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The Source of Antibody Globulin in Rabbit Milk and Goat Colostrum

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After the intravenous injection of radioactive amino acids into lactating rabbits, there is an extremely rapid and efficient incorporation of radioactive carbon into milk proteins. The blood plasma-protein radioactivity reached its maximum value 6 or 7 hr. after the injection, but it is only about one-tenth the activity of a milk protein sample collected at the same time (Campbell & Work, 1951, 1952). It has been concluded that blood plasma protein is not a major and direct precursor of milk protein and that most of the milk protein is synthesized in the mammary gland from amino acids or small peptides. Amino acids isolated from radioactive rabbit casein were, however, of slightly higher specific activity than the corresponding amino acids from whey protein, and it seemed possible that this difference might be due to the direct transfer from blood to milk of a fraction of the plasma protein. Campbell & Work (1952) calculated that up to 25% of the total whey protein would have to be so transferred to account for the lowered radioactivity of the whey. Experiments have, therefore, been directed towards identification of the protein transferred from blood to milk in the lactating rabbit. More recently we have been interested in the biosynthesis of milk proteins in the goat, and it was hoped to extend the work to this animal.

Extensive studies on the transmission of immunity from parent to offspring have suggested that antibody may pass across the placental barrier unchanged; high concentrations of antibody being found in colostrum and traces in milk (Ehrlich, 1892; Ratner, Jackson & Gruhl, 1927; Marrack, 1947; McMeekin & Polis, 1949; McCarthy & McDougall, 1953). According to Smith (1948) the immune-globulin fraction of cow colostrum is identical with the immune-globulin of cow milk and

closely similar to, but not quite identical with, the T-globulin of cow plasma. The immune-globulins are, however, proteins of high molecular weight, rabbit γ -globulin having a molecular weight of 160 000 (Kabat, 1939; Nichol & Deutsch, 1948) and there has been considerable doubt as to whether this whole protein passed across cell barriers or whether it was partially degraded and resynthesized (Cohen, 1950; Calman & Murray, 1951; Brambell, Hemmings & Henderson, 1951).

From the work on immune-globulin, it was clear that in so far as proteins could be transferred directly from rabbit blood to milk, they would probably be found in the immune-globulin fraction. Moreover, any protein transferred directly to the milk during the hours immediately following intravenous injection of a radioactive amino acid should have the radioactivity characteristic of the plasma proteins and, 6 hr. after injection, should have not more than about one-tenth the activity of protein synthesized in the mammary gland. Accordingly, a lactating rabbit was immunized with pneumococcus (type III) and was then given an injection of [35 S]methionine. Blood and milk samples were withdrawn at intervals and pneumococcus capsular polysaccharide (SSS III) was used to precipitate the specific antibody from blood and milk. The antibodies thus obtained were compared with one another and with the other milk and blood proteins. A preliminary account of this investigation has been published (Campbell, Humphrey & Work, 1953).

An attempt was then made to reproduce in a goat the experimental conditions already used with rabbits. However, even after a prolonged course of intravenous injections of formalin-treated pneumococcus (type III) the serum antibody level did not rise above 0.4 mg./ml. The level in the milk was much lower, and was too low to permit accurate

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quantitative work. We therefore used an alternative approach and measured the distribution of radioactivity in goat colostrum proteins after injection of radioactive methionine.

Colostrum differs from milk not only in its higher protein content but also in the relative proportions of the constituent proteins (Crowther & Raistrick, 1916). Smith (1946) calculated from electrophoretic data that 55% of the protein in cow colostrum taken 1 hr. *post partum* consists of an immune-globulin fraction rich in antibodies, whereas normal milk contains only about 10% of immune-globulin. The goat has been less extensively studied, but Deutsch (1947) showed that there was a similar and rapid change in the composition of proteins secreted by the mammary gland immediately *post partum*.

In the present experiments a goat was injected with [³⁵S]methionine shortly after parturition and the colostrum samples taken at 10 and 13 hr. after the injection were separated into casein, lactoglobulin and immune-globulin fractions. The distribution of radioactivity in these proteins was consistent with the assumption that in the goat as in the rabbit immune-globulin passes from blood to milk without degradation and resynthesis.

EXPERIMENTAL

A lactating rabbit with a litter 1 week old was injected intravenously with a suspension of formalin-treated pneumococcus type III. Eight injections were given during a period of 17 days, the total number of cells injected being 8×10^{10} . The antibody content of the serum was measured by the method outlined below and was 4.5 mg./ml. after 2 weeks, rising to 10 mg./ml. at 3 weeks. Antibody concentrations are expressed as mg. dry weight of antigen-antibody complex containing about 4% of antigen (Fig. 1).

The rabbit was then given, by intravenous injection, 6.7 mg. of [³⁵S]DL-methionine containing about 25 μ C of ³⁵S (supplied by the Radiochemical Centre, Amersham) on the fourth day after the last injection of antigen. The animal was separated from its litter and milked after 6 hr. Immediately after milking, a blood sample was taken from the ear. Milking and bleeding were repeated at 30 hr. and 9 days.

The animal was kept in full lactation throughout the experimental period by replacement of the litter every 14 days by another litter 7 days old.

Treatment of rabbit milk. After removal of the bulk of the fat by low-speed centrifugation, most of the casein was separated by centrifugation at 1° for two periods of 1 hr. in an angle head at 33000 g. The clear supernatant fluid was treated with 'Merthiolate' (Eli Lilly and Co. Ltd., Basingstoke) 1:10000 and stored at 2°. It showed no tendency to spontaneous precipitation. Antibody was precipitated by addition of SSS III at optimum proportions (Dean & Webb, 1926), and after allowing 24 hr. at 2° for precipitation, the antigen-antibody complex was centrifuged down, washed twice with 0.9% NaCl at 2°, and once with water. It was dried with ethanol and ether and weighed. The antigen-antibody complex was calculated to contain approximately 4% of antigen. The milk contained about one-twentieth of the concentration of antibody in the plasma, and the yields varied between 8.7 mg. from 17 ml. milk at the height of immunization and 3.0 mg. from 14 ml. milk during the declining phase.

The whey protein remaining in solution after removal of antibody was precipitated by heat.

Treatment of rabbit blood. The serum was separated and antibody to SSS III was precipitated from a 3.5 ml. sample by addition of SSS III at optimum proportions and collected in the same way as for milk.

The supernatants after precipitation of the antibody were treated with 2 vol. of 27% (w/v) Na₂SO₄ dissolved in Na phosphate buffer, pH 7.8 (*I* = 0.1), to produce a final concentration of 18% (w/v). The precipitated globulins, which consisted of about 95% of γ - and 5% β -globulins, were redissolved and reprecipitated at 18% (w/v) Na₂SO₄. After dialysis against distilled water this 'globulin' fraction was coagulated with trichloroacetic acid, washed and dried. The proteins remaining in solution at 18% Na₂SO₄ were coagulated by heat, washed and dried. They are termed the 'albumin' fraction, although some α - and β -globulins are included with them.

Specificity of precipitation. Since differences in the radioactivity of the immune-globulin and the other plasma proteins were to be measured it seemed desirable to arrange a control experiment to indicate to what extent proteins might be bound to an antigen-antibody precipitate by non-specific adsorption. This was achieved by adding ovalbumin to the milk whey and blood serum and precipitating it by the addition of antiovalbumin antibody. The radioactivity of this precipitate then gave a measure of the degree of non-specific adsorption of radioactive protein. The experiment was conducted as follows.

A sample of whey (17 ml.) diluted with an equal volume of 0.9% NaCl was mixed with 30 mg. (dry wt.) of rabbit γ -globulin, of which one-third was antiovalbumin antibody,

Table 1. *Precipitation of pneumococcus antibody from rabbit serum and milk by specific soluble polysaccharide type III. Effect of addition of antiovalbumin followed by ovalbumin on yield and radioactivity of pneumococcal antibody*

(For conditions see text.)					
Material	Time (hr.)	Direct precipitation with SSS III		After treatment antiovalbumin-ovalbumin	
		Yield (mg./ml.)	Radioactivity (counts 1 min./0.3 sq.cm. of infinite thickness)	Yield (mg./ml.)	Radioactivity (counts 1 min./0.3 sq.cm. of infinite thickness)
Milk	6	0.505	10	0.49	7
	30	0.535	20	0.525	17
Serum	6	10.7	19	10.4	16
	30	9.9	22	9.4	17

and treated with 1 mg. crystallized ovalbumin dissolved in 0.9% NaCl (0.5 ml.). The precipitate, which was not significantly radioactive, was removed after 24 hr. at 2° and the pneumococcus antibody then precipitated as before. This procedure diminished the yield of anti-SSS III by about 5% and lowered the radioactivity about 20%, compared with the product obtained from the untreated sample.

Duplicate samples of blood serum (3.8 ml.) were also treated with the antiovalbumin-ovalbumin system in the same way. The effect upon the yield and radioactivity of the anti-SSS III precipitates was very similar to that on the milk samples (Table 1).

It is difficult to know what importance to attach to the diminution of radioactivity in anti-SSS III precipitates from the pretreated milk and serum samples. Such a diminution might be expected if the pretreatment process prevented non-specific co-precipitation of traces of some more highly radioactive material, e.g. casein in the milk—but in blood serum the main protein fractions would all have radioactivities of the same order and this explanation is therefore unlikely. Since the counts and consequently their accuracy, were relatively low, and since the findings do not affect our main conclusions, we did not pursue the matter further.

Goat colostrum

A British Saanen goat gave birth to three kids over a period of approximately 1 hr. About 2 hr. after the birth of the first kid the mammary gland was milked dry by an experienced milker and about 1 l. of very thick colostrum obtained. The goat was then given intravenously 17 mg. of DL-methionine containing approximately 40 μ C of 35 S. The kids were allowed to remain with the mother, and 10 hr. after the injection 35 ml. of colostrum were obtained; 13 hr. after the injection 5 i.u. of posterior pituitary extract were given intravenously, a blood sample taken and 290 ml. of colostrum were withdrawn.

Treatment of goat colostrum. The colostrum was fractionated according to Smith (1946). After removal of the fat by centrifugation, the casein was precipitated at pH 4.5. The casein was washed twice with distilled water, the first washing being added to the whey fraction. After the casein had been dissolved in water by the addition of *n*-NaOH to pH 6.5, the solution was clarified by filtration through kieselguhr and again precipitated. The twice-washed casein was freeze-dried.

The whey was clarified by filtration through a pad of paper pulp, adjusted to pH 6.0 with 0.5*N*-NaOH and solid $(\text{NH}_4)_2\text{SO}_4$ was added to 0.49 sat. (30.4 g./100 ml.). The precipitate, which will be referred to as the immune-globulin fraction, was separated by centrifugation. The supernatant was filtered until completely clear and then saturated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate will be referred to as the lactoglobulin fraction. The immune-globulin and lactoglobulin precipitates were dissolved in water, clarified by filtration and again precipitated at the appropriate concentration of $(\text{NH}_4)_2\text{SO}_4$. After dissolving the precipitates in water the solutions were extensively dialysed at 1° and then freeze-dried.

Treatment of goat blood. The blood sample was fractionated into plasma protein and red cells as previously described (Campbell & Work, 1952).

Methionine and cystine in goat colostrum proteins. Comparison of the radioactivity of whole proteins could be mis-

leading if these differed greatly in amino-acid composition. To check that methionine-labelled immune-globulin could be compared with methionine-labelled lactoglobulin, a rough estimate was made of cystine and of total sulphur in the two proteins. A sample (15 mg.) of each protein was digested with Pirie's (1932) reagent followed by HCl as described by Simpson, Tarver & Rutman (1950) and sulphur estimated as benzidine sulphate (Scott & Furman, 1939). Immune-globulin was found to contain about 0.7% sulphur by weight and lactoglobulin about 1.3%. Another sample of each protein (10 mg.) was hydrolysed for 24 hr. at 110° with 6*N*-HCl. Excess of acid was removed by evaporation *in vacuo* and the amino acid mixture treated with excess of bromine water to oxidize cystine and cysteine to cysteic acid (Friedmann, 1903). A comparison between the amount of cysteic acid in the two hydrolysates was then made by paper chromatography followed by reaction with ninhydrin and visual comparison of the cysteic acid spots. This is a very roughly quantitative method but as the differences in radioactivity were sixfold or more it was sufficiently accurate for our purpose. No significant difference was found in the cysteic acid concentration in the two protein hydrolysates.

Assay of radioactivity

All protein samples were transferred to polythene disks, rabbit proteins to 0.3 sq.cm. disks, goat proteins to 1 sq.cm. disks, and counted at infinite thickness, using a standard end-window counter. Since all the proteins were dried in a similar manner, no special precautions were taken to control moisture content during counting.

RESULTS

Experiments with the rabbit. Fig. 1 shows the variation in concentration of rabbit-serum antibody over the period of the experiment. It will be seen that the injection of radioactive methionine took place when antibody concentration was at its maximum, and that during the following 9 days of milking the antibody level in the serum was falling rather rapidly.

Fig. 1. Antibody content of serum and milk of rabbit immunized with pneumococcus. ●—●, blood serum antibody; ○—○ milk antibody. The times of the intravenous injections of pneumococci are indicated by arrows on the time axis.

Table 2. *Activity of milk and blood protein after administration of [³⁵S]methionine to a lactating immune rabbit*

Results as counts/min./0.3 sq.cm. of infinite thickness.

Material	Time after injection		
	6 hr.	30 hr.	9 days
Serum antibody	16	17	15
Milk antibody	7	17	14
Casein	108	30	6
Whey protein (after removal of antibody)	91	38	8
Serum, globulin	17	16	10
Serum, albumin	18	13	7

Table 3. *Distribution of proteins in goat colostrum*

Time after parturition (hr.)	Total amount of protein (g.)	% of each component		
		Casein	Immune-globulin	Lactoglobulin
1	105	24	62	14
11	2.15	59	25	16
14	13.10	52	26	22

Table 4. *Radioactivity of blood and colostrum proteins obtained from a lactating goat after injection of [³⁵S]DL methionine*

Results as counts/min./sq.cm. of infinite thickness.

Fraction	Time after injection	
	10 hr.	13 hr.
Casein	58	48
Immune-globulin	6	4
Lactoglobulin	37	40
Plasma protein	—	14
Red cells	—	1

In Table 2 a comparison is made of the radioactivity of the milk and serum proteins of the rabbit. It is clear that milk-antibody radioactivity follows closely the serum-antibody activity and fails to show the steep rise in activity characteristic of casein and of whey protein.

Experiments with the goat. The amount of protein isolated from each of the colostrum fractions is shown in Table 3, while in Table 4 a comparison is made of the radioactivities of the various proteins isolated. The casein and lactoglobulin fractions are notably more radioactive than the immune-globulin.

It seemed just possible that this difference might be due to a difference in the methionine content of the proteins but this is not so. To account for the higher activity of lactoglobulin and casein we should have to assume that these proteins contained between 6 and 10 times as much methionine as immune-globulin but roughly quantitative measurement of cystine and of total sulphur in lactoglobulin and immune-globulin showed no difference of this order between the two proteins (see Experimental).

DISCUSSION

We have previously shown that, 6 hr. after the injection of a radioactive amino acid to a lactating rabbit, the protein synthesized in the mammary gland had up to 10 times the radioactivity of the blood protein. In the present experiments (Table 2) 6 hr. after injection of [³⁵S]methionine the casein was 15 times more active than the milk antibody and the whey protein not precipitable by pneumococcus antigen was 13 times as active as the milk antibody.

These results are so striking as to leave no doubt that, during normal lactation, rabbit pneumococcus antibody passes through the mammary gland without degradation and resynthesis. The results obtained during the remainder of the experimental period served to confirm this deduction. Both the 30-hr. and the 9-day samples of milk antibody were of the same radioactivity as the serum antibody, moreover, at 9 days the milk antibody was twice as active as the casein or the residual whey protein, and also twice as active as the serum albumin and more active than the serum globulin (Table 2).

The high radioactivity of antibody at 9 days is probably explained by the shape of the curve for total antibody (Fig. 1). It is known that although specific antibody is synthesized independently of other plasma globulins, all the γ -globulins, including antibody globulin, are catabolized at the same rate, equal to 10–12½% per day (Humphrey & MacFarlane, 1952). Synthesis of new antibody slowed down rapidly during the experimental period so that antibody globulin labelled with ³⁵S on the first day

was diluted at rapidly diminishing speed with new antibody globulin, whereas other labelled globulins were diluted at a steady rate governed by the constant production of new plasma protein. It is not surprising that the 6-hr. sample of milk antibody was less radioactive than the 6-hr. sample of serum antibody. The milk collected at 6 hr. had accumulated in the mammary gland over, at least, a 6-hr. period so that it would not be in equilibrium with serum collected at 6 hr. but would represent an average value for the 6 hr. period. During part of this time unlabelled or weakly labelled antibody protein must have been passing into the gland.

The results with goat colostrum are not as clear cut as those with rabbit milk. This was not unexpected since the proportions of different proteins in colostrum varies very considerably with time (Table 3). Thus, at 1 hr. the immune globulin accounted for 62% of the milk protein, whereas at 11 and 14 hr. it was only 25% of the total protein. In spite of this difficulty the results are consistent with those obtained in the rabbit and suggest that in the goat, as in the rabbit, immune-globulin passes unchanged through the mammary gland. How this can occur without (in colostrum at any rate) a corresponding accumulation of other plasma proteins raises another problem upon which our results, shed no light.

Smith and his colleagues have shown that immune-globulin of bovine colostrum is very similar to the T-globulin of blood plasma, but they find differences between the two in amino acid composition and in absorption spectrum (Smith, Greene & Bartner, 1946; Smith & Coy, 1946; Smith, 1948). They suggest that the mammary gland alters the protein as it passes through the gland but, in the present work, if any such alteration does take place it does not involve rupture and reformation of methionine peptide linkages.

In view of the demonstration that plasma protein can pass into the milk without degradation and resynthesis, the results quoted by Campbell & Work (1952) for unequal specific radioactivities of amino acids isolated from rabbit casein and rabbit-whey protein after injection of radioactive amino acids, could be supposed to be due to such transfer. It has thus become necessary to repeat these experiments with a larger animal and to fractionate the milk-whey proteins before determination of radioactivities. The results of such an experiment will be reported later.

SUMMARY

1. The antibody concentration in the milk of an immune rabbit (type III pneumococcus) was about one-twentieth of that in the blood.

2. After the injection of radioactive [³⁵S]-methionine the distribution of radioactivity in blood and milk proteins was measured.

3. Antibody was selectively precipitated from blood serum and from milk whey by addition of type III specific soluble polysaccharide.

4. Serum antibody and milk antibody acquired a similar degree of radioactivity. Casein and lactoglobulin collected 6 hr. after injection of methionine had 6–10 times the radioactivity of milk or serum antibody collected at the same time.

5. The results show that, in the rabbit, antibody protein passes from blood to milk without degradation and resynthesis.

6. Antibody protein also passes from blood to colostrum in the goat without degradation and resynthesis.

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