The Use of Glutamine and Glutamic Acid by the Mammary Gland for Casein Synthesis

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(Received 3 January 1956)

During lactation the secretory cells of the mammary gland continuously take compounds from the blood stream and convert them into the components of milk, and the milk is continuously secreted into the alveoli and ducts of the gland where it is stored until milking. The mammary gland is specially suited for investigating the components of the blood that are used for the synthesis of proteins in body cells (assuming that casein synthesis is typical), because after synthesis casein becomes separated from its precursors and can be drawn off in the milk a few hours later. There is thus no 'turnover' of casein: the casein molecules in a small milk sample, unlike the protein molecules in most samples of other body fluids or of tissues, have all been synthesized at approximately the same time. Therefore, the precursor in the blood stream of an amino acid in casein is easily identified by experiments with isotopic tracers: the specific activity of the amino acid from the casein of a sample of milk secreted over, say, 1 hr. will equal the mean specific activity of the precursor during the hour of its uptake by the mammary gland.

Previous work showed that the mammary gland takes free lysine and tyrosine and inorganic phosphorus from the blood stream to provide the lysine, tyrosine and phosphorus of casein; the lysine and tyrosine of plasma proteins were not used for casein synthesis (Barry, 1952). Work and his colleagues also found that certain other amino acids of casein are derived from the free amino acids of plasma, and not from plasma proteins (Campbell & Work, 1952; Askonas, Campbell, Godin & Work, 1955). The results provide evidence against the suggestion that while plasma proteins are synthesized from free amino acids, other proteins are synthesized from plasma proteins (Madden & Whipple, 1940).

The purpose of the present work was to discover the compounds taken from the blood stream by the mammary gland to provide the glutamine and glutamic acid of casein. It was thought that this might throw light on the function of the large amount of glutamine in blood plasma, and show whether, in casein synthesis, glutamine is involved in transpeptidation reactions as suggested by Waelsch (1952).

The results of experiments with labelled glutamine and glutamic acid demonstrate that the glutamine residues of casein are derived from the free glutamine of blood plasma, and the glutamic acid residues from the free glutamic acid. Thus glutamine and glutamic acid are incorporated into casein as two independent amino acids, and an important new function of glutamine is therefore revealed. The results also suggest that glutamine is not involved in transpeptidation reactions during casein synthesis.

Note: In this paper 'casein glutamine' refers to the glutamine residues in casein, 'casein glutamic acid' to the glutamic acid residues in casein, and 'casein glutamic acid (acid hydrolysis)' to the glutamic acid in an acid hydrolysate, which is derived from both the casein glutamine and casein glutamic acid.

EXPERIMENTAL

Isotopic compounds

DL-[1-¹⁴C]Glutamic acid and D-[¹⁴C]glucose (uniformly labelled) were bought from the Radiochemical Centre, Amersham, and the [*ammonium*-¹⁵N]ammonium nitrate from A.E.R.E., Harwell.

DL-[1-¹⁴C]Glutamine. This was synthesized from DL-[1-¹⁴C]glutamic acid as described by King & Kidd (1949), except that after the phthalylglutamine had reacted with hydrazine the solution was acidified with 2*N*-HI instead of HCl; this avoided removing the Cl⁻ ions with silver carbonate.

The product, when eluted from 100 cm. columns of Dowex-50 with citrate buffer at pH 3.40, emerged at the same effluent volume as an authentic sample of L-glutamine, and gave no glutamic acid; a sample of isoglutamine emerged at a different volume. The product, when treated with glutamic decarboxylase in a Warburg manometer, yielded carbon dioxide at the same rate as half its weight of L-glutamine.

Yield (calculated from DL-glutamic acid) 30.2%.

DL-[Amide-¹⁵N]glutamine. This was made by the same method, but with the following modified preparation of phthalyl-DL-glutamine. Phthalyl-DL-glutamic anhydride (3 g.) was dissolved in dry dioxan (100 ml.) and dry ether (100 ml.) added. [*Ammonium*-¹⁵N]ammonium nitrate (1.9 g.) was dissolved in a few millilitres of water and excess of saturated KOH solution added; the ammonia generated was slowly drawn in a stream of air through CaO, and then through the solution of phthalylglutamic anhydride. Dry ether (100 ml.) was then added and phthalylglutamine

isolated as before. After the phthalylglutamine had been filtered off, $^{15}\text{NH}_3$ was recovered from the $^{15}\text{NH}_4\text{Cl}$ in the filtrate and used in a second synthesis.

Yield [% of original ^{15}N recovered in DL- ^{15}N]glutamine] 31.6%.

Procedure in experiments on goat

The experiments were performed on a goat which was giving about 1 l. of milk/day, and was in good health. Compounds were injected, and blood and milk samples taken, as described by Barry (1952).

Determination of ^{14}C specific activities

C-1 of the free glutamine and glutamic acid of blood plasma. A sample of glutamine and glutamic acid was isolated from each sample of plasma, the amount of each was determined, and to each a known weight of inactive glutamine or glutamic acid was added. C-1 was then removed by adding glutamic decarboxylase, and its specific activity was corrected for the dilution.

Free glutamine and glutamic acid of plasma were separated by chromatography on columns of Dowex-50 resin as described by Moore & Stein (1951), but at room temp. instead of 37.5°. At this temperature glutamine is not completely destroyed, and about 65% is recovered, together with asparagine, in a single peak (Barry, 1953). The procedure was as follows. Plasma (30 ml.) was dialysed overnight against water (150 ml.) on a shaker at 0°, and the diffusate freeze-dried. The residue was dissolved in a few millilitres of water, brought to pH 3.0 with HCl, and eluted from columns of Dowex-50 resin (100 × 1.15 cm.) as described by Moore & Stein (1951), but at room temperature. Glutamine and asparagine emerged at 160–185 ml., and glutamic acid at 260–300 ml., and were determined by the quantitative ninhydrin method. The asparagine concentration of the goat's plasma was found to average 0.95 mg./100 ml. and to vary very little; it was therefore responsible for about 25% of the ninhydrin colour of the glutamine and asparagine fraction. The glutamine concentration in the fraction could therefore be found, with little error, by subtraction. The glutamine and asparagine fraction and the glutamic acid fraction were shown by paper chromatography to contain no other ninhydrin positive compounds (Sheldon-Peters & Barry, 1956).

To the glutamine and asparagine fraction, L-glutamine (30 mg.) and conc. HCl (5 ml.) were added, the solution was slowly evaporated on a water bath, and the residue dissolved in a few millilitres of water. To the glutamic acid fraction, L-glutamic acid (30.0 mg.) was added and the solution rapidly evaporated to a small volume.

The solutions were brought to pH 4.5 and mixed with glutamic decarboxylase (4 ml.) (Krebs, 1950) in the outer chamber of a microdiffusion unit (Conway, 1950), specially designed to hold 40 ml.; the carbon dioxide evolved was absorbed in NaOH in the inner chamber.

The carbon was precipitated as barium carbonate, centrifuged on to an aluminium disk, and counted with a thin-windowed counter, to give less than 3% probable error. Each count was corrected to give a value equal to that of an infinitely thick sample of the same area, and the specific activity calculated by the use of a previously determined factor. From this specific activity, that of the undiluted glutamine or glutamic acid of plasma was calculated.

C-1 of casein glutamine and glutamic acid. Casein was hydrolysed by a pancreas suspension prepared as follows. Fat-free and minced pig pancreas (250 g.) was suspended in 25% (v/v) ethanol (1 l.). Before use the suspension was dialysed against water to remove alcohol, and incubated for 24 hr. at 37°, dialysed overnight, and diluted to twice its original volume with water. The product, when incubated alone at 37° for 24 hr., produced no free glutamine or glutamic acid; when incubated with glutamine, no glutamic acid was formed; when incubated with casein and radio-active glutamine, or with casein and active glutamic acid, only the glutamine in the first hydrolysate and the glutamic acid in the second were radioactive.

Casein (700 mg.), precipitated by acid (Barry, 1952), was dissolved in 0.1 N-NaOH (5 ml.) and the pancreas suspension (25 ml.), and a little toluene added, and the mixture brought to pH 7.0–7.5 and left overnight at 37°. The amount of free glutamine and glutamic acid formed was determined with glutamic decarboxylase; it was 60–70% of the possible amount. The digest was dialysed overnight against water (150 ml.) on a shaker, and the diffusate freeze-dried. Glutamine and glutamic acid were then separated from one another on columns of IR-4B (Hirs, Moore & Stein, 1952) or Dowex-1 (Hirs, Moore & Stein, 1954), and the specific activity of C-1 was determined after decarboxylation.

C-1 of casein and plasma-protein glutamic acid (acid hydrolysis). After separating the free amino acids from plasma, the proteins were dialysed overnight against running water and then precipitated with tungstic acid. Plasma proteins and casein were hydrolysed with HCl (Barry, 1952) and the hydrolysates evaporated and treated with glutamic decarboxylase (2 ml.), and the specific activity of the carbon dioxide was determined.

Total carbon of lactose and of casein glutamic acid (acid hydrolysis). Lactose was isolated as described by Reiss & Barry (1953). Glutamic acid was isolated from acid hydrolysates of casein by chromatography on Dowex-1 (Hirs *et al.* 1954). They were burnt in a stream of oxygen and the specific activity of the carbon in the carbon dioxide was measured.

Determination of ^{15}N

A known weight of the DL-[1- ^{14}C :amide- ^{15}N]glutamine used for injection was diluted with a known weight of inactive DL-glutamine, and part of this hydrolysed with N-HCl for 2 hr. in the outer chamber of a microdiffusion unit. After evaporating to dryness, saturated KOH solution (2 ml.) was added, and the ammonia absorbed in 0.25 N-HCl (1 ml.) in the inner chamber. Nitrogen was prepared (Rittenberg, 1946) and the ^{15}N determined in a mass spectrometer. ^{14}C was also determined after decarboxylation, and the ^{15}N and ^{14}C in the original compound could then be calculated.

The amide nitrogen of casein glutamine was liberated by heating a diffusate of casein, hydrolysed by pancreas suspension, at pH 7 and 100° for 4 hr., which converts glutamine into pyrrolidonecarboxylic acid and ammonia (Vickery, Pucher, Clark, Chibnall & Westall, 1935). The hydrolysate was then mixed with saturated potassium carbonate solution in the outer chamber of a microdiffusion unit, and the ammonia collected and converted into nitrogen as before. This treatment did not release amide nitrogen from asparagine.

The ammonia released on hydrolysis of casein with 20% (w/v) HCl was collected in the same way; about 90% comes

from the amide nitrogen of the glutamine and asparagine of casein, and the remainder from the decomposition of other amino acids (Rees, 1946). The remaining nitrogen in the acid hydrolysate was isolated by the Kjeldahl method.

RESULTS

Goat's milk contains about 3% of casein, in which the amino acid concentrations are very similar to those in cow's casein (Davis & Macdonald, 1953). On acid hydrolysis, 100 g. of cow's casein yields 22.4 g. of glutamic acid, which is derived from the glutamine and glutamic acid residues, the relative proportions of which are not known, and 1.6 g. of amide nitrogen from glutamine and asparagine residues (Gordon, Semmett, Cable & Morris, 1949). Since 100 g. of casein yields 7.1 g. of aspartic acid, it can be calculated that the maximum possible amount of the glutamic acid (acid hydrolysis) which can have come from glutamine residues is 16.8 g./100 g. of casein (assuming that there are no asparagine residues), and the minimum possible is 8.9 g. (assuming that there are no aspartic acid residues).

Goat's blood plasma contains about 5 mg. of free glutamine/100 ml. and 1.5 mg. of free glutamic acid/100 ml. The glutamine and glutamic acid of casein could therefore be derived from the free glutamine or glutamic acid of plasma, or from plasma proteins, or could be synthesized within the gland from some other compounds absorbed from the blood.

Fig. 1 shows the results of an experiment designed to find if either the glutamine or glutamic acid of casein is derived from the free glutamine of plasma. The curve *A* passes through each value for the specific activity of casein glutamine, in the last three milk samples, at a time half-way through the period in which the milk was secreted. Since milk secretion is continuous, this curve shows the specific activity of casein glutamine, at various times, in milk which has just reached a point in the mammary gland from which it can be drawn off by milking. If the curve was drawn back to zero, it would rise to a peak at about 1.5 hr. and fall to zero specific activity at zero time, with the area under the curve equal to the total area under the histogram; it is impossible to plot the complete curve accurately.

It can be seen that after injecting [^{14}C]glutamine the glutamine and glutamic acid residues of each casein sample have very different specific activities. The curve *A* shows that, after 2½ hr., the specific activity of casein glutamine is always equal to that of the plasma glutamine 1–2 hr. earlier, and is always many times higher than that of the free glutamic acid of plasma, or of the plasma-protein glutamic acid (acid hydrolysis) the values for which were too low to plot. The relation between the specific activities of casein glutamine and plasma

glutamine is similar to that between the phosphorus of milk and the inorganic phosphorus of plasma, and between the casein lysine and tyrosine and the free lysine and tyrosine of plasma (Barry, 1952).

If the rate of absorption of milk precursors from the blood stream remains constant during an experiment, the area under the specific activity-time histogram of a component of milk will equal that under the curve of its precursor in the blood. It was found previously (Barry, 1952) that, after injecting labelled inorganic phosphorus intravenously into a lactating goat, the area under the specific-activity histogram for the inorganic phosphorus of milk up to 7 hr. was, within the limits of

Fig. 1. DL-[1- ^{14}C]Glutamine (101 mg.; 9.04 μC) was injected intravenously into a lactating goat. The goat was milked immediately before injection, and blood and milk samples were taken at intervals up to 7 hr. after. ●, Free glutamine of plasma; ○, free glutamic acid of plasma. The histograms show the specific activities of casein glutamine (broken lines) and casein glutamic acid (solid lines) in each milk sample, the width of each rectangle occupying the time during which the milk sample was secreted. The significance of curve *A* is described in the text. The values for plasma-protein glutamic acid (acid hydrolysis) were too low to plot.

experimental error, equal to that under the curve for plasma inorganic phosphorus up to 5 hr. (the time difference allows for the 2 hr. lag found between blood and milk). Since all the inorganic phosphorus of milk must come from blood, the fact that these two areas were equal is good evidence that the rate of absorption of milk precursors was constant, and that the precursor of a component of milk can in fact be identified in this way.

In Fig. 1 the area under the casein-glutamine histogram up to 7 hr. is 335 units; that under the plasma-glutamine curve (if extrapolated back to the ordinate) up to 5.5 hr. is 325 units. It may, therefore, be concluded that 1-2 hr. before an average casein molecule reaches a point in the mammary gland from which it can be drawn off in the milk, all of its glutamine existed as free glutamine in the blood.

These results strongly suggest that the mammary gland takes free glutamine from the blood to provide the glutamine residues of casein. The only alternative is that the plasma glutamine is rapidly incorporated into a derivative such as a peptide or protein, in some other part of the body, and this is absorbed by the mammary gland for casein synthesis, the transfer from free glutamine to casein still taking only 1-2 hr. However, no glutamine peptide has been found in plasma in the extensive analyses of Stein & Moore (1954); and it is shown in the accompanying paper (Sheldon-Peters & Barry, 1956) that when blood flows through the lactating mammary gland of the cow, its glutamine concentration falls by the amount to be expected if it is used in protein synthesis. It may, therefore, be concluded that the mammary gland takes free glutamine from the blood to provide the glutamine residues of casein.

The specific activities of casein glutamic acid in this experiment were many times those of plasma-protein glutamic acid (acid hydrolysis). Therefore casein glutamic acid cannot have been derived from any major component of the plasma proteins. The area under the histogram for casein glutamic acid is roughly the same as that under the curve for the free glutamic acid of plasma; this suggests that the casein glutamic acid was derived from plasma glutamic acid, but is not conclusive since several compounds in the blood may have had the same low specific activities as plasma glutamic acid.

In Table 1 are shown the specific activities of glutamine, glutamic acid, and glutamic acid (acid hydrolysis) in each casein sample from this experiment. From these figures the percentage of the glutamic acid (acid hydrolysis) derived from casein glutamine has been calculated. The results suggest that there are about equal amounts of glutamine and glutamic acid in casein. For this calculation it must be assumed that the specific activities of glutamine and glutamic acid, which were determined on 60-70% of the total glutamine and glutamic acid of casein, would have been identical after complete hydrolysis. This is almost certainly true. The same specific activities were found in the casein taken at 1.5 hr. whether there was 20% or 62% hydrolysis. Askonas *et al.* (1955) have found that, in the casein of milk taken 4 hr. after injecting a number of [^{14}C]amino acids into a goat, each amino acid had the same specific activity whether isolated from a complete hydrolysate, or from different peptides obtained after partial hydrolysis.

The values for plasma-protein glutamic acid (acid hydrolysis), if plotted in Fig. 1, would be scarcely separated from the axis. The values found in another similar experiment are shown in Table 2.

Table 1. *Specific activities of amino acids from casein of milk samples taken at intervals after injecting DL-[1- ^{14}C]glutamine (101 mg.; 9.04 μC) intravenously into a lactating goat*

Time of sampling (hr.)	...	...	...	...	1.5	3.0	4.5	7.0
Specific activity of	(a)	Glutamine			84.6	74.7	42.0	15.80
($\mu\text{mc/mmole of carbon}$)	(b)	Glutamic acid			11.76	14.55	4.99	2.38
	(c)	Glutamic acid			44.1	45.3	22.8	9.56
		(acid hydrolysis)						
	x				44.4	51.2	48.1	53.5
	Average x				49.3	—	—	—

x is the percentage of the glutamic acid (acid hydrolysis) derived from casein glutamine. It is calculated from

$$x = \frac{c-b}{a-b} \times 100.$$

Table 2. *Specific activities of plasma-protein glutamic acid (acid hydrolysis) after injecting DL-[1- ^{14}C]glutamine (425 mg.; 38.0 μC) intravenously into a lactating goat*

Time of sampling (hr.)	...	0.33	1.0	3.0	6.0	12.0
Specific activity of C-1		0.145	0.65	1.73	2.32	1.85
($\mu\text{mc/mmole of carbon}$)						

Table 3. DL-[1-¹⁴C:amide-¹⁵N]glutamine (563 mg.) injected intravenously into goat and milk taken 1.5 hr. later

	Atoms % excess ¹⁵ N	Specific activity of C-1 (μmc/mmole of carbon)	μmc/mmole of carbon Atoms % excess ¹⁵ N
Injected glutamine	25.8	1800	69.8
Casein glutamine	0.611	35.2	57.6
Amide N of casein (a)	0.444	—	—
Non-amide N of casein (b)	0.004	—	—

(a) Nitrogen released as ammonia on hydrolysis with 20% (w/v) HCl. (b) Nitrogen not released as ammonia on hydrolysis with 20% (w/v) HCl.

It has been suggested that γ -glutamyl peptides formed from glutamine by enzymic exchange reactions are important intermediates in protein synthesis (Waelsch, 1952). The results in Fig. 1 demonstrate that the glutamine residues of casein are derived from plasma glutamine. It seems unlikely that this glutamine has formed γ -peptides during its incorporation, since it would then probably have appeared in the casein as glutamic acid. But it is conceivable that γ -peptides were formed, and that glutamine was resynthesized from the glutamic acid of these peptides. If so, the amide-nitrogen atom of each glutamine residue in casein will not be the same as the amide-nitrogen atom it had when in the plasma. Whether it is the same can be tested by labelling the plasma glutamine with DL-[1-¹⁴C:amide-¹⁵N]glutamine. Then, if the glutamine has formed intermediate γ -peptides, the ratio ¹⁴C:¹⁵N will be higher in the casein glutamine than in the plasma glutamine (provided that the labelled nitrogen which was lost on γ -peptide formation has been diluted with unlabelled nitrogen before resynthesis).

In Table 3 are shown the results of an experiment in which DL-[1-¹⁴C:amide-¹⁵N]glutamine was injected into the goat. It can be seen that the ¹⁵N concentration in the amide nitrogen of casein glutamine is over 150 times that in the non-amide nitrogen of casein, and that the ratio ¹⁴C:¹⁵N is approximately the same in the casein glutamine as in the injected glutamine. (The ratio is, in fact, slightly lower in the casein glutamine, showing that more of the injected ¹⁵N than ¹⁴C has appeared in casein; this could be due to partial breakdown of the injected glutamine and resynthesis of glutamine with a lower ¹⁴C:¹⁵N ratio before its absorption by the mammary gland.) The results therefore suggest that glutamine does not exchange its amide nitrogen during its incorporation into casein, and so does not pass through intermediate γ -glutamyl peptides.

Hanes, Hird & Isherwood (1952) have suggested that glutathione enters into transpeptidation reactions which are important in protein synthesis, and it was thought that if glutathione is present in milk some information might be gained by measur-

Fig. 2. DL-[1-¹⁴C]Glutamic acid (31 mg.; 169 μc) was injected intravenously into a lactating goat and blood samples were taken at intervals after injection. Change in specific activity with time is shown for free glutamic acid of plasma (▲) and for free glutamine of plasma (○). A rough value for the specific activity of glutamic acid at zero time has been calculated assuming that the blood stream contained 2 l. of plasma with 1.5 mg. of free glutamic acid/100 ml.

ing the specific activity of its glutamic acid residue in each milk sample. However, by determining glutamic acid in a protein-free filtrate of goats' milk before and after hydrolysis, it was found that glutathione, if present in milk, had a concentration of less than 2 mg./100 ml., and its isolation was not attempted. Gould (1940) was also unable to detect glutathione in milk.

It was hoped that when [¹⁴C]glutamic acid was injected into the goat, the specific activity of the

free glutamic acid of plasma would remain higher than that of the plasma glutamine for several hours, and it would then be possible to decide whether the glutamic acid residues of casein are derived from the glutamic acid of plasma. However, when [^{14}C]glutamic acid was injected, the values in Fig. 2 were found for the specific activities of plasma glutamic acid and glutamine. It is seen that before 20 min. the specific activities of glutamine and glutamic acid had become equal, and thereafter the specific activity of glutamine was higher than that of glutamic acid. It is clear that glutamine is rapidly formed from glutamic acid.

Although, after injecting [^{14}C]glutamic acid, the specific activity of plasma glutamine does not, for long, remain lower than that of glutamic acid, it was still possible to discover the origin of the glutamic acid of casein. It can be deduced from Fig. 2 that if casein glutamic acid comes from the free glutamic acid of plasma, then it should become radioactive more rapidly than the casein glutamine (which Fig. 1 showed to come from plasma glutamine), but its activity should also fall off more rapidly. A further experiment (Fig. 3) shows that these results are in fact found, and it may be concluded that the mammary gland takes free glutamic acid from the blood to provide the glutamic acid residues of

casein. This conclusion is confirmed by the fall in concentration of free glutamic acid when blood flows through the lactating mammary gland of the cow (Sheldon-Peters & Barry, 1956).

The areas under the casein glutamic acid and glutamine histograms in Fig. 3 are roughly equal, being 94 and 99 units respectively. This suggests that the areas under the specific activity-time curves of plasma glutamic acid and glutamine, if they had been measured, would also have been found equal. (In Fig. 2 there are too few experimental points to determine these areas accurately.) It follows that glutamic acid is probably the sole precursor of plasma glutamine, and glutamine in equilibrium with it (Siri, 1949), a result which would be expected from the work of Elliot (1951) and Speck (1949). Fig. 2 shows that glutamine is rapidly metabolized, and, from the slopes of the curves, it seems that at the end of about every 35 min. one-half of the plasma glutamine, and glutamine in equilibrium with it, which was present at the start of the 35 min., has been metabolized and replaced by glutamine newly synthesized from glutamic acid. Therefore, the average life of a glutamine molecule is apparently about 50 min.

It has been shown here that the glutamine and glutamic acid of casein come from the glutamine and glutamic acid of the blood. This finding is confirmed by the results of a further experiment (Fig. 4),

Fig. 3. DL-[^{14}C]Glutamic acid (2.8 mg.; $15.2\mu\text{C}$) was injected intravenously into a lactating goat. The goat was milked immediately before injection and at intervals up to 7 hr. after. The histograms show the specific activities of casein glutamic acid (solid lines) and casein glutamine (broken lines) in each milk sample, plotted as in Fig. 1. The specific activities of glutamic acid (acid hydrolysis) in each casein sample are not shown; they always lay between those of glutamine and glutamic acid.

Fig. 4. D-[^{14}C]Glucose (uniformly labelled) (224 mg.; $71\mu\text{C}$) was injected intravenously into a lactating goat. The goat was milked immediately before injection and at intervals up to 7 hr. after. The histograms, plotted as in Fig. 1, show the specific activities of lactose (broken lines) and casein glutamic acid (acid hydrolysis), solid lines, in each milk sample.

which shows that there was little synthesis of casein glutamine or glutamic acid from blood glucose within the mammary gland. It has been demonstrated previously that blood glucose is the principal compound used for lactose synthesis by the mammary gland of the goat (Reiss & Barry, 1953). Therefore, after injecting [^{14}C]glucose, the area under the specific activity-time histogram of a compound in milk which is synthesized solely from blood glucose must be at least as large as the area under the lactose histogram. In Fig. 4 the area under the casein-glutamic acid (acid hydrolysis) histogram is only about one-fifth that under the lactose histogram; therefore not more than one-fifth of the casein glutamic acid (acid hydrolysis) can have been synthesized in the mammary gland from glucose. In fact, it is probably less than one-fifth, the activity of casein glutamic acid (acid hydrolysis) being, at least in part, due to the synthesis of blood glutamine and glutamic acid from the injected glucose outside the mammary gland.

DISCUSSION

The results demonstrate that the glutamine of casein is derived from the free glutamine of blood. For a number of years it has been known that glutamine is present in blood plasma in higher concentration than any other free amino acid, but hitherto its only function in living animals which was definitely known was to provide urinary ammonia from its amide nitrogen (Van Slyke *et al.* 1943; Waelsch, 1952). If, as seems probable, glutamine is used in the synthesis of other proteins than casein, another important function has been revealed.

The results also provide some evidence against the theory of Waelsch (1952) that an important mechanism by which amino acids are incorporated into peptide chains is their reaction with glutamine to form γ -peptides, followed by their transfer to the carboxyl groups of other amino acids or peptides, to form α -peptide links. For each α -peptide link formed by transpeptidations, one molecule of glutamine will be converted into glutamic acid. Therefore, if in the mammary gland the glutamine used in these reactions was absorbed from the plasma, and if a significant portion of the total peptide bonds in casein were formed by transpeptidation, a significant amount of glutamic acid would be released, and, it seems probable, incorporated into casein. For example, if only one in ten of the peptide bonds in casein were formed by transpeptidation with plasma glutamine, more than enough glutamic acid would be released to provide all the glutamic acid of casein. As a result, at least part of the glutamic acid residues of casein would probably be derived from plasma glutamine, whereas in fact they were not.

It is conceivable that glutamine absorbed from the blood forms γ -peptides, but, after transpeptidation, is reconverted into glutamine and incorporated into casein. But, if this happened, each glutamine molecule would exchange its amide nitrogen in the process, and the experiment with doubly labelled glutamine suggested that it does not.

It is possible, on the other hand, that the glutamine from which γ -peptides are formed is continually synthesized from glutamic acid within the secretory cells; but, if so, this glutamine must be kept separate from the glutamine absorbed from the blood, because if it was not kept separate the casein glutamine would be partly derived from glutamic acid, and the experimental results showed that it is not. Suppose, for example, that the number of glutamine molecules absorbed from the blood for incorporation into casein was equal to the number synthesized for transpeptidation reactions (which could, therefore, initiate the formation of less than one in ten of the peptide bonds of casein). Then, if the glutamine from these two sources became mixed before use, one-half of the casein glutamine would be derived from glutamic acid. The separation of glutamine synthesized in the gland from that absorbed from the plasma seems improbable.

From previous work it can be concluded that all the essential amino acids of casein are derived completely from the free amino acids of plasma (Askonas *et al.* 1955; Barry, 1952). With the present results the source in the blood of about 72% of the casein nitrogen is known. The source of the other non-essential amino acids of casein, including aspartic acid and asparagine, is now being investigated.

SUMMARY

1. From experiments with glutamine labelled with ^{14}C and ^{15}N , and glutamic acid labelled with ^{14}C , it is concluded that the lactating mammary gland of the goat takes free glutamine and glutamic acid from the blood plasma to provide the glutamine and glutamic acid residues of casein respectively. Glutamine and glutamic acid are thus incorporated into casein as two independent amino acids.

2. This conclusion is supported by the finding that there is little synthesis of casein glutamine or glutamic acid from glucose within the mammary gland.

3. Evidence is presented that transpeptidation reactions, involving γ -glutamyl peptides formed from glutamine, are not important in casein synthesis.

4. The results also suggest that the glutamine of blood plasma is synthesized from plasma glutamic acid, and that the average life of a plasma glutamine molecule is about 50 min.

I am indebted to Professor D. D. Woods for providing the glutamic decarboxylase and pancreas suspension; to Dr R. P. Martin for designing an automatic fraction collector; to Dr D. H. Tomlin for making the determinations of ^{15}N ; and to the Agricultural Research Council for a research grant.

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The Uptake of Glutamine and other Amino Acids from the Blood Stream by the Lactating Mammary Gland

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(Received 3 January 1956)

Work with isotopic tracers has demonstrated that the mammary gland takes glutamine and glutamic acid from the blood stream for the synthesis of casein (Barry, 1956). The results reported in this paper show that the concentrations of glutamine and glutamic acid in the mammary venous blood of lactating cows are markedly lower than in the arterial blood; in dry cows these arteriovenous differences were not found. Arteriovenous differences for serine and threonine are also reported.

The fall in concentration of many metabolites across the mammary gland is a small percentage of their total concentration in blood, and is therefore difficult to demonstrate convincingly. However, arteriovenous differences in free amino acids should be very marked, because their concentrations in blood plasma are low in comparison with their concentrations, as protein, in milk. In fact, the concentrations of the amino acids measured were up to 55% lower in mammary venous blood than in arterial blood.

EXPERIMENTAL

Sampling in blood

The cows were Shorthorns in the herd of the National Institute for Research in Dairying. The lactating cows were giving about 4 gallons of milk/day at the time of sampling. They were milked as usual at 7 a.m. and the blood samples were taken 4 hr. later. The dry cows were in the interval between successive lactations; their milk yields during lactation were similar to those of the other cows.

It has been emphasized that the cow should not be excited while the blood samples are taken, and it has been shown in particular that excitement can cause the mammary venous-blood concentration to change by as much as 6%, and so may cause errors of about this magnitude in arteriovenous differences, especially of non-diffusible compounds (Graham, Kay & McIntosh, 1936; Shaw & Petersen, 1939). The arteriovenous differences we have found are between 11 and 55% of the arterial concentrations, and therefore errors due to excitement are not so important as in most arteriovenous measurements. However, care was taken that the cows should not be excited.