Metabolism of [^{14}C]Citrulline in the Perfused Sheep and Goat Udder

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1. A lactating-sheep mammary gland was perfused for 12h in the presence of L-[2- ^{14}C]citrulline and received adequate quantities of glucose, acetate and amino acids. Two lactating-goat udders were similarly perfused in the presence of either L-[carbamoyl- ^{14}C , 2- ^{14}C]citrulline or L-[carbamoyl- ^{14}C , 1- ^{14}C]citrulline and L-[4- ^3H]arginine. 2. In these experiments, [^{14}C]citrulline was substantially oxidized to CO_2 and converted into arginine and proline of casein. 3. The specific radioactivities of arginine, ornithine and proline of the plasma increased after passage through the udders, demonstrating that [^{14}C]citrulline is metabolized by the mammary gland. 4. The presence of two unknown radioactive metabolites of [^{14}C]citrulline was detected in the perfusate. These substances were not found after incubation *in vitro* of oxygenated blood in the presence of the radioactive precursor. 5. From these experiments, it is concluded that citrulline is metabolized in mammary tissue by way of arginine to urea, ornithine and proline.

In the plasma of ruminants, free citrulline occurs in concentrations ranging from 0.44 to 1.50mg/100ml (Verbeke & Peeters, 1965). In studying the uptake of free amino acids from plasma by the lactating udder of cows *in vivo* by using the technique of arteriovenous differences, it was found that citrulline was absorbed by the udder in some cows but not in others. The mean arteriovenous difference, although positive, was small but not significant (Verbeke & Peeters, 1965). In analogous experiments carried out on lactating goats by Mephram & Linzell (1966), a definite absorption of citrulline similarly could not be established. As citrulline does not occur in milk, it is obvious that this substance, if absorbed, will be broken down or converted into other amino acids.

A high arginase (EC 3.5.3.1) activity is present in the rat mammary gland (Folley & Greenbaum, 1947; Yip & Knox, 1972), but the urea cycle does not operate in this tissue. After the addition of radioactive arginine to the perfusion blood of isolated goat and sheep udders, radioactivity was detected in arginine and proline of milk casein, as well as in proline and ornithine of the blood plasma (Verbeke *et al.*, 1965, 1968; Mephram & Linzell, 1967). Moreover, in the perfusion experiment carried out by Mephram & Linzell (1967), the presence of radioactive urea was demonstrated in the plasma, but no radioactivity was detected in plasma citrulline. Neither was radioactivity detected in the arginine residues of the milk protein (Verbeke *et al.*, 1965, 1968), after addition of [^{14}C]ornithine to the perfusion blood. It has been

shown recently that both carbamoyl phosphate synthetase 1 (EC 2.7.2.5) and ornithine carbamoyl-transferase (EC 2.1.3.3) (each of which is required in the urea cycle for the step after arginase) are absent from the rat mammary gland (Yip & Knox, 1972).

In the experiments described here, the metabolism of [^{14}C]citrulline was studied in isolated perfused sheep and goat udders. Citrulline was converted into arginine, suggesting the presence of another part of the urea cycle in the mammary gland.

Two unknown radioactive metabolites were detected in the blood plasma, with structural similarities to cyclized argininosuccinic acid. To obtain more information about the origin of these metabolites, [^3H]arginine was added to the perfusate in the last experiment. A preliminary account of part of this work has been published (Roets *et al.*, 1973).

Experimental

Materials

Purification of L-[carbamoyl- ^{14}C]citrulline. L-[carbamoyl- ^{14}C]Citrulline was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Purification of 50 μCi of this substance was performed on a column (0.6cm $\times$ 55cm) of Aminex A6 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.; Li^+ form). Elution of the column was started with buffer at pH 2.80 (0.2M- Li^+), this buffer being changed 150min later to pH 4.15 (0.2M- Li^+). Effluent fractions of volume 6ml were collected. The radioactivity of each fraction was measured by liquid-scintillation. The

fractions containing citrulline were pooled, desalted and evaporated to dryness; 1.3% of the radioactivity in L-[carbamoyl- ^{14}C]citrulline was eluted as a small peak in the region of urea.

Preparation of L-[2- ^{14}C]citrulline. L-[2- ^{14}C]Citrulline was prepared by incubating DL-[2- ^{14}C]ornithine (Calbiochem, Los Angeles, Calif., U.S.A.) with carbamoyl phosphate, in the presence of ornithine carbamoyltransferase (EC 2.1.3.3). This enzyme was purified from rat liver by the method of Reichard (1957). The incubation mixture contained in a total volume of 10 ml: 175.4 μmol of DL-[2- ^{14}C]ornithine (200 μCi); 130 μmol of dilithium carbamoyl phosphate; 30 units of carbamoyltransferase activity; 1 mmol of Tris-HCl buffer, pH 7.4. The reaction was stopped by immersion in a boiling-water bath for 3 min. Carbamoyl phosphate was removed from the reaction mixture by adsorption on a column (0.9 cm $\times$ 6 cm) of Dowex 1X4 (Fluka A.G., Buchs, Switzerland; acetate form). Citrulline and unreacted ornithine were eluted with water, and the eluate was concentrated to 1 ml. Separation of citrulline from ornithine (mainly D-[2- ^{14}C]ornithine) was performed on a column (0.9 cm $\times$ 50 cm) of Dowex 50X12 (Fluka A.G., Buchs, Switzerland; H^+ form) by using a gradient of 1–6 M-HCl. The radioactivity of a portion of each effluent fraction was assayed by liquid-scintillation. From this column 98.4 μCi of L-[2- ^{14}C]citrulline, with a specific radioactivity of 1.15 nCi/mmol, was obtained.

The citrulline obtained was chromatographically pure as tested on an amino acid analyser, and the radioactivity of the effluent fractions. Analysis of L-[2- ^{14}C]citrulline on thin layers of cellulose in propan-2-ol-formic acid-water (75:12.5:12.5, by vol.) or phenol-water (80:20, v/v) in the presence of added CN^- showed only one ninhydrin-positive spot coinciding with the radioactivity measured along the thin-layer plate.

Preparation of L-[1- ^{14}C]citrulline. L-[1- ^{14}C]Citrulline was prepared by incubation of DL-[1- ^{14}C]ornithine (The Radiochemical Centre) as described for the preparation of L-[2- ^{14}C]citrulline (DL-[2- ^{14}C]ornithine was no longer available from The Radiochemical Centre).

Purification of L-[4- ^3H]arginine. Purification of 1000 μCi of L-[4- ^3H]arginine (CEA-IRE-SORIN, Mol, Belgium) was performed on a column (0.6 cm $\times$ 20 cm) of Dowex 50X8 (Fluka A.G., Na^+ form) as described by Verbeke *et al.* (1972). The total column effluent was directed into a fraction collector. After determining the radioactivity of the effluent fractions, arginine was pooled, desalted and evaporated to dryness. With this method, 660 μCi of L-[4- ^3H]arginine was recovered.

Methods

Perfusion of isolated mammary glands. Three

perfusion experiments were carried out. In the first experiment a mammary gland, yielding 720 ml of milk per day, was taken from a sheep; in the second and third, mammary glands yielding 658 and 704 ml of milk respectively, were taken from two goats. The surgical and perfusion techniques used for the preparation of the experiment have been described previously (Verbeke *et al.*, 1972). The isolated glands were perfused separately at a constant pressure of 140 cm H_2O . The temperature at the onset of perfusion was 33°C and was gradually increased to 37°C; this temperature was then maintained during perfusion (Hardwick & Linzell, 1960). The perfusion fluid consisted of 1.3 litres of heparinized homologous blood, to which was added bromolysergic acid diethylamide (BOL 148, Sandoz, Basle, Switzerland; 1 $\mu\text{g}/\text{ml}$) in order to block vasoconstriction induced by 5-hydroxytryptamine released from blood platelets. The perfusate was oxygenated by a mixture of air + CO_2 (93:7) and O_2 + CO_2 (93:7). The perfusion circuit included a blood reservoir, artificial lung, heart and kidney systems, an outflow recorder and a blood pressure-regulation device. In the artificial kidney, blood was circulated through a 11 m length of dialysis tubing immersed in 20 litres of modified Krebs solution (Hardwick *et al.*, 1961). At the onset of perfusion, the glands were emptied by milking after injection of oxytocin *inter alia*. A priming dose of the radioactive precursor was added to the blood and to the buffer solution of the kidney. A substrate solution containing plasma proteins, glucose, sodium acetate, amino acids and radioactive precursor was infused into the perfusate by means of a peristaltic pump, at a rate adjusted to the intensity of milk secretion. Another solution containing Ca^{2+} and Mg^{2+} ions was similarly infused. The glands were milked with the aid of oxytocin at 1 h intervals.

In the sheep udder experiment (Expt. 1), 49 μCi of L-[2- ^{14}C]citrulline was dissolved in 25 ml of 0.9% (w/v) NaCl; 1.55 ml and 14.00 ml of this solution were added to the perfusion blood and to the kidney fluid respectively, and 9.45 ml was added to 250 ml of substrate solution. The kidney fluid and the substrate solution contained respectively 1.31 and 36.5 mg of unlabelled citrulline/100 ml. The perfusion was continued for 12 h and 184 ml of substrate solution was infused into the perfusate. In total 44.11 μCi of L-[2- ^{14}C]citrulline was added to the system. The total milk production was 79.5 ml.

In the first goat udder experiment (Expt. 2), 48.26 μCi of L-[carbamoyl- ^{14}C]citrulline plus 47.78 μCi of L-[2- ^{14}C]citrulline were dissolved in 25 ml of 0.9% NaCl. This solution was divided as follows: 1.7 ml was added to the blood, 17.5 ml to the kidney fluid and 5.8 ml to 250 ml of substrate solution. The content of L-citrulline in the substrate solution and kidney fluid amounted to respectively 36.5 and 1.31 mg/100 ml. We infused 242 ml of the former

solution during the perfusion, bringing the total radioactivity to 95.05 μCi of L-[carbamoyl- ^{14}C ,2- ^{14}C]citrulline. The experiment was sustained for 10h, and a total volume of 115.4ml of milk was collected.

In the second goat udder experiment (Expt. 3), 26.12 μCi of L-[carbamoyl- ^{14}C]citrulline plus 26.12 μCi of L-[1- ^{14}C]citrulline were dissolved in 50ml of 0.9% (w/v) NaCl; 3.4, 35 and 11.6ml of this solution were added respectively to the blood, kidney fluid and to 250ml of substrate solution. The concentration of L-citrulline in the substrate solution and kidney fluid was respectively 35 and 1.31mg/100ml. Another solution, containing 330 μCi of L-[4- ^3H]arginine in 5ml of 0.9% NaCl was divided as follows: 0.2ml was added to the blood, 3.16ml to the kidney fluid and 1.64ml to 250ml of substrate solution. During the perfusion 200ml of substrate solution was infused. In this way, the total radioactivity added was 307 μCi of L-[4- ^3H]arginine, and 49.8 μCi of L-[carbamoyl- ^{14}C ,1- ^{14}C]citrulline. The perfusion was continued for 9h, and a total of 89.9ml of milk was collected.

Incubation experiment in vitro. Blood was drawn aseptically from a lactating sheep into a sterile bottle containing heparin. A quantity of 4.19 μCi of L-[carbamoyl- ^{14}C ,2- ^{14}C]citrulline was incubated with 500ml of the blood, in the presence of 30mg of penicillin and 7.5mg of neomycin, for 7h at 35°C. The mixture was carefully stirred and oxygenated by $\text{O}_2 + \text{CO}_2$ (95:5). No bacterial growth was observed. A 50ml blood sample was taken after termination of the incubation period, and the plasma was prepared for analysis.

Analytical methods. The measurement of expired $^{14}\text{CO}_2$ and the quantitative analysis of the milk components were carried out as described by Massart-Leën *et al.* (1970). Milk fat, lactose, citric acid and casein were isolated by the methods of Verbeke *et al.*, (1968). The extraction of milk fat and the precipitation of casein were performed in the presence of non-radioactive L-citrulline. The incorporation of ^{14}C into casein amino acids was measured after hydrolysis and chromatographic separation (Verbeke *et al.*, 1959).

Isolation of amino acids from plasma and kidney fluid. After perfusion or incubation, the plasma was prepared for analysis as described by Verbeke & Peeters (1965); the sample volume for each determination was 5ml of plasma. Kidney fluid was desalted by passage through a column (2cm $\times$ 3cm) of Dowex 50X7 (H^+ form). The column was washed with water until neutral and then eluted with 2M- NH_3 . Approx. 7.5ml of kidney fluid was used for each determination. The free amino acids in plasma and kidney fluid were determined by using an automatic amino acid analyser, employing a single chromatographic column (0.6cm $\times$ 135cm) of Aminex A6 (Bio-Rad Laboratories; Li^+ form) analogous to the

method described by Percy *et al.* (1968). A discontinuous buffer change was used; the elution was carried out at 30ml/h. Column elution was started with buffer at pH 2.80 (0.2M- Li^+). Buffer changes were performed at 7.5h (buffer pH 3.80; 0.2M- Li^+), 12h (buffer pH 4.20; 0.8M- Li^+) and 17h (buffer pH 6.10; 1.2M- Li^+). Column elution was completed 24h after the beginning of analysis. This chromatographic procedure was useful, as we had to determine quantitatively urea and several amino acids in limited blood samples. Amino acids were isolated from the plasma by directing 50% of the column effluent into a fraction collector. The radioactivities of the fractions containing urea, proline, citrulline, ornithine and arginine were measured by liquid-scintillation.

Isolation of an unknown metabolite from plasma. During the amino acid analysis of the plasma samples taken in Expt. 2, a highly radioactive peak was detected in the column effluent. This fraction was ninhydrin-positive and was eluted between the position of urea and aspartic acid.

Alkaline hydrolysis was performed on samples of the unknown radioactive peak, the fractions being pooled, freeze-dried and desalted on a column (0.9cm $\times$ 6cm) of Dowex 50WX8 (H^+ form). The radioactive substances could not be eluted with 0.1M-HCl, but were quantitatively desorbed by 5 column volumes of 1M-HCl (15ml). The 1M-HCl eluate was evaporated to dryness, dissolved in 0.5ml of water and hydrolysed in a sealed tube in the presence of 750mg of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ at 110°C for 15h. The hydrolysate was quantitatively transferred to a diffusion flask, acidified with 0.5M- H_2SO_4 , and the CO_2 trapped in 0.5ml of Hyamine hydroxide. After 24h of diffusion, the hyamine solution was assayed for radioactivity. The acidified hydrolysate was filtered, adsorbed on a column (0.9cm $\times$ 6cm) of Dowex 50 (H^+ form) and eluted successively with 15ml of 0.1M-, 1M-, 2M-, 3M- and 4M-HCl. Most of the radioactivity in the acidified hydrolysate solution was recovered in the 3M-HCl fraction. This fraction was concentrated and the ornithine content determined by the Chinard (1952) reaction.

A $\text{Ba}(\text{OH})_2$ hydrolysis was performed on a second sample. After trapping the CO_2 in Hyamine hydroxide, the hydrolysate was applied to the amino acid analyser. A portion of the hydrolysate was dansylated, and the dansyl-amino acids were separated by two-dimensional t.l.c. (Zanetta *et al.*, 1970).

Isolation of amino acids from udder tissue. After perfusion, the goat udders (Expts. 2 and 3) were immediately cooled on ice. Skin, teat and adipose tissue were removed by dissection, and the gland was cut into pieces. Some 100g wet wt. of udder tissue was homogenized at 2°C in 500ml of ice-cold 0.3M- HClO_4 . The mixture was stored at 2°C for 24h, the proteins removed by centrifugation, then resuspended in 200ml of 0.3M- HClO_4 and re-centrifuged. The

combined supernatants were adjusted with 5M-KOH to give a final pH of 4-5, and the mixture was stored at 2°C for 48h. The precipitated KClO_4 was filtered off, and the supernatant was passed through a column (2cm×25cm) of Dowex 50 (H^+ form). The column was washed with water until neutral and eluted with 2M- NH_3 . The eluate was evaporated to dryness, and then dissolved in 5ml of Li^+ buffer, pH2.2. The amino acids were isolated by chromatography on a column (2cm×120cm) of Amberlite IR-120 (BDH Chemicals Ltd., Poole, Dorset, U.K.; Li^+ form) by stream splitting of the column effluent, by using an automatic amino acid analyser. A discontinuous buffer gradient was used, as described for the isolation of amino acids from plasma. A flow rate of buffer of 98ml/h was maintained throughout the analysis: 80% of the column effluent was directed into a fraction collector. The radioactivity of the fractions containing urea, proline, citrulline, ornithine and arginine was measured by liquid scintillation. The

fractions containing the investigated amino acids were pooled and desalted. Urea and citrulline were quantitatively determined, as described by Hunninghake & Grisolia (1966). Ornithine and proline were analysed by the Chinard (1952) reaction, and the arginine concentration was determined by a modified Sakaguchi reaction (Izumi, 1965).

Radiochemical techniques. The radioactivity of the prepared L-[2- ^{14}C]citrulline was measured by cutting the t.l.c. cellulose foils into 1cm segments. Each segment was assayed for radioactivity with 10ml of scintillation fluid consisting of 2,5-diphenyloxazole (15g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (50mg)/litre of toluene in a Packard Tri-Carb 3003 liquid-scintillation counter. The radioactivity of the isolated compounds was assayed as described by Massart-Leëen *et al.* (1970). The ^{14}C and ^3H radioactivity of the unknown metabolite(s) in Expt. 3 was measured in a single radioactivity-counting procedure (Hendler, 1964).

The product quotient, giving the percentage of milk product derived from the precursor in the plasma, was calculated from the ratio of the specific radioactivity of the isolated product to the mean specific radioactivity of the precursor in the plasma at steady state.

Results

(A) L-[2- ^{14}C]Citrulline experiment (Expt. 1) with a sheep udder

Milk composition. As compared with the composition of the milk from the living animal, normal values were obtained for the concentration of citric acid in milk produced during perfusion (Fig. 1). However, the casein concentration was always lower, although higher concentrations for lactose were found. The triglyceride concentration, which was high during the first hours of the experiment, reached normal values at the end of perfusion.

Concentration and specific radioactivities of amino acids and urea in the plasma. The plasma concentration of citrulline during the perfusion was within the physiological concentration range found *in vivo* (Table 1). The arteriovenous differences for citrulline were variable, and negative values were found after 2h of perfusion. The specific radioactivity of the precursor in blood plasma was relatively constant during the experiment. At the end of perfusion 24.6 μCi of the added citrulline (44.1 μCi) was recovered from the blood and kidney fluid, so that a maximum of 19.5 μCi of citrulline was consumed during the experiment.

The concentrations of arginine, ornithine and proline generally showed positive arteriovenous differences across the perfused mammary gland (Table 1). The specific radioactivities of these amino acids were usually higher in the venous plasma than

Fig. 1. Perfusion of a sheep mammary gland with L-[2- ^{14}C]citrulline (Expt. 1)

The gland weighed 495g wet wt. and yielded 30ml of milk/h before the experiment (average for 5 days, shown as 100%). The infusion of substrates in presence of L-[2- ^{14}C]citrulline was started at 0h. The mean blood flow during perfusion was 69 ml/min per 100 g wet wt. of tissue; the maximum O_2 consumption was about 1.43 ml/min per 100 g wet wt. of tissue. (a) Specific radioactivities of casein (○) and expired CO_2 (△); (b) hourly milk yield (%) of previous hourly yield over the previous 5 days).

Fig. 2. Perfusion of a goat mammary gland with L-[carbamoyl-¹⁴C,2-¹⁴C]citrulline (Expt. 2)

The gland weighed 590g wet wt. and yielded 27.4ml of milk/h before the experiment (average for 6 days, shown as 100%). The infusion of substrates with L-[carbamoyl-¹⁴C,2-¹⁴C]citrulline was started at 0h. The mean blood flow during perfusion was 46ml/min per 100g wet wt.; the maximum O₂ consumption was about 1.65ml/min per 100g wet wt. (a) Specific radioactivities of casein (O) and expired CO₂ (Δ); (b) hourly milk yield (% of previous hourly yield over the previous 6 days).

in the corresponding arterial samples. The specific radioactivity of ornithine in the plasma was higher than that of arginine. At the end of the experiment 0.082μCi of arginine, 0.242μCi of proline and 0.128μCi of ornithine were recovered from the blood and kidney fluid. No measurable radioactivity was detected in plasma urea.

Carbon dioxide. The specific radioactivity of ¹⁴CO₂ was practically constant during the perfusion (Fig. 1a). In total, 2.08μCi (10% of the consumed citrulline) was recovered as ¹⁴CO₂, indicating that citrulline is metabolized by the isolated sheep mammary gland. The product quotient (Table 2, Expt. 1), however, indicates that a very low percentage of the total expired CO₂ was derived from plasma citrulline.

Synthesis of milk components. The specific radio-

Fig. 3. Perfusion of a goat mammary gland with L-[carbamoyl-¹⁴C,1-¹⁴C]citrulline and L-[4-³H]arginine (Expt. 3)

The gland weighed 462g wet wt. and yielded 29.3ml of milk/h before the experiment (average for 7 days, shown as 100%). The infusion of substrates in the presence of L-[carbamoyl-¹⁴C,1-¹⁴C]citrulline and L-[4-³H]arginine was started at 0h. The mean blood flow during perfusion was 34ml/min per 100g wet wt.; the maximum O₂ consumption was about 2.03ml/min per 100g wet wt. (a) Specific radioactivities of casein (O) and expired CO₂ (Δ); (b) hourly milk yield (% of previous hourly yield over the last 7 days).

activity of milk fat was very low (Table 3): a total of only 0.024μCi was recovered in this fraction. In milk collected after 8h of perfusion, only traces of ¹⁴C were found in lactose. A higher radioactivity was detected in citric acid. The highest radioactivity was found in casein, and we calculated that 2.79μCi was recovered in this fraction. The specific radioactivity of casein increased linearly during the first 4h of perfusion, and remained constant during the final hours (Fig. 1a). As shown by the product quotient (Table 2, Expt. 1), a significant amount of the casein carbon originated from citrulline.

Table 1. Concentrations and specific radioactivities of some amino acids and urea in blood plasma during perfusion of sheep udder with L-[2-¹⁴C]citrulline (Expt. 1)For experimental details see the text. Values are means of three individual measurements $\pm$ S.E.M. —, Sample lost; n.d., not determined.

Time of perfusion (h)	Blood sample	Concentration (μ mol/100 ml of plasma)					Specific radioactivity (nCi/mg of C)				
		Citrulline	Arginine	Ornithine	Proline	Urea	Citrulline	Arginine	Ornithine	Proline	Urea
2	Arterial	3.33	5.36	3.99	27.55	101	234 $\pm$ 12	8.4 $\pm$ 0.4	15.7 $\pm$ 0.8	1.2 $\pm$ 0.1	0.0 $\pm$ 0.05
	Venous	3.57	4.22	3.52	20.53	71	240 $\pm$ 12	11.2 $\pm$ 0.6	18.1 $\pm$ 0.9	2.1 $\pm$ 0.2	0.0 $\pm$ 0.07
6	Arterial	3.36	2.61	3.80	18.35	n.d.	301 $\pm$ 15	8.2 $\pm$ 0.6	9.4 $\pm$ 0.5	4.4 $\pm$ 0.2	n.d.
	Venous	3.34	2.02	2.28	16.50	n.d.	275 $\pm$ 14	—	17.2 $\pm$ 0.9	4.9 $\pm$ 0.2	n.d.
8	Arterial	3.72	2.17	1.77	18.07	94	264 $\pm$ 13	5.8 $\pm$ 0.9	18.0 $\pm$ 0.9	3.2 $\pm$ 0.2	0.0 $\pm$ 0.05
	Venous	1.17	2.12	2.00	17.18	97	272 $\pm$ 14	8.9 $\pm$ 1.0	—	3.1 $\pm$ 0.2	0.0 $\pm$ 0.05

Table 2. Transfer of ¹⁴C from L-[2-¹⁴C]citrulline (Expt. 1), L-[carbamoyl-¹⁴C, 2-¹⁴C]citrulline (Expt. 2) or L-[carbamoyl-¹⁴C, 1-¹⁴C]citrulline (Expt. 3) to CO₂, urea and milk constituents

For details see the text. In Expt. 1, perfusion was carried out on a sheep udder; Expts. 2 and 3 were performed on udders from separate goats. n.d., Not determined.

Expt. no. ...	Product quotient (% of product formed from precursor)		
	1	2	3
CO ₂	0.3	0.20*	0.12*
Casein	1.31	0.19	0.28
Citric acid	0.29	n.d.	n.d.
Lactose	0.0	n.d.	n.d.
Fat	0.007	n.d.	n.d.
Plasma urea	n.d.	7.4†	3.8†
Casein arginine	18.7	3.9	4.3
Casein proline	3.92	1.10*	1.9*
Casein glutamic acid	0.40	0.04*	0.03*
Casein aspartic acid	0.17	0.02*	0.0
Casein glycine	0.05	n.d.	0.0
Casein alanine	0.02	n.d.	0.0

* Corrected for radioactivity loss as [¹⁴C]urea during metabolism.

† Calculated from the ratio of the specific radioactivity of guanidine-C of tissue arginine to the specific radioactivity of carbamoyl-C of citrulline.

Table 3. Specific radioactivities (nCi/mg of C) of different milk components and of amino acids isolated from casein after perfusion with L-[2-¹⁴C]citrulline (Expt. 1)

In Expt. 1, the perfusion was performed on an isolated sheep udder. Milk components and amino acids from casein were isolated and assayed for radioactivity as described in the text. n.d., Not determined.

	Time of perfusion (h)			¹⁴ C recovered in amino acids (%)
	2	3	8	
Citric acid	n.d.	n.d.	0.79	
Lactose	n.d.	n.d.	0.002	
Fat	0.0	0.002	0.018	
Casein	1.46	2.66	3.60	100
Casein arginine	21.1	39.6	51.4	51
Casein proline	4.2	7.5	10.8	43
Casein glutamic acid	0.33	0.64	1.11	5
Casein aspartic acid	0.16	0.33	0.47	0.9
Casein glycine	0.02	0.13	0.14	
Casein alanine	0.003	0.03	0.06	

Table 4. Concentrations and specific radioactivities of some amino acids and urea in blood plasma during perfusion of goat udder with L-[carbamoyl-¹⁴C,2-¹⁴C]citrulline (Expt. 2)

For experimental details see the text. Values are means of three individual measurements ± S.E.M. —, Sample lost.

Time of perfusion (h)	Blood sample	Concentration (μmol/100ml of plasma)				Specific radioactivity (nCi/mg of C)			
		Citrulline	Arginine	Ornithine	Urea	Citrulline	Arginine	Ornithine	Urea
2	Arterial	5.80	10.51	4.62	52.40	443 ± 22	16.3 ± 0.8	6.9 ± 0.3	9.2 ± 0.5
	Venous	3.26	8.63	4.00	53.7	353 ± 18	20.0 ± 1.0	—	18.0 ± 0.9
4	Arterial	2.49	5.61	2.54	49.1	479 ± 24	17.5 ± 0.9	8.9 ± 0.7	17.0 ± 0.9
	Venous	5.97	5.38	2.27	39.2	462 ± 23	15.1 ± 0.8	11.1 ± 0.7	23.0 ± 1.2
6	Arterial	5.56	5.45	2.60	43.2	470 ± 24	8.1 ± 0.4	7.3 ± 0.5	30.5 ± 1.5
	Venous	4.89	3.50	2.02	42.0	458 ± 23	14.6 ± 0.7	11.3 ± 0.8	32.5 ± 1.6
7	Arterial	4.95	5.50	2.75	47.5	470 ± 24	8.1 ± 0.4	6.5 ± 0.6	26.5 ± 1.3
	Venous	8.27	3.82	1.65	64.8	469 ± 24	10.3 ± 0.5	8.1 ± 0.9	32.5 ± 1.6

Table 5. Concentrations and specific radioactivities of some amino acids and urea in blood plasma during perfusion of goat udder with L-[carbamoyl-¹⁴C,1-¹⁴C]citrulline (Expt. 3)

For experimental details see the text. Values are means of three individual measurements ± S.E.M. —, Sample lost.

Time of perfusion (h)	Blood sample	Concentration (μmol/100ml of plasma)				Specific radioactivity (nCi/mg of C)			
		Citrulline	Arginine	Ornithine	Urea	Citrulline	Arginine	Ornithine	Urea
0.5	Arterial	2.04	7.66	3.72	26.31	365 ± 20	3.8 ± 0.7	—	0.88 ± 0.57
	Venous	4.57	—	—	28.84	294 ± 15	—	—	3.40 ± 0.15
2	Arterial	9.61	11.16	5.81	56.82	216 ± 11	2.4 ± 0.3	1.7 ± 0.8	3.15 ± 0.16
	Venous	10.29	8.72	5.15	50.85	215 ± 11	6.5 ± 0.6	4.7 ± 0.5	3.17 ± 0.16
4	Arterial	5.63	6.64	3.22	38.01	262 ± 13	1.6 ± 0.4	4.3 ± 1.0	4.67 ± 0.23
	Venous	5.79	5.15	2.71	37.42	271 ± 14	6.3 ± 0.9	4.6 ± 0.6	4.64 ± 0.23
6	Arterial	5.55	8.40	4.34	52.73	216 ± 11	2.5 ± 0.6	5.6 ± 1.0	4.28 ± 0.21
	Venous	5.28	4.61	2.24	33.00	231 ± 12	8.1 ± 1.0	5.7 ± 1.2	4.69 ± 0.23
7	Arterial	4.82	7.40	1.51	48.65	206 ± 10	3.8 ± 0.6	1.7 ± 0.5	4.90 ± 0.25
	Venous	10.34	8.62	—	52.11	181 ± 9	3.2 ± 0.6	—	4.44 ± 0.22
8	Arterial	—	6.02	3.42	39.30	—	2.8 ± 0.6	1.2 ± 0.5	4.27 ± 0.21
	Venous	—	—	—	—	—	—	—	—

Synthesis of casein amino acids. The amino acids were isolated from hydrolysates of casein obtained after 2, 3 and 8 h of perfusion (Table 3). In each of the samples, the highest specific radioactivity was observed in arginine, followed by proline, the specific radioactivity of the former being approx. 5 times higher than that of the latter. The specific radioactivity of glutamic acid was about 1/50th of that found in arginine. Practically all of the radioactivity found in casein (99%) was recovered in these three amino acids. Only low amounts of radioactivity were found in aspartic acid, glycine and alanine; no radioactivity was found in any other amino acid. According to the product quotients (Table 2, Expt. 1), 19% of the arginine residues of casein and 4% of the casein proline would be derived from plasma citrulline.

(B) L-[carbamoyl- ^{14}C , 2- ^{14}C]Citrulline (Expt. 2) and L-[carbamoyl- ^{14}C , 1- ^{14}C]citrulline experiment (Expt. 3) with separate goat udders (Figs. 2 and 3)

Milk composition. Similar variations were observed in the concentrations of citric acid, casein and lactose as mentioned for Expt. 1. The milk triglyceride concentration at first declined, however, but rose again towards the end of perfusion.

Concentration and specific radioactivities of amino acids and urea in the plasma. The concentrations of citrulline in arterial plasma was within normal (2.5–8.6 μM /100 ml of plasma) values (Tables 4 and 5). In Expt. 2, positive arteriovenous differences were observed for citrulline after 2 and 6 h of perfusion, but not found after 4 h of perfusion (Table 4). However, in Expt. 3 no evidence of a positive arteriovenous difference for citrulline could be obtained (Table 5). In Expt. 2 the specific radioactivity of the precursor in arterial blood plasma varied less than 8%, and remained constant after 4 h. The specific radioactivity of citrulline in the venous blood after 2 h of perfusion was 20% lower than that in the artery, suggesting that the citrulline pool within the mammary gland had not reached its maximum radioactivity at that time. Although in Expt. 3 the specific radioactivity of citrulline was more variable, values fluctuated within narrow limits during the major part of perfusion (2–7 h). At the end of Expts. 2 and 3 a total of 61.1 and 37.5 μCi of citrulline respectively were recovered from blood, kidney fluid and udder tissue extract. Since 95.05 μCi of citrulline was infused during Expt. 2, it was calculated from the amount of [^{14}C]citrulline recovered (61.1 μCi) that a maximum quantity of 37% of the added dose could have been metabolized by the goat gland. Similarly in Expt. 3, it was calculated that 25% of the added dose (49.8 μCi) was metabolized by the goat gland. As in the sheep udder experiment (Expt. 1, Table 1), positive arteriovenous differences for arginine, ornithine and proline were usually found across the goat udder (Tables 4

and 5). Generally higher specific radioactivities were also noted for these amino acids and for urea in the venous samples, as compared with the corresponding arterial plasma. However, in contrast with Expt. 1 (Table 1), lower specific radioactivities were found for ornithine than for arginine in all plasma samples of Expt. 2 (Table 4), as well as in the venous samples of Expt. 3 (Table 5). At the end of Expt. 2, 0.303 μCi of arginine, 0.615 μCi of proline, 0.045 μCi of ornithine and 2.66 μCi of urea were recovered in blood, kidney fluid and udder tissue extract. In Expt. 3 the following amounts were recovered: 0.535 μCi of arginine, 0.030 μCi of ornithine, 0.378 μCi of proline and 0.523 μCi of urea.

Specific radioactivity and analysis of the unknown metabolite isolated from plasma. After $\text{Ba}(\text{OH})_2$ hydrolysis of the unknown substance in Expt. 2, 50% of the total radioactivity was trapped as $^{14}\text{CO}_2$. By analysis with column chromatography of the hydrolysate, 76% of the remaining radioactivity was recovered in ornithine. From the Chinard (1952) reaction, and from the radioactivity observed in this fraction, the specific radioactivity of ornithine was calculated to be 1.62 nCi/ μmol . Since half of the radioactivity of the original product was lost as $^{14}\text{CO}_2$, this substance should have had a specific radioactivity of at least 3.24 nCi/ μmol . This specific radioactivity is 1.3 times higher than that of arginine, which had been isolated from the goat udder tissue, suggesting that arginine is not the direct precursor of the unknown metabolite.

Amino acid analysis of a second alkaline hydrolysate showed four distinct peaks on the chromatogram: aspartic acid, ornithine and one peak (G_1) eluted at the position of glutamic acid, followed by a second (G_2). The specific radioactivity of ornithine was 1.92 nCi/ μmol , suggesting that the original substance had a specific radioactivity of 3.84 nCi/ μmol . Thus the specific radioactivities of ornithine, isolated from both hydrolysates, show a reasonable agreement, when the low amount of ornithine isolated ($\pm 0.05 \mu\text{mol}$) is taken into account. The relative molar ratio (G_1 and G_2 expressed as leucine equivalents) of aspartic acid, peak G_1 , peak G_2 and ornithine was calculated as 1:0.6:0.4:2. Since the first three compounds contained no measurable radioactivity, the original molecule might have been split by $\text{Ba}(\text{OH})_2$ into one part of ornithine and one part of either aspartic acid, or G_1 , or G_2 . T.l.c. of the dansylated derivatives of the hydrolysates indicated the presence of aspartic acid, ornithine, glutamic acid and at least one unknown spot. Considering the elution characteristics of the unknown radioactive substance and the radiochemical results obtained, it may be suggested that this unknown peak contains more than one metabolite. One of these substances may be an acidic cyclization product of arginino-succinate.

Fig. 4. Elution pattern of the unknown substances and of urea (Expt. 2) on a column (0.6 cm $\times$ 135 cm) of Aminex A6 (Li^+ form)

Details of the experimental conditions are described in the Materials and Methods section. ●, Radioactivity (c.p.m.); ▲, ninhydrin colour (E_{570}). (a) Artery; (b) vein.

Lee & Pollitt (1972) studied different, new derivatives of argininosuccinic acid in the urine of a patient with argininosuccinaciduria. The elution characteristics of substances A^1 on Zeocarb 225, described by Lee & Pollitt (1972), are quite similar to those of the unknown substance which we observed during amino acid analysis. Peak A^1 contained a ninhydrin-positive (structure IX), and a ninhydrin-negative (yellow spot) substance. However, alkaline hydrolysis of these substances showed ornithine and aspartic acid as the only components. The presence of two additional ninhydrin-positive substances (G_1 and G_2) in our hydrolysates suggests that the radioactive peak, which we detected, may contain two substances with similar chromatographical behaviour.

By applying a lower sample load to the column, the unknown substance was eluted as two distinct radioactive peaks (X_1 and X_2) during analysis of the venous plasma (Fig. 4). These peaks emerged at 22 and 30 ml respectively after the elution position of urea. In the corresponding arterial sample, the radioactivity of the second peak was relatively low and appeared as a shoulder on peak 1. Both peaks were Sakaguchi-negative and did not react with the carbamoyl reagent of Hunninghake & Grisolia (1966). These results suggest that the first radioactive, ninhydrin-positive peak is similar to the argininosuccinate derivative IX of Lee & Pollitt (1972). The second radioactive peak may yield, besides ornithine, products G_1 and G_2 after alkaline hydrolysis.

These two ^{14}C radioactive peaks were also found in plasma of Expt. 3, but no ^3H was detected in these substances.

Carbon dioxide. Almost constant values for the specific radioactivity of the expired $^{14}\text{CO}_2$ were

reached after 2 h of perfusion in Expt. 2 (Fig. 2a) and from the beginning of Expt. 3 (Fig. 3a). The specific radioactivity of the expired CO_2 was always higher in the venous plasma than in the corresponding arterial plasma. In total, 0.91 and 0.18 μCi respectively were collected during Expts. 2 and 3. These amounts are approx. 4 and 12 times smaller in comparison with the previous sheep udder experiment (Expt. 1, Fig. 1a), considering the dose of labelled precursor used in Expts. 2 and 3. The product quotients (Table 2, Expts. 2 and 3) suggest that a very low percentage of the total expired CO_2 was derived from citrulline.

Synthesis of milk components. Since in Expt. 1 with sheep udder, only a very low incorporation of ^{14}C was found in lactose, fat and citric acid, these components were not isolated in Expts. 2 and 3 with goat udder. Only 0.59 μCi (Expt. 2) and 0.39 μCi (Expt. 3) were recovered in the casein fraction. The radioactivity of this protein rose during the first hours of the experiments, reaching a constant value during the last 2 (Expt. 2; Fig. 2a) and 3 h (Expt. 3; Fig. 3a) of perfusion. The product quotient (Table 2, Expts. 2 and 3) suggests that the ^{14}C incorporation of citrulline into casein was much lower in Expt. 2 and 3 as compared with Expt. 1.

Synthesis of casein amino acids. In Expt. 2, two samples of casein were hydrolysed, followed by chromatographic isolation of the amino acids (Table 6). In the second goat experiment (Expt. 3), the amino acids were isolated from one sample of casein, obtained after 8 h of perfusion (Table 6). In each of the casein samples almost all of the ^{14}C (98–99%) was recovered in the arginine and proline residues. Table 6 shows that the specific radioactivities of

Table 6. *Specific radioactivities (nCi/mg of C) of casein amino acids and free amino acids extracted from goat udder tissue after perfusion with L-[carbamoyl- ^{14}C ,2- ^{14}C]citrulline (Expt. 2) or L-[carbamoyl- ^{14}C ,1- ^{14}C]citrulline (Expt. 3)*

For experimental details see the text. Values are means of three individual measurements $\pm$ S.E.M. n.d., Not determined.

Time of perfusion (h) ...	Casein amino acids			^{14}C recovered in casein amino acids (%)		Udder tissue amino acids	
	Expt. 2		Expt. 3			Expt. 2	Expt. 3
	5	9	8	Expt. 2	Expt. 3	Expt. 2	Expt. 3
Casein	0.343	0.905	0.63	100	100		
Arginine	7.84	18.55	9.59	45	34	34.6 ± 1.7	8.5 ± 1.2
Proline	1.03	2.60	2.10	53	65	10.2 ± 0.5	1.5 ± 0.2
Glutamic acid	0.031	0.084	0.035	1.8	1	0.45 ± 0.02	0.53 ± 0.03
Aspartic acid	0.028	0.062	n.d.	0.5	—	n.d.	n.d.
Citrulline						n.d.	268 ± 14
Urea						38 ± 2	7.2 ± 0.4

arginine were 7 and 5 times greater respectively, than those found in proline. Compared with proline, only low amounts of radioactivity were found in glutamic acid and aspartic acid. The product quotients observed here (Table 2; Expts. 2 and 3) are much smaller than in the previous sheep udder experiment, suggesting that citrulline is metabolized to a much smaller extent by the isolated goat udder.

Incubation experiment. In the analysis of a plasma sample taken at the end of incubation carried out in the presence of L-[carbamoyl- ^{14}C ,2- ^{14}C]citrulline (Expt. 2), the radioactivities of urea, ornithine and arginine were measured by liquid-scintillation. The radioactivity was also determined in the region of component X_1 and X_2 of argininosuccinate. No measurable radioactivity could be detected in any of the components examined.

Discussion

The results of these perfusion experiments demonstrate that citrulline of the blood plasma is metabolized to arginine. In the isolated goat udder (Expts. 2 and 3), only 4% of the casein arginine was derived from plasma citrulline (Table 2). On the other hand, this value amounted to 19% in the sheep experiment (Expt. 1). Since duplicate perfusion experiments carried out on glands from the same animal species yielded reproducible product quotients (the goat udder experiments described here and previous sheep udder experiments; Verbeke *et al.*, 1968), the differences in the intensity of citrulline metabolism observed here may be related to the animal species.

In the present experiments, our results indicate the metabolism of citrulline by the udder. Radioactive carbon was transferred from [^{14}C]citrulline to CO_2 and milk constituents. Generally, the specific radioactivity of the arginine, ornithine and proline of the venous plasma were significantly higher than those of the corresponding amino acids in the artery,

indicating that citrulline metabolism occurs in the udder itself. Analysis of the radioactive citrulline content in the whole perfusion system indicates that a substantial amount of the infused citrulline disappeared during the experiment. Incubation experiments in the presence of [^{14}C]citrulline indicate the absence of citrulline metabolism by oxygenated sheep blood.

Since radioactive citrulline disappeared from the perfusion system as a result of its metabolism, a significant arteriovenous difference for citrulline across the udder would be expected. In the goat udder experiments (Expts. 2 and 3), the rather low incorporation of [^{14}C]citrulline into milk constituents would not allow detection of any net uptake of citrulline by the gland. In Expt. 1 with sheep udder, an arteriovenous difference of $0.2 \mu\text{M}$ -citrulline/100ml of plasma would be expected, considering the extent of ^{14}C incorporation, as well as the value of blood flow through the gland. However, only one positive arteriovenous difference was observed in three pairs of samples analysed (Table 1). Previous arteriovenous difference measurements failed to demonstrate a statistically significant absorption of citrulline in the cow udder (Verbeke & Peeters, 1965) and the goat udder (Mephram & Linzell, 1966) *in vivo*. However, it should be noted that arteriovenous differences reflect only momentary uptakes; owing to the very marked fluctuating absorption pattern of citrulline (Mephram & Linzell, 1974), a net absorption of this amino acid may be detected only by measuring arteriovenous differences, and by studying blood samples collected over longer intervals of time. This was done in recent balance-experiments on the udder of a lactating cow *in vivo* (G. Peeters, E. Roets, R. Verbeke & A. Houvenaghel, unpublished work). The results obtained (Table 7) show that the mammary uptake of citrulline, measured over periods of 1–2 min duration, still varies considerably within the same experimental period. However, the mean uptake of citrulline measured during each experiment is positive.

Table 7. Concentrations of citrulline in arterial and venous plasma of the udder, arteriovenous differences across the udder and uptake from the plasma in a cow

Three experiments (A, B, C) were performed on the same cow. In each experiment, blood samples were taken simultaneously from the artery and from the vein of the udder for 1–2 min. The cow was surgically prepared so that arterial and venous blood samples truly representative for the udder were collected. The uptake of citrulline was calculated from the arteriovenous difference $\times$ plasma flow at the time of sampling. Values are means of each experiment $\pm$ s.e.m.

		Concentration (mg/100 ml of plasma)				
	Sample no.	Time	Artery	Vein	Arteriovenous difference	Uptake (mg/min)
A.	65	0	1.230	1.183	0.047	1.04
	66	1 min 30s	1.176	1.148	0.028	0.62
	69	32 min 22s	0.677	0.999	-0.322	-7.90
	70	44 min 30s	0.916	0.982	-0.066	-1.58
	71	50 min	1.018	0.617	0.401	10.35
	Mean				0.018 ± 0.116	0.51 ± 2.93
B.	72	0	1.272	0.964	0.308	7.17
	73	9 min	1.168	1.213	-0.045	-0.99
	74	18 min	1.076	0.809	0.267	6.34
	Mean				0.177 ± 0.111	4.17 ± 2.59
C.	78	0	1.189	0.878	0.311	7.73
	79	3 min 32s	1.099	0.919	0.180	4.54
	80	10 min 36s	0.972	1.127	-0.155	-3.85
	81	15 min 10s	1.019	1.014	0.005	0.12
	Mean				0.085 ± 0.102	2.14 ± 2.53

From these results obtained *in vivo*, it is not surprising that a considerable variation of the momentary arteriovenous differences was observed in our perfusion experiments (Tables 1, 4 and 5).

Previous perfusion experiments (Verbeke *et al.*, 1965; Mepharm & Linzell, 1967; Verbeke *et al.*, 1968) have shown that mammary tissue converts arginine by way of ornithine into casein proline and, to a lesser degree, to casein glutamic acid. In the present experiments, the relatively high radioactivity of proline as compared with arginine in casein confirms these results. The high specific radioactivities observed in plasma arginine, ornithine and urea, during perfusion with L-[carbamoyl- ^{14}C , 2- ^{14}C]-citrulline (Expt. 2) and L-[carbamoyl- ^{14}C , 1- ^{14}C]-citrulline (Expt. 3), also substantiate the view that citrulline is metabolized via arginine to ornithine and proline. The conversion of ornithine, by way of glutamic acid, into α -oxoglutaric acid and catabolism of the latter via the tricarboxylic acid cycle would explain the incorporation of ^{14}C into glutamic acid, aspartic acid, citric acid and CO_2 in the [^{14}C]citrulline experiments.

In Expts. 2 and 3 the ^{14}C recovered in the urea fraction amounted to approx. 7.8 and 4.3% of the total citrulline activity consumed during perfusion. If the free arginine, isolated from udder tissue (Table 6), is accepted as the immediate precursor of ornithine and urea, then the product quotient of urea can be determined. Calculation on the basis of

the specific radioactivity in the guanidine group of tissue arginine, to the specific radioactivity of the carbamoyl group in plasma citrulline, shows that at least 7.4 (Expt. 2) and 3.8% (Expt. 3) of the urea originates from plasma citrulline (Table 2). The latter values correlate well with the quantity of ^{14}C recovered in the urea fraction. The high specific radioactivity of plasma arginine observed in the first hours of Expts. 2 and 3, points to a very rapid efflux of radioactive arginine from mammary tissue. In Expt. 1, the higher specific radioactivity of plasma ornithine, as compared with plasma arginine, suggests that this sheep mammary gland exchanges intracellular ornithine more readily than arginine. The relative amounts of ^{14}C recovered from arginine, ornithine and proline in plasma and kidney fluid substantiate these findings.

As the metabolism of citrulline to arginine is known to proceed via argininosuccinate in liver tissue (see Ratner, 1962), we tried to isolate this substance from blood plasma or udder tissue. During the analysis of the free amino acids in blood plasma or in udder tissue after perfusion, no radioactivity was detected on the elution position of argininosuccinate or its normal anhydrides. However, in Expt. 2, two highly radioactive substances were detected on the chromatogram, the first substance being ninhydrin-positive (Fig. 4). The total radioactivity of these components was higher in venous than in arterial plasma, suggesting that these substances are formed

within the mammary gland. Moreover, after incubation of oxygenated sheep blood in the presence of L-[carbamoyl- ^{14}C ,2- ^{14}C]citrulline, no radioactivity was found on the elution position of these components during chromatographical analysis of the amino acids.

Radiochemical analysis of the products obtained by degradation with alkali of the unknown metabolites, suggests their relation to at least one of the ninhydrin-positive argininosuccinate derivatives, which were described by Lee & Pollitt (1972). These authors obtained different derivatives by heating argininosuccinic acid at pH 5, and also found appreciable quantities of these substances in the urine of a patient with argininosuccinic aciduria. In view of the moderate conditions employed during the preparation of our extracts, it is highly improbable that the substances found here would be artifacts of argininosuccinic acid.

In Expt. 3, these unknown ^{14}C substances were also found in plasma. Since in this experiment [^3H]-arginine was added to the perfusate, and no ^3H radioactivity could be detected in these metabolites, it must be concluded that these compounds are derived from [^{14}C]citrulline, and not from arginine.

It will be interesting to study the production of these metabolites by the isolated mammary gland so that their role in the metabolism of citrulline may be defined.

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