The Source of Proline for Casein Biosynthesis

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Previous work has shown that the mammary gland takes the free essential amino acids from the blood stream to provide the essential amino acids of casein (Barry, 1952; Campbell & Work, 1952; Askonas, Campbell, Godin & Work, 1955); that it takes free glutamine and glutamic acid to provide the glutamine and glutamic acid respectively of casein (Barry, 1956; Sheldon-Peters & Barry, 1956); and that it takes free asparagine to provide asparagine, but not aspartic acid, residues of casein (Sansom & Barry, 1958).

Experiments described in this paper show that the mammary gland of the goat takes free proline from the blood to provide at least 50% of the proline residues of casein. It is calculated from this and previous work that at least half of the residues of non-essential amino acids in casein are not synthesized within the mammary gland, but come directly from the blood stream.

EXPERIMENTAL

L-[¹⁴C]Proline (uniformly labelled). This was bought from the Radiochemical Centre, Amersham, Bucks.

Experiments on goat. The experiments were done 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).

Isolation of free proline of blood plasma. Blood plasma (40 ml.) was dialysed overnight against distilled water (200 ml.) on a shaking machine, and the diffusate was freeze-dried. The residue was dissolved in a little water, brought to pH 3 and eluted from a column of Dowex-50 (100 cm. $\times$ 1.5 cm.) as described by Moore & Stein (1951), but at room temperature. The proline fractions were combined, their proline content was accurately determined by the quantitative ninhydrin method (Moore & Stein, 1948) and a known weight of inactive proline added. Salts were then removed (Sheldon-Peters & Barry, 1956) and the solution was evaporated to give a crystalline sample of proline. From the specific activity of this sample, and the dilution by inactive proline, the specific activity of the free proline of plasma was calculated.

Isolation of proline from casein. Casein was isolated and hydrolysed as before (Barry, 1952), and a sample of its proline was isolated for ¹⁴C determination in the same way as the free proline of blood.

Isolation of glutamic acid from casein. The glutamic acid in an acid hydrolysate of casein was isolated by elution from a column of Dowex-1 (acetate form; 20 cm. $\times$ 1.6 cm.) with 0.5N-acetic acid (Hirs, Moore & Stein, 1954). Evaporation

of the fractions containing glutamic acid gave a crystalline sample.

Determination of ¹⁴C specific activities. The crystals of proline or glutamic acid were packed in aluminium cups (1.5 cm. diameter) to give samples of infinite thickness, and put in a standard position under the window of a Geiger counter. They were counted to give less than 3% probable error, and the counts were converted into specific activities with a factor determined previously.

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 hydrolysis, 100 g. of cow's casein yields 11.3 g. of proline (McMeekin & Polis, 1949). Human blood plasma contains about 2 mg. of free proline/100 ml. (Stein & Moore, 1954), and the concentrations in the plasma of the goat were found to be similar. The proline of casein might therefore come directly from the free proline of blood, or from that of plasma proteins, or might be synthesized within the mammary gland from other compounds absorbed from the blood.

The experiment shown in Fig. 1 was designed to find the proportion of the proline of casein which is derived from the free proline of blood. [¹⁴C]Proline was injected intravenously into a goat, thus labelling the free proline of its blood, and blood and milk samples were taken at intervals after the injection. It can be seen that the specific activity of the proline of casein rapidly became comparable with that of the free proline of blood plasma. The curve *A* passes through each value for the specific activity of casein proline, in the last three milk samples, at a time half-way through the period in which the milk was secreted, and shows the specific activity of casein proline, at various times, in milk which has just reached a point in the mammary gland from which it can be drawn off by milking. It can be seen that after 2½ hr. the specific activity of casein proline was always equal to that of the free proline of blood plasma 1–2 hr. earlier.

In an experiment of this type, the area under the specific activity–time histogram of a component of milk, divided by the area under the specific-activity curve of the labelled component of blood, gives the proportion of the component of milk

which is derived from that of blood, provided that only one constituent of the blood has significant radioactivity (Barry, 1956). In this experiment, significant radioactivity would be gained by any component of the blood only if the component were continually and rapidly synthesized from free proline. It can be predicted from previous studies (Barry, 1952, 1956; Sansom & Barry, 1958) that the proline of plasma proteins would have gained

Fig. 1. Use of proline for synthesis of casein. L- ^{14}C -Proline (0.32 mg.; 25 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 proline of plasma; ▲, curve A, the significance of which is described in the text. The histograms show the specific activities of casein proline in each milk sample, the width of each rectangle occupying the time during which the sample was secreted.

Table 1. *Specific activity of glutamic acid in acid hydrolysates of casein samples*

Samples were taken at intervals after injecting L- ^{14}C -proline (0.32 mg.; 25 μC) into a lactating goat.

Time of sampling (hr.) ...	1.5	3.0	4.5	7.0
Specific activity ($\mu\text{C}/\text{mole of carbon}$)	0.36	0.84	0.51	0.31

little activity during this experiment, and that the area under a curve showing the specific activity of the proline of plasma proteins would have been less than 5% of that under the curve for the free proline of plasma. The only other component of blood, a significant part of which might be continually and rapidly synthesized from free proline, is the free glutamic acid (Stetten, 1955). In a previous similar experiment (Barry, 1956) it was shown that the area under the specific-activity curve for the free glutamic acid of blood equals the area under the specific-activity histogram for the glutamic acid of casein. In the present experiment the activity of the glutamic acid in an acid hydrolysate of each casein sample was measured, and the results are shown in Table 1. It can be calculated that the area under the specific-activity histogram is only 3.3 units. Although the glutamic acid in the acid hydrolysate came from both glutamic acid and glutamine residues, it may be concluded that the area under the specific-activity curve for the free glutamic acid of blood was also about 3.3 units, and was therefore not significant compared with the area under the curve for the free proline. It is clear that an insignificant portion of the glutamic acid of blood is synthesized from proline, although proline is apparently metabolized via glutamic acid (Stetten, 1955).

In Fig. 1 the area under the casein-proline histogram up to 7 hr. is 408 units, and that under the plasma-proline curve (if extrapolated back to the ordinate) up to 5.5 hr. is 580 units. (The time difference allows for the lag of about 1.5 hr. between curve A in Fig. 1 and the curve for the specific activity of the free proline of plasma.) The ratio (0.703) suggests that 70% of the proline of casein came from the free proline of the blood. This figure may not be exact, since the area under the plasma-proline curve cannot be measured accurately; but it may be concluded with certainty that over half the proline residues of casein came from the free proline of the blood. The possibility that the free proline is converted into a derivative, such as a peptide, before being absorbed by the mammary gland may be excluded for reasons similar to those mentioned before (Barry, 1952, 1956).

DISCUSSION

The results which have been described show that the mammary gland takes free proline from the blood to provide at least half of the proline residues of casein. The evidence for the origin of the other non-essential amino acids of casein is as follows. Similar experiments with ^{14}C glutamine and ^{14}C glutamic acid, in which areas under specific-activity curves and histograms were compared, gave the result that 100% of the glutamine

and glutamic acid of casein come from the free glutamine and glutamic acid respectively of blood. Allowing for experimental error, we may conclude with certainty that over 70 % of the glutamine and glutamic acid of casein come directly from the blood. An experiment with [^{14}C]glucose also showed that less than 20 % of the glutamine and glutamic acid of casein were synthesized from glucose within the mammary gland (Barry, 1956). From similar experiments it may be concluded that over 70 % of the tyrosine of casein comes from the free tyrosine of blood, and is not derived from plasma proteins, or synthesized from phenylalanine within the gland (Barry, 1952). A further experiment showed that at least 50 % of the asparagine residues of casein come from the free asparagine of blood, and that less than 17 % are synthesized from glucose within the mammary gland. No significant part of the aspartic acid of casein comes from the asparagine of blood, nor is it synthesized within the gland from glucose; however, its precise origin remains unknown (Sansom & Barry, 1958). Arterio-venous differences in free serine, of up to 48 % of the arterial concentrations, were found across the mammary glands of lactating, but not dry, cows (Sheldon-Peters & Barry, 1956). These results suggest that at least part of the serine of casein comes from the free serine of blood. The source in the blood of the cystine, alanine and glycine of casein is unknown, but these amino acids make up only about 6 % by weight of the casein molecule.

From these findings it can be calculated that about half, at least, of the residues of non-essential amino acids in casein are derived directly from the blood. It would be interesting to know whether any synthesis of non-essential amino acids occurs in the mammary gland, since it is possible that only a few organs of the body can synthesize some

of them, as has been suggested by Eagle, Oyama & Levy (1957). Experiments of the type described in this paper, however, will never exclude the possibility that small amounts of non-essential amino acids are synthesized within the mammary gland.

SUMMARY

1. From an experiment with [^{14}C]proline it is concluded that the mammary gland of the goat takes free proline from the blood to provide at least half of the proline residues of casein.

2. From this and previous work it is calculated that at least half the residues of non-essential amino acids in casein are not synthesized in the mammary gland, but come directly from the blood stream.

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The Reactivity of Free Amino Groups in Native and Denatured Ovalbumin Towards Fluorodinitrobenzene

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Porter (1950) showed that ovalbumin contained no *N*-terminal residue able to react with 1-fluoro-2,4-dinitrobenzene (Sanger, 1945), but subsequent work by Niu & Fraenkel-Conrat (1955) showed that it did contain a *C*-terminal residue (proline). Linderstrøm-Lang (1952) suggested that ovalbumin had one of two possible structures. Anfinsen & Redfield (1956) preferred one of these structures

and considered ovalbumin to be a cyclic molecule (probably held by S-S bonds) from which protruded a peptide chain containing the *C*-terminal residue. They suggested that the terminal α -amino group was either masked sterically or chemically bound in a linkage which may be intramolecular or involve the carbohydrate moiety known to be present in ovalbumin (Neuberger, 1938).