the increased production of milk. No enzymes were detected to carry out the reactions of proline oxidation or reduction of glutamate to pyrroline-5-carboxylate. Minces of the gland converted ornithine into proline and into glutamate plus glutamine. These conversions increased during the cycle of lactation in proportion to the increased milk production and to the content of the necessary enzymes. The minced gland did not convert labelled ornithine into citrulline, confirming the absence from the gland of a functioning urea cycle, and did not convert labelled proline or glutamate into ornithine. A metabolic flow of labelled arginine to proline and glutamate in mammary gland was confirmed in intact animals with experiments during which the specific radioactivity of proline in plasma remained below that of the proline being formed from labelled arginine within the gland. It was concluded that arginase in this tissue had a metabolic role in the biosynthesis of extra proline and glutamate needed for synthesis of milk proteins.

Several metabolic roles have been considered for arginase (L-arginine amidinohydrolase, EC 3.5.3.1) in those extrahepatic mammalian tissues, such as lactating mammary gland (Yip & Knox, 1972), which are deficient in enzymes of the urea cycle (Ratner, 1973). One possibility is the conversion of ornithine from the arginase reaction into proline and glutamate through the common intermediate, Δ^1-pyrroline-5-carboxylate. Interconversions between glutamate, proline and ornithine through Δ^1-pyrroline-5-carboxylate are characteristic of many animal tissues (Meister, 1965; Adams, 1970), but the direction of flow in particular tissues is uncertain. Labelled arginine or other intermediates of the urea cycle have given rise to labelled proline in several animal and insect tissues (Eagle *et al.*, 1965; Reddy & Campbell, 1969; Shimazaki *et al.*, 1973; Mepham & Linzell, 1967; Verbeke *et al.*, 1968; Ratner, 1973), and the reverse conversions of glutamate into citrulline (Windmueller & Spaeth, 1974) and of proline into ornithine (Smith *et al.*, 1967) have been demonstrated in intestine and liver respectively. The enzymes that catalyse the steps of these conversions, several of which are unidirectional, are associated in many rat tissues in ways favouring conversions of ornithine into proline and of proline into glutamate (Herzfeld *et al.*, 1977).

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Various observations suggest that arginine may be a significant biosynthetic source of proline and glutamate in lactating mammary gland. The first two of the necessary enzymes, arginase and ornithine aminotransferase (L-ornithine–2-oxo acid aminotransferase, EC 2.6.1.13), develop co-ordinately in the rat gland during lactation and are unaccompanied by other enzymes of the urea cycle (Yip & Knox, 1972). Lactating goat mammary gland takes from the blood more arginine and less proline and glutamine than it puts out in milk (Mepham & Linzell, 1966). Perfusion of the goat gland with labelled arginine gave results consistent with the conversion of arginine into proline (Mepham & Linzell, 1967; Verbeke *et al.*, 1968; Roets *et al.*, 1974) in amounts sufficient to cover the proline deficit.

In the present study the activities of the several enzymes needed for the conversion of arginine into proline were measured in rat mammary gland throughout the lactation period, and the metabolic interconversions were also examined in minces of the gland and in the living animal. There was a significant metabolic flow only in the direction from arginine to proline and glutamate. This flow increased in proportion to milk production and was associated with parallel increases in the amounts of the necessary enzymes in the gland.

Materials and Methods

DL-Δ^1-Pyrroline-5-carboxylate was prepared from DL-hydroxylysine by periodate oxidation (Mezl &

Knox, 1976). U-^{14}C-labelled amino acids (260mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.); they were checked for chemical and radioisotopic contaminants by amino acid analysis. Uniform distribution of radioactivity in the arginine was verified by decomposing it with rat liver arginase and comparing the specific radioactivities of the separated urea and ornithine.

Kx syngeneic rats (MRC Laboratory Animal Centre registration) were used from the colony maintained here. Pregnancies were dated. Lactating mammary glands were taken only from dams with a second or later litter numbering seven to nine pups. Pups were weaned at 22 days old. Lactose (Slater, 1957) contents of all mammary glands were determined and used to correct the tissue weight for milk content on the basis of 30mg of lactose/ml of milk (Yip & Knox, 1972; Kuhn, 1972).

Dams for experiments *in vivo* were anaesthesized with 60mg of pentobarbital (Abbott Laboratories, South Pasadena, CA, U.S.A.)/kg and injected with 25μCi of [U-^{14}C]arginine in 0.75ml of 0.9% NaCl into the inferior vena cava through an abdominal incision. Then 15min later a blood sample was taken by heart puncture and the tissues were removed rapidly. Tissues were homogenized in an equal volume of water and deproteinized (see below). Extract samples equivalent to 0.6–0.9g of mammary gland (about 100000d.p.m.) or 0.8–1.1ml of plasma were used for each amino acid analysis.

To make comparisons between the experiments *in vivo* and between the different compounds derived from labelled arginine in these experiments, the radioactivities of each compound per g of tissue have been expressed relative to the specific radioactivity (d.p.m./μmol) of the arginine carbon atoms from which the compound is derived as measured in the plasma arginine at 15min. These relative radioactivities have values equivalent to plasma arginine content (μmol) expressed either as the total radioactivity/g of tissue or as specific radioactivity/μmol of derived compound.

For tissue minces 1g (wet wt.) of an inguinal mammary gland was cut into 1–2mm cubes with a blade and put in a 60mm×15mm Falcon dish containing 3ml of 0.15M-KCl, 0.3mM-proline and 0.2mM-ornithine. After the addition of 1μCi of one of the uniformly labelled precursors, the covered dish was gently shaken in air at room temperature (25°C) for 1h. The reaction was terminated by deproteinization (see below) and centrifugation. The soluble fraction equivalent of about 0.2g of tissue (300000d.p.m.) was used for each amino acid analysis.

All tissue samples were deproteinized before analysis with a final concentration of 10% (w/v) sulphosalicylic acid. Amino acid analyses were carried out with a Beckman 120C analyser by using the sodium citrate buffer system described for physiological fluids (Benson & Patterson, 1965). To give maximum resolution of proline from glutamate the acidity of the first buffer was increased to pH3.16. This procedure did not separate glutamine from asparagine. The effluent from the amino acid analyser was collected for radioactivity measurements in fractions that were synchronized with the recorder by allowing for the time-lag between the photometer and the collection point (Olson *et al.*, 1968), and for the lag (0.2min) due to the ^{14}C isotope effect (Gaitonde & Nixey, 1972). Resolution of closely spaced radioactive amino acids was confirmed by collecting multiple fractions between such peaks. Samples (0.5–2ml) were counted for radioactivity in 10ml of Instagel (Packard, Downers Grove, IL, U.S.A.) at an efficiency of 80–85% (automatic external-standard-ratio method). Corrections were made for loss of radioactive CO_2 in the ninhydrin reaction (Peterson *et al.*, 1976) by using the following recoveries of radioactivity obtained from uniformly labelled amino acids in the amino acid-analyser effluent: 5-oxoproline, 97%; urea, 92%; ornithine, proline and Δ^1-pyrroline-5-carboxylate, 80%; arginine, 67%; glutamate and glutamine, 60%.

Enzymes were assayed in fresh tissue homogenates made in 0.25M-sucrose. Adult male rat liver was assayed with each group of samples as the reference standard. Average activities in this standard tissue were 2500, 3.3, 31.5 and 33.5μmol/min per g respectively, with the assays used for arginase (Herzfeld & Raper, 1976), ornithine aminotransferase (Herzfeld & Knox, 1968), Δ^1-pyrroline-5-carboxylate reductase [L-proline–NAD(P)$^+$ 5-oxidoreductase, EC 1.5.1.2) and Δ^1-pyrroline-5-carboxylate dehydrogenase (L-pyrroline-5-carboxylate–NAD$^+$ oxidoreductase, EC 1.5.1.12) (Herzfeld *et al.*, 1977). Samples for assay of Δ^1-pyrroline-5-carboxylate reductase were handled throughout at room temperature because of inactivation in the cold. In the ornithine aminotransferase assay the product was determined with *o*-aminobenzaldehyde by using $\varepsilon = 2580$ litre·mol^{-1}·cm^{-1} (Mezl & Knox, 1976). Enzyme reaction rates were all proportional to enzyme concentrations and linear with time. Results are expressed as units (μmol/min) per g of milk-free tissue.

Results

Milk production by rat mammary glands increases in close proportion to the accumulated weight of the suckled pups and reaches about 40ml/day (Cowie & Folley, 1947; Walters & McLean, 1967). Activities of those enzymes in the gland that are needed to convert arginine into proline (and glutamate) also increase substantially during the lactation period and then decrease precipitously after weaning (Fig. 1). Insets in Fig. 1 show the 5–10-fold increases in activity of arginase and ornithine aminotransferase

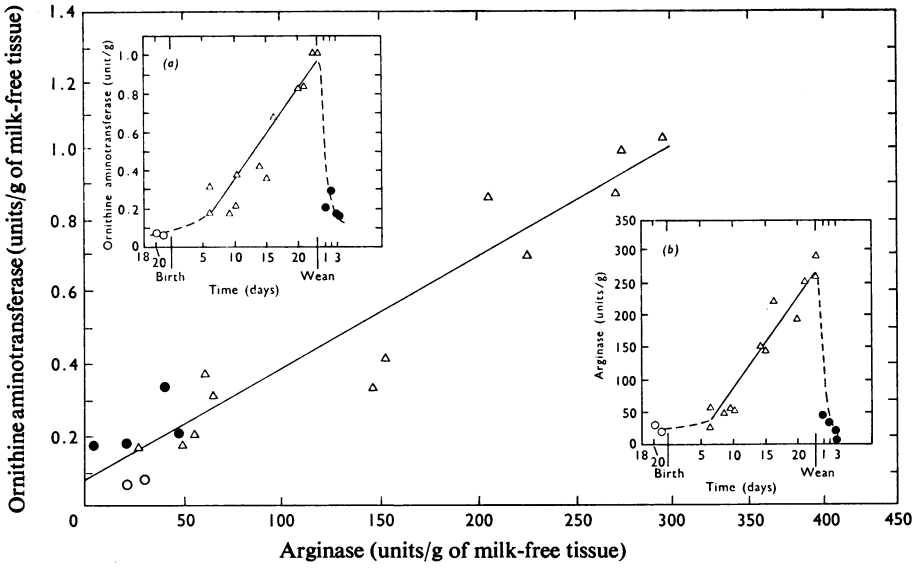

Fig. 1. *Correlation between the activities of ornithine aminotransferase and arginase in rat mammary gland throughout the lactation cycle*

Inserts show the changes with time of ornithine aminotransferase (insert *a*) and arginase (insert *b*) per g of milk-free tissue. Points represent individual glands in which both activities were measured as μmol reacted/min. In the curve these two activities (during pregnancy, ○; lactation, △; involution, ●) are plotted against one another. The correlation coefficient (R) is 0.95 ($n = 18$, $P < 0.005$).

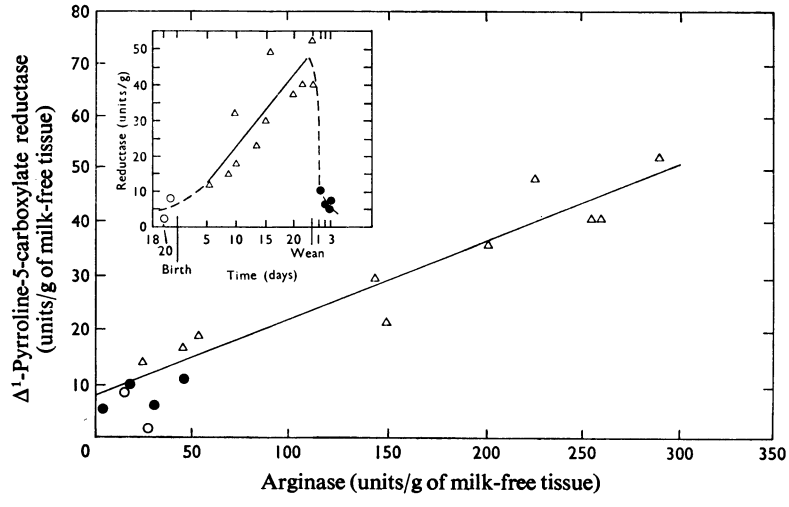

Fig. 2. *Correlation between the activities of Δ¹-pyrroline-5-carboxylate reductase and arginase in rat mammary gland throughout the lactation cycle*

The insert shows the change with time of Δ¹-pyrroline-5-carboxylate reductase. Other data are the same as in Fig. 1. For the two enzyme activities the correlation coefficient (R) is 0.93 ($n = 17$, $P < 0.005$).

per g of milk-free glandular tissue. These rose to maxima at the time of weaning and then returned to basal values within 3 days during the subsequent involutional period (Folley & Greenbaum, 1947; Yip & Knox, 1972). The main curve in Fig. 1 shows that the activities of these two enzymes, both

measured in the same glands, were highly correlated with each other throughout the cycle of lactation.

Fig. 2 shows the similar time course of the activity of Δ^1-pyrroline-5-carboxylate reductase, and its equally significant correlation throughout the cycle of lactation with the activity of arginase in the same glands. The three enzyme activities, two of which are each correlated with the third, are therefore correlated with each other and change their activities co-ordinately. The gland of late lactation was among the most active of rat tissues for each of these enzymes (Herzfeld *et al.*, 1977). Δ^1-Pyrroline-5-carboxylate dehydrogenase activity also increased in the glands from late pregnancy (0.34 unit) to late lactation (0.75 unit) per g of milk-free gland. Only six of 30 tissues surveyed had activities as high as this (Herzfeld *et al.*, 1977). No significant proline oxidase activity and no conversion of glutamate into Δ^1-pyrroline-5-carboxylate was detectable with sensitive chemical tests.

Of the four enzymes demonstrated to be present in the gland, all but ornithine aminotransferase function unidirectionally from arginine toward proline (or glutamate). Although the enzymic potentials for such conversions increased substantially during the course of lactation, an actual metabolic flow and its direction must be demonstrated in the glandular itself.

Conversions between ornithine, proline and glutamine by mammary gland in vitro

Minces and homogenates of lactating mammary gland, when incubated as described in the Materials and Methods section, caused the same interconversions of labelled amino acids. Minced glands showed severalfold greater activity in this respect and were studied in detail. After incubation of a mince for 1 h with labelled ornithine, proline or glutamate and with concentrations of these free amino acids comparable with those in the tissues, the amino acid concentrations and absolute radioactivities were those shown in Table 1. This gland, though it was from the late lactation period and was rich in arginase, did not convert labelled ornithine into detectable amounts of citrulline or arginine and therefore lacked a functional urea cycle. The same had been concluded from the incomplete urea cycle present in this tissue (Yip & Knox, 1972). In control experiments with similarly treated rat liver, arginine and citrulline were heavily labelled from ornithine by means of the functioning urea cycle in that tissue. The other interconversions in minced liver were qualitatively similar to those shown for mammary gland in Table 1.

Reactions in a gland during late lactation as described in Table 1 resulted in definite labelling of glutamate, glutamine and proline from ornithine. There was also the expected, incomplete equilibration of label between glutamate and glutamine. But there were only dubious conversions between glutamate and proline and virtually none from these compounds into ornithine. Δ^1-Pyrroline-5-carboxylate was not detectable among the products either by the ninhydrin reaction or by radioactivity in the appropriate fraction from the amino acid analyser.

Since it has been suggested that the role of arginase in mammary gland was to provide ornithine for the biosynthesis of spermidine (Oka & Perry, 1974), the polyamine fraction from the amino acid analyses of this tissue mince was also examined. It contained less than 0.3% of the total radioactivity, and this was not different if the added radioactive precursor was ornithine or proline. Labelled L-pyrrolidonecarboxylate (5-oxoproline) added to similar mammary gland minces was converted into glutamate and glutamine, as previously observed in other tissues (Meister, 1974), but it was also not converted into proline or ornithine.

To compare the conversions demonstrated in minced mammary gland with the changing enzyme pattern in this tissue during lactation, the experiment of Table 1 was repeated on glands at successive stages of the lactation cycle (Table 2). The same pattern of

Table 1. *Radioactivities of ^{14}C-labelled amino acids in minced lactating mammary gland incubated with precursors*

Amino acid analyses and radioactivities were determined after 1 h incubation of a mince of mammary gland from day 17 of lactation as described in the Materials and Methods section. Fractions not significantly above background radioactivity are marked 'negl.'. The listed amino acids accounted for 90% of the total radioactivity in the deproteinized filtrate. Concentrations of amino acids are means ± S.D.

Amino acid analysed	Concentration (μmol/dish)	10^{-3} × Radioactivity (d.p.m./dish) with precursors		
		[U-^{14}C]Ornithine	[U-^{14}C]Proline	[U-^{14}C]Glutamate
Ornithine	0.70 ± 0.05	1180	negl.	5
Proline	2.12 ± 0.05	183	2500	10
Glutamate	5.17 ± 0.15	108	30	2140
Glutamine	2.35 ± 0.05	131	4	153
Citrulline	0.06 ± 0.04	negl.	negl.	—
Arginine	0.32	negl.	—	—
Pyrroline-5-carboxylate	negl.	negl.	negl.	negl.

Table 2. *Interconversions of ornithine, proline and glutamate in minced mammary gland at different stages of lactation*
Amino acids formed are calculated by dividing the radioactivity found in an amino acid by the specific radioactivity (d.p.m./μmol) of the precursor at the end of the incubation. Results are expressed as μmol per g of milk-free tissue. Conditions were those of Table 1.

	State of mammary gland				
	21 days pregnant	2 days lactating	11 days lactating	12 days lactating	17 days lactating
Proline from [^{14}C]ornithine	0.035	0.105	0.069	0.071	0.230
Glutamate+glutamine from [^{14}C]ornithine	0.015	0.061	0.18	0.13	0.290
Ornithine from [^{14}C]proline	0.0028	0.0033	—	<0.0007	negligible
Ratio of proline/(glutamate+glutamine) from [^{14}C]ornithine	2.38	1.73	0.39	0.56	0.77

conversions occurred in glands at all stages. These are shown in Table 2 as μmol of product formed per μmol of precursor. The main conversions were from ornithine into proline and glutamate (plus glutamine). The total amounts of proline and glutamate formed increased later in lactation in the same manner as did the concentrations of enzymes (Figs. 1 and 2), but the ratio of proline/glutamate formed decreased in the later stages. The total flow was substantial and was equivalent at the latest (17-day) stage to about one-half of the ornithine (0.7 μmol/g; Table 1) still present at the end of the 1 h incubation. The conversion of proline into ornithine was very small at all stages, and that from glutamate into ornithine was even less (results not shown).

Intermediate formation of Δ^1-pyrroline-5-carboxylate

A relationship between the rising activities of the appropriate unidirectional enzymes and the increased conversions of labelled ornithine into proline and glutamate in minced gland during lactation must remain inferential unless the reactions by which the conversions take place can be identified. The key intermediate in the postulated enzymic pathway is Δ^1-pyrroline-5-carboxylate (Adams, 1970), which was not detectable in the incubated minced tissues. In fact, the only direct evidence for participation of Δ^1-pyrroline-5-carboxylate in mammalian metabolism is its excretion in the rare genetic disorder of type II hyperprolinaemia (Goodman *et al.*, 1974; Applegarth *et al.*, 1974). To demonstrate its occurrence as an intermediate, DL-Δ^1-pyrroline-5-carboxylate was added to minces like those of Tables 1 and 2 in the presence of labelled ornithine. About one-half the added Δ^1-pyrroline-5-carboxylate remained at the end of the incubation, as well as some polymer (Mezl & Knox, 1976) formed from it (Table 3). A substantial amount of the Δ^1-pyrroline-5-carboxylate was converted by the minced gland into proline and glutamate, and some into ornithine, presumably by the enzyme reactions described above for this tissue. The pattern of labelling from ornithine was not substantially changed despite grossly altered concentrations of metabolites, except that the low specific radioactivity of the enlarged pool of glutamate minimized the labelling of glutamine. A significant amount of labelled Δ^1-pyrroline-5-carboxylate formed from the labelled ornithine was trapped. Its specific radioactivity, after allowing for the metabolically inactive D-isomer (Herzfeld *et al.*, 1977), was only slightly less than those of proline and glutamate presumably formed from it. The radioactivities usually found in proline and in glutamate (plus glutamine) were halved in the presence of the added Δ^1-pyrroline-5-carboxylate, which is consistent with its being a common precursor for both of these products.

Conversions of ornithine into proline and glutamate in mammary gland in vivo

It was possible to demonstrate the formation of free proline in mammary gland *in vivo* during a 15 min period by intravenous injection of [U-^{14}C]arginine. During this period the specific radioactivity of proline in plasma remained below that in the mammary gland itself. Labelled arginine disappeared from plasma with a half-life of 2.4 min [cf. 3–6 min (Rogers *et al.*, 1972)]. The rate of arginine disappearance did not vary between rats at different stages of lactation. Arginine and ornithine still accounted for most of the radioactivity in plasma 15 min after the injection (Table 4). The specific radioactivity of proline in plasma, though low, was the next highest after that of components of the urea cycle, as it also was after a longer period (Ratner, 1973). The labelled proline in plasma was equivalent to the formation of 0.002 μmol/μmol of the labelled arginine present in plasma after 15 min. There was an active uptake of labelled compounds into mammary gland, liver and kidney during this period, so that they contained more radioactivity per g than was present per ml of plasma. In mammary gland and liver about one-half of this radioactivity,

Table 3. *Effect of added* Δ^1*-pyrroline-5-carboxylate on metabolism of* [^{14}C]*ornithine in minced mammary gland*

Mince of 11-day lactating mammary gland was incubated for 1 h with [U-^{14}C]ornithine under the same conditions as in Table 1. In the trapping experiment, the medium contained 240 μmol of DL-Δ^1-pyrroline-5-carboxylate/dish. N.D., None detected..

Additions	Ornithine		Δ^1-Pyrroline-5-carboxylate		Proline		Glutamate		Glutamine	
	(d.p.m./dish)	(μmol/dish)	(d.p.m./dish)	(μmol/dish)	(d.p.m./dish)	(μmol/dish)	(d.p.m./dish)	(μmol/dish)	(d.p.m./dish)	(μmol/dish)
[U-^{14}C]Ornithine	1750000	0.97	N.D.	N.D.	97600	1.72	115000	4.5	136000	1.6
Unlabelled Δ^1-pyrroline-5-carboxylate+[U-^{14}C]-ornithine	1980000	2.72	87230	118	46000	15.3	110000	23.9	8400	2.2

Table 4. *^{14}C-labelled amino acids in plasma of a lactating rat after injection of* [*U-^{14}C*]*arginine*

Results are averages (± S.D., with number of determinations in parentheses) of similar measures in rats at days 8, 11, 15 and 19 of lactation. Blood was taken from the heart 15 min after injection of 25 μCi of [U-^{14}C]arginine. The listed compounds accounted for 93% of the total soluble radioactivity in plasma (91900 ± 23000 d.p.m./ml).

Amino acid	Radioactivity in plasma (%)	10^{-2} × Specific radioactivity (d.p.m./μmol)
Arginine	53 ± 14 (4)	4500 ± 820
Ornithine	17 ± 4 (4)	3620 ± 1060
Urea	10.3 ± 4.5 (3)	16 ± 2
Citrulline	2.2 ± 1.1 (3)	690 ± 254
Glutamine	1.1 ± 0.6 (3)	24 ± 20
Proline	0.8 ± 0.2 (3)	58 ± 20
Glutamate	<0.4	<90

but 90% of that in kidney, was acid-soluble. Mammary gland under these conditions was therefore exposed to highly labelled arginine and ornithine precursors and unlabelled products, just as in the mince experiments.

Radioactivities of the free ^{14}C-labelled amino acids within the mammary gland, relative to the specific radioactivity of the carbon atoms of plasma arginine at the same time, showed more rapid interconversions than were evident in the plasma (Table 5; cf. Table 4). There was no significant radioactivity in citrulline, confirming the virtual absence of the urea cycle in this tissue *in vivo*. Most of the label remained in arginine, ornithine and urea. The ratio of radioactivities of the carbon in urea to that in the C_5 compounds was approx. 1.0 in the several experiments, indicating that the radioisotope had entered the gland mainly as [U-^{14}C]arginine, which was there stoicheiometrically degraded to urea and the C_5 compounds that accumulated in the gland. Proline and glutamate (plus glutamine) were substantially labelled. Formation of proline was least at the earliest stage of lactation examined (8 days), but even then was equivalent to 0.006 μmol of labelled plasma arginine. This was three times as much new proline as was formed, on the average (Table 4), in plasma in the same period. The amount of label in glutamate (and glutamine) in the gland was greater than that in proline. From early to late lactation the total of proline and glutamate that was formed increased. The proportion of glutamate also rose, as was observed in the experiments with minced gland. Formation of these two products from arginine was quantitatively correlated with the amount of milk being produced, which was measured by the accumulated weight of the litters at the time of the measurements (Fig. 3).

Table 5. *^{14}C-labelled amino acids formed in vivo in lactating mammary gland from [U-^{14}C]arginine*

Amino acid fractions in the glands and arginine in plasma of anaesthetized lactating rats were measured 15min after intravenous injections of 25μCi of [U-^{14}C]arginine. Radioactivities (relative) and amounts (μmol, in parentheses) of amino acids are given per g of milk-free tissue. Measured radioactivities are expressed relative to the specific radioactivity of plasma arginine at 15min, as given at the lactation stages described.

	Relative radioactivities and amounts			
Day of lactation ...	8	11	15	19
Litter wt. (g) ...	80	107	262	233
Plasma arginine (d.p.m./μmol)	506000	330000	457000	501000
Arginine	0.103	0.115	0.096	0.102
	(0.19)	(0.29)	(0.16)	(0.26)
Ornithine	0.148	0.098	0.153	0.240
	(0.17)	(0.11)	(0.24)	(0.21)
Proline	0.006	0.005	0.021	0.012
	(0.36)	(0.34)	(0.24)	(0.22)
Glutamate	0.012	0.007	0.049	0.039
	(1.86)	(1.46)	(2.13)	(1.67)
Glutamine	0.003	0.006	0.025	0.014
	(0.47)	(1.02)	(0.96)	(0.57)
Urea	0.120	0.149	0.21	0.22
	(11.1)	(5.7)	(9.6)	(7.3)
Ratio of radioactivities				
Proline/(glutamate+glutamine)	0.39	0.40	0.28	0.23
Urea/C_5 amino acids	0.7	1.3	0.8	0.7

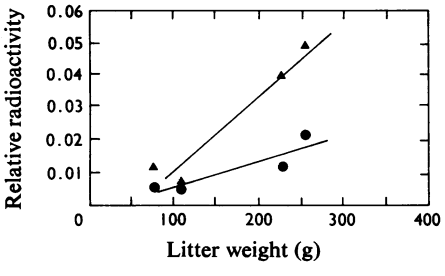

Fig. 3. *Formation of proline and glutamate from arginine in lactating mammary glands in vivo, in proportion to milk production*

Milk production is measured by the accumulated total weight of the litter of pups. The total relative radioactivities (per g of milk-free tissue) of proline (●) and glutamate (▲) are standardized ones from Table 5. Correlation coefficients (R) for $n=4$ are greater than 0.9 and are significantly different ($P<0.025$ for glutamate; $P<0.05$ for proline) from zero.

Table 6. *Relative specific radioactivities of amino acids formed in vivo in lactating mammary glands from [U-^{14}C]arginine*

Data are from experiments described in Table 5. These relative specific radioactivities represent the total relative radioactivities divided by the concentrations in μmol/g of milk-free tissue.

	Relative specific radioactivities per μmol			
Day of lactation ...	8	11	15	19
Arginine	0.56	0.40	0.61	0.39
Ornithine	0.87	0.88	0.66	1.15
Proline	0.016	0.014	0.086	0.052
Glutamate	0.006	0.005	0.023	0.023
Glutamine	0.006	0.006	0.025	0.024
Urea	0.011	0.026	0.021	0.030
Ratio of specific radioactivities of proline in gland/plasma	1.9	—	4.9	3.0

Relative specific radioactivities of the metabolites in mammary glands from experiments *in vivo* at different stages of lactation are shown in Table 6. Proline had the highest specific radioactivity after arginine and ornithine, indicating an appreciable conversion of these compounds into proline. Its specific radioactivity in the glands was at least twice that of proline in the plasma in the three instances when both were measured. This proline must have been formed in the gland. That glutamate and glutamine were formed only within the glands was less certain because their relative specific radioactivities were lower. The enzyme measurements and the metabolism in minced glands, however, demonstrated that this conversion was plausible.

Discussion

In lactating rat mammary gland there was a significant metabolic flow from arginine to proline and glutamate (and glutamine). This flow increased as milk production rose. The conversions occurred in minced glands and in the living rat. There was a coordinated rise in the activities in the glands of the four enzymes needed to effect these conversions, which also paralleled milk production. The unidirectional nature of one of the enzyme reactions in each branch of the pathway, the Δ^1-pyrroline-5-carboxylate reductase and Δ^1-pyrroline-5-carboxylate dehydrogenase (Herzfeld *et al.*, 1977), and the failure to demonstrate the reverse of these reactions in minced glands, would seem to preclude any significant metabolic flow from glutamate to proline or ornithine in this tissue. Δ^1-Pyrroline-5-carboxylate, the common intermediate in these reactions, was shown to be a metabolite and the precursor of both proline and glutamate by trapping it as it was formed from labelled ornithine.

Balance experiments on the uptake of amino acids from blood and their output in milk by the lactating goat mammary gland (Mepham & Linzell, 1966) were consistent with the conversions found here in the lactating rat mammary gland. Much more arginine and much less proline and glutamate plus glutamine were taken from the blood than appeared in goat milk (Mepham & Linzell, 1966). The deficiency of uptake over output for glutamate (plus glutamine) was twice as great as for proline. Since less than 20% of the glutamate and glutamine of casein is synthesized in the gland from glucose (Barry, 1956), the deficiencies in glutamate and proline uptake, and the excess in the uptake of arginine, can be accounted for by conversion of arginine in the gland into proline and glutamate, as shown here.

It can be concluded that synthesis of proline and glutamate in the amounts necessary for milk production is the major metabolic role of the arginase isoenzyme that appears in mammary gland during lactation. This arginase is unaccompanied by a functioning urea cycle (Folley & Greenbaum, 1947; Yip & Knox, 1972; Glass & Knox, 1973). A small proportion of the ornithine produced is undoubtedly channelled into polyamine synthesis (Oka & Perry, 1974; Russell & McVicker, 1972), but the amounts of polyamines present in the gland do not change in parallel with the arginase amounts and are apparently not controlled by it. In other extrahepatic tissues, containing significant arginase activity and possibly an incomplete urea cycle (Ratner, 1973), the metabolic role of arginase is less clear.

The rate of thes several sequential enzyme reactions in cell-free preparations of mammary gland differed, with ornithine aminotransferase and Δ^1-pyrroline-5-carboxylate dehydrogenase being the slowest. Under physiological conditions the limiting step may be the conversion of ornithine into Δ^1-pyrroline-5-carboxylate by ornithine aminotransferase, because arginine and ornithine were labelled more than later products, and there were only undetectably low concentrations of Δ^1-pyrroline-5-carboxylate in the minces. However, the minced glands formed glutamate in excess of proline, in opposition to the relative activities of the respective enzymes forming these products.

Proline was converted at a low rate into ornithine in liver (Smith *et al.*, 1967), and this was enhanced by conditions designed to reverse the ornithine aminotransferase reaction. The forward flow from ornithine to proline was not determined in those experiments. Ratner's (1973) observation of proline labelling from citrulline in liver, and our own control experiments with liver, suggest that the main metabolic flow in adult rat liver is also from ornithine to proline, as it is in mammary gland. It is otherwise in some other organs. In small intestine the flow is clearly from glutamate to proline and to citrulline (Windmueller & Spaeth, 1974, 1975). In fibroblasts from human lung, glutamate was better than arginine as a precursor for proline (Shen & Strecker, 1975). Since Δ^1-pyrroline-5-carboxylate dehydrogenase, Δ^1-pyrroline-5-carboxylate reductase and arginase (in the absence of a functioning urea cycle) all catalyse unidirectional reactions, there must be additional specialized enzymes in some tissues to effect the conversion of glutamate into proline and ornithine.

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