ethanolamine or dimethylethanolamine would be deaminated to any great extent and these substances would therefore be freely available for choline synthesis. The slight increase in choline synthesis observed when glycine was fed may be due to the ability of the α -carbon atom of glycine to act as a precursor of ethanolamine and the methyl groups of choline. The loss of betaine in some of these experiments is difficult to explain. The seed of wheat contains approx. 0.092 % of betaine (Guggenheim, 1940) and as the young seedling does not appear to synthesize betaine from choline or other possible precursors it is assumed that the betaine present in the seedling is translocated from the seed. As the fate of betaine was not determined in these experiments it is impossible to do more than suggest that on the analogy of the behaviour of betaine in animal tissues, this substance can also act as a methyl donor in plants. In experiments in which a loss of betaine was recorded, a methyl group derived from betaine might have made some contribution to the methylation of *N*-methylethanolamine and dimethylethanolamine to choline.

SUMMARY

1. Experiments on the effects of feeding possible precursors of choline to etiolated wheat seedlings grown under sterile conditions have been described.

2. The biosynthesis of choline was stimulated to a marked degree when *N*-methylethanolamine or dimethylethanolamine were fed alone and in combination with methionine and/or formate. *N*-Methylethanolamine or dimethylethanolamine used in combination with formate stimulated choline synthesis to a greater extent than the combination of these precursors with methionine.

3. The feeding of ethanolamine either alone or in combination with methionine and/or formate did not result in a significant increase in the choline content of the tissues.

4. The feeding of glycine alone and in combination with methionine brought about a slight increase in the choline content of the tissues but a slight decrease in the content of betaine. The feeding of dimethylglycine alone or in combination with methionine resulted in slight losses of choline but a slight increase in the content of betaine. A slight increase in the content of betaine was observed when dimethylglycine was fed in combination with formate.

5. Conversion of choline into betaine did not appear to take place in etiolated wheat seedlings under the conditions of the experiments. The possible significance of losses of betaine which occurred in some experiments is discussed.

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The Biosynthesis of Proteins

2. SYNTHESIS OF MILK PROTEINS BY THE GOAT

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In the first paper of this series (Campbell & Work, 1952) it was shown that the lactating rabbit synthesized milk protein mainly from the free amino acids of the blood. The distribution of radioactivity between casein and whey proteins showed,

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however, that if casein is synthesized entirely from free amino acids then a portion of the whey nitrogen must be supplied as bound amino acid, probably in the form of whole protein or peptide.

It has been shown recently (Askonas, Campbell, Humphrey & Work, 1954) that the immune globulin of rabbit milk is not synthesized in the mammary

gland; thus, the presence of this protein in whey could account in part for the low radioactivity of whey protein as compared to casein. The quantity of immune globulin in milk is low and it seemed doubtful whether this protein alone could account for all the 'bound' amino acids of whey and, particularly, for the low specific radioactivity of whey serine as compared to casein serine after injection of radioactive glycine.

Since whey contains a complex mixture of proteins it was undesirable to make further comparison between casein and whey protein and it was decided to compare instead casein and β -lactoglobulin. It was not possible to obtain sufficient rabbit milk to permit isolation of the lactoglobulin fraction and the present series of experiments have therefore been made on a goat. A method for the crystallization of goat β -lactoglobulin is given in another paper (Askonas, 1954) along with details of its physical properties. In the present experiments milk was collected after injection of radioactive glycine, valine, methionine and lysine. Comparison of the specific radioactivities of the amino acids from casein and from β -lactoglobulin showed (as with the rabbit) a substantial difference between casein serine and lactoglobulin serine but no significant difference of specific radioactivity between the lysine, valine and glycine from casein and the corresponding amino acids from β -lactoglobulin. A preliminary account of some of this work has been published (Work, Campbell & Askonas, 1952).

MATERIALS AND METHODS

Animal. A British Saanen goat was used, 2 years old and in her first lactation period. Her milk yield was about 5 lb. daily.

Radioactive amino acids. Glycine was supplied as [2- 14 C]-glycine by the Radiochemical Centre, Amersham; DL-[35 S]methionine and DL-[4- 14 C]valine were obtained from the same source. DL-[1- 14 C]lysine was kindly supplied by Dr Hunter (Arnstein, Hunter, Muir & Neuberger, 1952).

Administration of radioactive amino acids. A suitable quantity of amino acid was dissolved in isotonic saline and injected into the jugular vein within 1 hr. of the morning milking.

Collection of milk samples. When it was necessary to obtain the maximum yield of radioactive protein, the animal was milked by hand in the normal way until no more milk could be obtained; it was then given an intravenous injection of 5 i.u. of posterior pituitary extract (British Drug Houses Ltd.) and milked dry.

Fractionation of proteins from goat milk

Casein. Milk (1 l.) was centrifuged to remove fat, and the casein precipitated by slow addition of N-HCl and N acetic acid (1:2, v/v) to the stirred solution until the pH was 4.5 (Van Slyke & Baker, 1918). The casein was centrifuged down, washed twice with water and the first washings were added to the whey fraction. The casein was

Table 1. Fractionation of goat whey proteins

For conditions see text.

Defatted whey (1000 ml.)	
pH 6.5 (NH ₄) ₂ SO ₄ (38 g./100 ml. 0.58 satd.)	
Precipitate A (Discarded)	Supernatant (NH ₄) ₂ SO ₄ (19 g./100 ml. 0.32 satd.) Centrifuged after 1 hr.
	Precipitate C (0.46 g.)
	Supernatant (NH ₄) ₂ SO ₄ (11.4 g./100 ml. 0.5 satd.) Centrifuged
	Precipitate D (Immune globulins, 0.55 g.)
Supernatant (Dialysed and freeze-dried) Fraction E (0.31 g.)	
Filtered through Celite 545 (Johns Manville Co., London), adjusted pH to 5.8, added (NH ₄) ₂ SO ₄ until saturated. Stood overnight	
pH 6.5 (NH ₄) ₂ SO ₄ (38 g./100 ml. 0.58 satd.)	
Supernatant	
Precipitate B (3.4 g.)	
(3.4 g.) Dissolved in water (0.6% w/v), dialysed, added NaCl to 0.03 M and ethanol to 34% (w/v)	
Precipitate F (1.7 g.)	
(3.4 g.) Fractionated with (NH ₄) ₂ SO ₄ using method of Askonas (1954)	
Crystals (0.3 g.) Fraction G (β -Lactoglobulin)	

redissolved by suspension in water (2–3% protein) and dropwise addition of N -NaOH to pH 6.5. The solution was shaken with an equal volume of ether to remove residual lipid and the casein in the aqueous layer reprecipitated at pH 4.5, washed twice and freeze-dried. The yield was about 26 g.

Whey protein. The whey, adjusted to pH 5.5, was defatted by shaking with an equal volume of ether. The aqueous layer was freed from ether *in vacuo* at 30° and the proteins were fractionated with $(NH_4)_2SO_4$, following, with minor modifications, the procedure of Smith (1946) for bovine whey (see Table 1). Solid $(NH_4)_2SO_4$ (A.R.) was added at room temperature with continuous stirring and all mixtures were allowed 2–3 hr. to reach equilibrium unless otherwise stated. All precipitates (Table 1) were dissolved in the minimum amount of water, dialysed against running tap water and then for 48 hr. at 1° against distilled water and freeze-dried. The yields given in Table 1 represent an average for several experiments.

Electrophoretic analyses of whey fractions were carried out in a Tiselius electrophoresis apparatus (A. Hilger and Co., London) in various buffers ($I=0.1$) at 1° and at a concentration of 1–2% protein.

Purification of lactoglobulin. The crude β -lactoglobulin fraction (B, Table 1) was crystallized by the method of Askonas (1954). Fraction B was also purified by using the last step in the ethanol fractionation of Bain & Deutsch (1948). A 0.6% (w/v) solution of B was dialysed 24 hr. against distilled water, the pH was adjusted to 6.55 and 3M-NaCl added to a final concentration of 0.03M. Absolute ethanol was added at –6° and the fraction insoluble in 34% ethanol (v/v) was collected (precipitate F).

Isolation of amino acids

The protein samples were hydrolysed in sealed tubes for 18 hr. at 105° with 6N-HCl, taken to dryness three times and dissolved in water. Preliminary fractionation of the amino acids was achieved in a column of Dowex 50 cation-exchange resin (200–400 mesh/in., Microchemical Specialties Co., Berkeley, California). The column (3 × 100 cm.) was packed in 1.5N-HCl and the amino acids from 1 g. protein were added to the column in about 50 ml. water. The column was eluted with increasing concentrations of HCl as described by Stein & Moore (1950). Using this method there is incomplete separation of the amino acids, but fractionation was followed by paper chromatography of the effluents, and those fractions which contained radioactive amino acids were collected separately. [^{14}C]Serine was mixed with threonine and glutamic acid, [^{14}C]glycine with alanine, [^{14}C]valine with a trace of proline and [^{14}C]lysine with a trace of tyrosine.

Glycine. The glycine-rich fraction was allowed to react with excess fluorodinitrobenzene (FDNB) in aqueous ethanol containing $NaHCO_3$ (Sanger, 1945). The product was fractionated by the method already described (Campbell & Work, 1952) and the dinitrophenylglycine (DNP-glycine) crystallized from a mixture of cyclohexane and ethyl acetate. Identity was confirmed by comparison with an authentic sample.

Serine and valine. The appropriate fraction from the Dowex column was treated as above and the DNP-amino acid purified by the appropriate method (Campbell & Work, 1952).

Lysine. The lysine-rich fraction was transferred to a column of Dowex 50 previously equilibrated with 0.2M ammonium acetate pH 5.5 (Hirs, Moore & Stein, 1952). The tyrosine was eluted with 0.2M ammonium acetate pH 5.5 and the lysine with 0.4M buffer pH 5.7. Ammonium acetate was removed from the effluent by sublimation and the lysine, after conversion into DNP-lysine, was purified on silica as described by Porter (1950). The final product crystallized from a mixture of formic acid and water.

Measurement of radioactivity

Proteins. Proteins were assayed for radioactivity on 1 or 2 sq.cm. disks at infinite thickness as described previously (Campbell & Work, 1952). When counting ^{35}S , corrections were made for radioactive decay.

Glycine, serine, valine and lysine. Radioactivities were measured on suitably diluted DNP-amino acids using 1 sq.cm. disks at infinite thickness; counting was continued to an error of less than 5%. All results are expressed as counts/min./sq.cm. of pure amino acid, allowance being made for the different molecular weights of the DNP-amino acids. Each DNP-amino acid was estimated colorimetrically before dilution, as described previously (Campbell & Work, 1952), and, as a check on the accuracy of this method, larger samples of some of the crystallized DNP-amino acids were also weighed and diluted. For example, 427 and 285 μ g. samples of DNP-glycine from casein measured colorimetrically and diluted as described previously gave specific activities of 10200 and 11700, while a larger weighed sample (4.036 mg.) gave a value of 11400. Similarly, valine radioactivity from colorimetric estimation was 1080 and 945 and from a large sample (14.449 mg.) was 1008. All estimations were made in duplicate and in all cases duplicate values differed by less than 15%.

RESULTS

Milk composition

In earlier experiments (Campbell & Work, 1952) the assumption was made that milk composition was constant over the period of a single experiment. This assumption was checked with the goat by milking dry at 7.30 a.m., 10.40 a.m., 12.40 p.m. and 2.40 p.m. on a single day. Milk fat was removed from each sample which was then analysed for total N (Kjeldahl), non-casein N (after removal of casein at pH 4.5) and non-protein N (after removal of whey proteins with 10% trichloroacetic acid). The results given in Table 2 show that there is no

Table 2. *Effect of repeated milking on the composition of samples of goat milk*

Results are expressed as g. N/100 ml. milk.

Time of milking	Non-protein N	Non-casein N	Total N
7.30 a.m.	0.036	0.16	0.70
10.40 a.m.	0.039	0.15	0.71
12.40 p.m.	0.038	0.15	0.68
2.40 p.m.	0.036	0.16	0.70
4.40 p.m.	0.039	0.15	0.70

change in the distribution of milk nitrogen during the course of multiple milkings on a single day.

Proteins of goat milk

When examined in the electrophoresis apparatus, casein separated at pH 7.0 (sodium phosphate, $I=0.1$) into three components corresponding to α -, β - and γ -casein with an approximate ratio $\alpha:\beta$ of 1.8:1 and with only traces of the γ -component. The different whey-protein fractions resulting from the fractionation outlined in Table 1 were also examined in the electrophoresis apparatus using veronal buffer, pH 8.6, $I=0.1$. Fraction *C* was a complex mixture. Fraction *D*, the immune globulin fraction, seemed to vary in composition with different preparations. In some experiments only two components separated at pH 8.6 but in other preparations three components could be distinguished. The yield of this fraction was too small to permit further analyses.

At pH 8.6 fraction *B* showed mainly one component with an asymmetric boundary and about 10% of a faster moving component (similar to bovine lactalbumin). In cow's milk this ammonium sulphate fraction is β -lactoglobulin (Smith, 1946). Bain & Deutsch (1948) have shown that goat lactoglobulin, like bovine β -lactoglobulin (Li, 1946), forms 2 or 3 boundaries at pH values below its isoelectric point. Our goat lactoglobulin fractions were therefore examined electrophoretically in sodium acetate buffer at pH 5.1, $I=0.1$. In fraction *B* four boundaries separated at pH 5.1. The picture of fraction *F* (obtained by ethanol fractionation of *B*) showed a major component with a mobility of $+1.95 \times 10^{-5}$ cm.²/v/sec. This fraction corresponded to the goat β -lactoglobulin of Bain & Deutsch (1948) obtained from milk by ethanol fractionations.

Fraction *E* (Table 1) at pH 5.1 gave a pattern closely similar to fraction *F*. The two components

present had the same mobilities as those of fraction *F* and the major component represented about 85% of the total. The two fractions *E* and *F* were also compared in an analytical ultracentrifuge in sodium acetate buffer, $I=0.1$ at pH 5.1 (1.2% protein concentration). Both fractions formed a single homogeneous boundary with the same sedimentation constant (S_{20} about 3.0).

As an additional check on the identity of *E* and *F*, a rabbit was immunized against fraction *E* by nine intravenous injections of increasing amounts of protein (1–10 mg.) over a period of 5 weeks. Immune serum from this rabbit was put in a centre well cut in a suitable agar plate as described by Ouchterlony (1953); solutions of proteins *E* and *F* were allowed to diffuse towards the centre well from equidistant subsidiary wells and the patterns of antibody–antigen precipitation in the agar were compared. Both fractions gave similar precipitation patterns (Fig. 1). Two components in fraction *E* appeared identical with those in fraction *F* but were present in different proportions, and both fractions showed traces of a third component.

It could thus be concluded that fractions *E* and *F* contained the same two protein components. The mobility of the main component is identical with that of crystalline β -lactoglobulin (Askonas, 1954). The crystalline protein (*G*) was used for isolation of the radioactive DNP-amino acids. As a check on the reliability of the radioactivity determinations fraction *E* was also hydrolysed and used for isolation of radioactive DNP-amino acids.

Non-specific adsorption of radioactivity on milk proteins

It was important to know whether non-specific adsorption of radioactive amino acids on milk proteins could be a source of serious error in estimation of radioactivity. Accordingly, 0.02 mg. of [³⁵S]methionine containing 1 μ C of ³⁵S was added to 100 ml. of fresh goat milk, and after gentle stirring for 30 min. the casein and whey proteins were precipitated in the usual way. The casein contained 0.0163 μ C of ³⁵S and the whey protein 0.0023 μ C of ³⁵S, thus only 2% of the added [³⁵S]methionine had been precipitated with the proteins and it was clear that non-specific adsorption was not a serious source of error.

Incorporation of radioactive methionine into milk proteins of goat

The goat was given a single intravenous injection of 2 mg. [³⁵S]methionine (50 μ C) and milked at frequent intervals for 8 hr. The distribution of radioactivity in casein, whey protein and blood plasma protein is shown in Fig. 2. The different proteins varied slightly in nitrogen content but the radioactivities in Fig. 2 have all been adjusted to the

Fig. 1. Antibody–antigen precipitation pattern obtained in agar gel using method of Ouchterlony. Immune serum from a rabbit immunized against protein *E* was placed in cup *X*. A solution of protein *B* (10 mg./ml.) was put in cup *Y* and a solution of protein *E* (10 mg./ml.) in cup *Z*. The plate was covered and left at 2° for 4 weeks before taking photograph (retouched).

same nitrogen value. Further samples of milk were obtained at 24, 32 and 48 hr. The general form of this curve is closely similar to that obtained for valine and lysine in rabbit milk (Campbell & Work, 1952) where it was found also that whey protein was slightly less radioactive than casein. The efficiency of incorporation is extremely high, about 50 % of the [^{35}S]methionine appearing as protein- ^{35}S within 8 hr. of injection.

*Incorporation of radioactive glycine,
valine and lysine into milk proteins*

Owing to the varying amino acid composition of different proteins, direct comparison of radioactivities of whole proteins may be misleading. Although [^{35}S]methionine is the cheapest available radioactive amino acid, it was found to be unsuitable for more exact comparison owing to difficulty in isolation of pure methionine and to the relatively short half-life of ^{35}S . For exact comparison of the specific activities of amino acids in casein and in whey proteins we therefore used the same three amino acids as we had previously used in rabbits. In order to reduce the possibility of comparisons being invalidated by biological variability, all three amino acids were injected simultaneously immediately after milking and the animal was again milked dry 4 hr. later. The radioactive proteins were separated by the method already described (Table 1) and the distribution of radioactivity in these protein fractions was determined. As might be expected from our earlier results on the transfer of immune globulin from blood to milk

Table 3. *Specific radioactivities of pure amino acids isolated from hydrolysates of milk proteins obtained 4 hr. after intravenous injection of 4.9 mg. [^{14}C]glycine (250 μC), 17.3 mg. [^{14}C]valine (25 μC) and 75.7 mg. [^{14}C]lysine (40 μC)*

Amino acid	Radioactivity (counts/min./sq.cm. at infinite thickness)		
	Casein	Fraction <i>E</i>	Cryst. lactoglobulin, <i>G</i>
Glycine	11 100	10 450	9900
Lysine	3 170	3 050	3000
Valine	1 010	1 000	950
Serine	1 510	1 040	1050

(Askonas, Campbell, Humphrey & Work, 1954) the immune globulin fraction *D* was significantly less active than the other whey fractions *B*, *C* and *E*.

*Comparison of specific radioactivities of
amino acids in casein and in β -lactoglobulin*

Pure DNP derivatives of valine, lysine, glycine and serine were isolated from casein and from fractions *E* and *G*. The results of radioactivity determinations are shown in Table 3. In crystalline β -lactoglobulin *G*, valine, lysine and glycine had, within experimental error, the same radioactivity as in casein. The less pure β -lactoglobulin *E* was indistinguishable from crystalline β -lactoglobulin in this respect. In both proteins *E* and *G* the serine was of considerably lower specific activity than in casein.

DISCUSSION

In an earlier paper (Campbell & Work, 1952) it was shown that if two proteins are synthesized entirely from free amino acids and if the ratio between the rates of synthesis of two proteins is constant, then, after a single injection of radioactive amino acid, this amino acid would have the same specific activity in the two proteins. In our earlier experiments with rabbits it was not practicable to demonstrate the constancy of the above ratio but in the present series of experiments we have shown (Table 2) that there is no change in the ratio of casein to whey protein during the course of an experiment. We also assumed in earlier experiments that there was no selective reabsorption of milk proteins and that at the instant of injection the ratio: casein pool/whey-protein pool was proportional to the ratio: rate of casein synthesis/rate of whey-protein synthesis. The shape of the radioactivity curves for casein and for whey in Fig. 2 show that this assumption also was justified; if it were not, the two curves would cross.

In our rabbit experiments (Campbell & Work, 1952) a significant difference was found between the specific activity of amino acids isolated from casein

Fig. 2. The appearance of radioactivity in the milk proteins after injection of 2 mg. [^{35}S]methionine (50 μC) into a goat. $\triangle$ — $\triangle$, casein; $\square$ — $\square$, whey proteins; $\bullet$ — $\bullet$, blood plasma proteins.

and from whey protein. This was taken to show that the amino acids of milk protein were obtained from two sources, the main source being free amino acids of the blood stream and the secondary source bound amino acids, either in the form of peptides or proteins. We have recently shown that the immune globulin of milk does not equilibrate with injected radioactive amino acid and that some of the bound amino acid transferred from blood to milk is thus accounted for as immune globulin (Askonas, Campbell, Humphrey & Work, 1954). It seemed possible that other blood proteins might be transferred to the milk in the same way and that this could account entirely for the differences in specific radioactivity between casein and whey protein. From the present comparison between the radioactivity of amino acids isolated from casein and β -lactoglobulin (Table 3) (neglecting for the moment the serine value which was obtained after injection of [^{14}C]glycine and not of serine) it would seem that the nitrogen pool for the synthesis of casein and of lactoglobulin is identical and that this pool is in equilibrium with free circulating amino acids. According to this result it is unlikely that either protein derives much nitrogen from blood peptides or from partial hydrolysis and rearrangement of blood proteins. Our results are, however, not conclusive on this point. We could suppose that bound amino acid in the form of peptide was used to an equal extent in the synthesis of casein and β -lactoglobulin. This assumption is improbable, partly because of the very different amino acid composition of casein and whey proteins and partly because we have been unable to find evidence for the incorporation of bound amino acids into casein after partial hydrolysis and isolation of the amino acids from different peptide sequences thus obtained (Askonas, Campbell & Work, 1954).

One point in the present results requires further comment, the low value of the specific radioactivity of serine in proteins *E* and *G* (Table 3). Serine was not one of the injected radioactive amino acids but was produced very rapidly from injected [^{14}C]glycine. Since no significant difference was found between the casein and the β -lactoglobulin for the [^{14}C]amino acids injected, the difference between these and serine might be attributed to the nature of the process involved in the conversion of glycine into serine *in vivo*. It may be suggestive that the serine of casein is present partly as serine and partly as serine phosphate.

SUMMARY

1. A lactating goat was injected with radioactive glycine, valine, lysine and methionine and the appearance of radioactivity in the milk proteins was followed.

2. After injection of [^{35}S]methionine, both casein and whey proteins reached maximum activity in about 1.5 hr. The whey activity curve followed closely the casein curve but was always slightly below it.

3. After simultaneous injection of [$2\text{-}^{14}\text{C}$]glycine, DL-[$4\text{-}^{14}\text{C}$]valine and DL-[$1\text{-}^{14}\text{C}$]lysine, milk whey was fractionated and a crystalline β -lactoglobulin was isolated. This protein and a sample of casein from the same milk were hydrolysed and the radioactive dinitrophenyl amino acids isolated in a pure state.

4. There was no significant difference in radioactivity between glycine, valine and lysine from casein and the corresponding amino acids from lactoglobulin. Casein serine was more active than lactoglobulin serine. The significance of these results is discussed.

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