calculating heritability percentages gives an estimate of the proportion of the variance in a segregating population which is genetic. There are errors associated with this method, as no distinction is made between dominance and epistasis effects which are not very heritable and additive genetic effects which are highly heritable. The heritability values for protein and niacin were high, being much greater than those reported earlier for these chemical components. The fact that the parenteral values for protein and niacin content were very homogeneous may be responsible for the high heritability percentages. The heritability percentages for riboflavin were 0 and 52%, respectively, for the Mindo × Colo and Colo × C. I. 5298 crosses. The second value corresponds very closely with those previously reported (*4*).

Table V. Correlation Coefficients of Protein, Niacin, and Riboflavin in Three Oat Crosses

	Cross		
Correlation of	Mindo × Colo	Colo × C. I. 5298	C. I. 5298 × C. I. 3656
Niacin and protein	0.50[a]	0.38[a]	0.42[a]
Riboflavin and protein	0.10	0.21	..

[a] Significance at 1% level.

Association of Characters

The correlation coefficients between protein and niacin and riboflavin in the three oat crosses are given in Table V. As in previous reports by Frey and Watson (*5*) and Frey and others (*4*), the correlation coefficients between niacin and protein were significant in each cross, while those between riboflavin and protein were significant in neither cross. To plant breeders this would mean that selection for higher protein content in oat strains would be accompanied by increases in niacin content. Nevertheless the correlation coefficients of niacin and protein are of a low enough magnitude to make it possible, if desired, to obtain with diligent selection a low protein–high niacin strain of oats.

Discussion

One of the most interesting and practical aspects of the data reported herein and those reported previously is the dominance of high riboflavin and high niacin content demonstrated in oat crosses. It seems possible that oat varieties can be selected with a much higher niacin or riboflavin content than those now being grown. The procedure to be followed in breeding for improved niacin and riboflavin content is open to question. Probably the most effective system would be recurrent selection in which the high niacin strains would be selected and intercrossed. Later the grain of the progeny of the intercrosses would be analyzed, again high niacin strains would be selected, and the cycle repeated until the desired niacin content was obtained. The heritability values for niacin are encouraging, as they indicate that selection for this characteristic should be rather effective.

The dominance for high or low protein percentage seems to depend upon the genetic background of the particular oat cross. In two of the crosses the high protein percentage appears to be dominant; in the other cross low protein percentage seems to be dominant. This could present a problem, as it could not be predicted from these data whether high protein percentage would be recessive or dominant in an oat cross until the cross had been tried; further data are needed.

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RUMEN CHEMISTRY

In Vitro Studies with Rumen Microorganisms Using Carbon-14–Labeled Casein, Glutamic Acid, Leucine, and Carbonate

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THE MICROORGANISMS OF THE RUMEN have a profound effect on the carbohydrate of the diet. The cellulose of the herbivorous ration is attacked in the rumen, releasing large quantities of readily fermentable substances which, together with similar products in the food, are largely converted to volatile fatty acids. It has been estimated that these short-chain fatty acids provide about 70% of the cow's daily caloric intake (*6*).

Previous studies have indicated that the metabolic activity of microorganisms in the rumen is not restricted to cellulose and fermentable carbohydrates like glucose but also involves amino acids and protein of the ration. Degradation of protein in the feed was indicated by appearance of ammonia following the addition of casein to in vitro rumen preparations (*2, 13, 19*). This ammonia might have come from amide nitrogen which could be released without destruction of amino acids. However, El-Shazly found (*7*) that the increase of ammonia was correlated with the increase of branched-chain fatty acids, indicating that essential amino acids had been metabolized.

The present study was undertaken to trace the fate of protein and amino acids in the rumen of sheep, using carbon-

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Previous studies have indicated that metabolic activity of microorganisms in the rumen of cattle and sheep is not limited to cellulose and fermentable carbohydrates but also involves proteins and amino acids. This investigation was undertaken to trace further the fate of proteins and amino acids in the rumen of sheep using compounds labeled with carbon-14. In vitro studies with rumen microorganisms incubated with carbon-14–labeled casein, glutamic acid, leucine, and carbonate indicated that proteolytic enzymes are active in rumen fluid and that deaminases and transaminases are present for decomposition of amino acids. The fixation of carbon dioxide in both essential and nonessential amino acids was demonstrated.

14–labeled materials. Amino acids, casein, and bicarbonate, labeled with carbon-14, were incubated in separate trials with rumen preparations in vitro. At the termination of the experiment, the isotope distribution was determined for the microbial cells, volatile fatty acids, and carbonate. The authors' results have demonstrated that protein and amino acids were metabolized by rumen microorganisms, resulting in some oxidation to carbon dioxide and some conversion to volatile acids.

Methods

Mature wethers from the University of California herd were surgically provided with permanent rumen fistulas, which were kept closed by removable plastic disks and through which samples of rumen fluids could be withdrawn as needed. The fluid mass was strained through 30-denier nylon woven fabric to remove coarse herbage, then lightly centrifuged, and the supernatant suspension of organisms was poured off. Suitable aliquots of the suspension were immediately measured into a series of prepared reaction flasks containing nutrient media plus the substance being tested. Anaerobic conditions were maintained throughout the incubation period by allowing a slow stream of nitrogen gas to flow through each flask, which was fitted with a two-holed rubber stopper and delivery tubes, one of which passed below the surface of the reaction medium. The flasks, connected in series, were incubated for 48 hours at 39° C. At the end of the experiment, microbial action was terminated by the addition of 10 ml. of 20% sulfuric acid through one of the aeration openings. Nitrogen gas streaming was continued and carbon dioxide which was evolved was trapped in sodium hydroxide for later assay.

Volatile fatty acids produced were removed from the digests by a modified variation of McClendon's steam distillation apparatus (*11*). Distillation flasks were maintained in a glycerol bath at 103° to 105° C. After titration of the volatile fatty acids, the neutralized solution was evaporated to dryness for radioassay.

Results with Labeled Casein. Carbon-14–labeled casein was used to study the alteration of a protein in the rumen system. The casein had been isolated from a milk sample collected 3 hours following intravenous injection of butyrate 2-carbon-14 into a dairy cow (*5*). The distribution of the isotope in the casein had been determined by separation of the amino acids, after hydrolysis, on an ion exchange column (*4*). It was found (*3*) that 72% of the carbon-14 was in glutamic acid, 11% in aspartic, 7% in proline, 6% in serine, 3% in alanine, 1% in glycine, and 0.5% in arginine. The essential amino acids did not contain appreciable quantities of isotope.

Weighed amounts of this preparation of carbon-14 casein (0.5 to 1.0-gram samples) were introduced into the reaction flasks which had previously been charged with 100 mg. of glucose, 10 ml. of mineral solution, 25 ml. of freshly obtained rumen fluid, and 25 ml. of distilled water. The composition of the mineral solution is 26.25 grams of monobasic sodium phosphate, 26.25 grams of sodium bicarbonate, 3.75 grams of sodium chloride, 1.13 grams of magnesium chloride, and 0.13 gram of calcium chloride in 1000 ml. of distilled water. Incubation was carried out at 39° C. in an atmosphere of nitrogen. Zero-hour and 48-hour blanks were obtained with each digestion trial. All trials were run in duplicate. Microbial action was stopped at 48 hours as described above, and the volatile fatty acid production determined. The utilization of casein for microbial "protein" synthesis was determined by measuring radioactivity of the microbial cell fraction after high speed centrifugation and washing.

The total activity of the casein employed together with the specific activity of the three fractions recovered, volatile fatty acids, protein, and carbon dioxide is given in Table I. Fatty acids accounted for 21% of the recovered activity and 7% was present in carbon dioxide. The activity of the protein fraction was less than 0.5%. No attempt was made to separate microbial protein from any casein protein which may have remained with the centrifuged material to separate the nonvolatile organic acids, or to account for the remaining activity.

The results with the carbon-14–labeled casein gave a composite picture of the fate of several nonessential amino acids. To obtain information on alterations of individual amino acids, glutamate-1-carbon-14 and leucine-3-carbon-14 were used.

Results with Glutamate-1-Carbon-14. Weighed quantities (1 to 3 mg.) of the labeled glutamate were introduced into the reaction vessels charged with nutrient media and fresh rumen fluid as before. The medium in this case

Table I. Distribution of Radioisotope among Fractions Isolated after in Vitro Incubation with Labeled Casein

Fraction	Total Activity Introduced, μc.	Specific Activity, μc. C^{14}/Gr. at ° C			Activity Recovered, μc.	Recovery, %
		Flask 1	Flask 2	Av.		
Casein	0.270	...	...	...	...	...
V.F.A.[a]	...	(5.28)	(5.32)	5.30	0.056	20.74
Protein[b]	...	(1.08)	(1.20)	1.14	0.001	0.37
CO_2	...	(3.96)	(3.95)	3.96	0.019	7.03

[a] Volatile fatty acids.
[b] Microbial cells obtained after high speed centrifugation.

Table II. Distribution of Radioisotope among Fractions Isolated after in Vitro Incubation with Glutamate-1-C^{14}

Fraction	Total Activity Introduced, μc.	Specific Activity, μc. C^{14}/Gr. at ° C			Activity Recovered, μc.	Recovery, %
		Flask 1	Flask 2	Av.		
Glutamate-1-C^{14}	12	...	...	...	...	...
V.F.A.[a]	...	(219)	(236)	227	1.73	11.92
Protein[b]	...	(42)	(42)	42	0.90	7.50
CO_2	...	(552)	(594)	573	2.14	17.83

[a] Volatile fatty acids.
[b] Tungstic acid precipitate.

contained, in addition to the glucose and mineral solution, 100 mg. of urea and 500 mg. of soluble starch. All other conditions were the same as in the trials with casein. The distribution of radioisotope among the fractions isolated after the incubation period is given in Table II.

The protein fraction in this case was the tungstic acid precipitate as described by Hudman and Kunkel (*8*). The carbon-14 was recovered mainly in the carbon dioxide and volatile fatty acid fraction with a smaller though significant amount in the protein fraction. Nearly one fifth of the total activity was recovered as carbon dioxide, showing appreciable oxidation of the carboxyl carbon adjacent to the amino group. The pathway may involve a glutamic decarboxylase which has been reported to be present in rumen suspensions (*18*) or it may have originated following deamination of the glutamic acid.

The appearance of about 12% of the added carbon-14 in the volatile fatty acids is difficult to explain with glutamate labeled on the 1-carbon. Removal of this carbon atom would be expected preliminary to any further alteration of the five-carbon molecule. The authors cannot account for the transfer by decarboxylation and subsequent carbon dioxide fixation, as their results on carbon-14 dioxide fixation presented below show that very little carbon-14 dioxide was fixed into volatile fatty acids.

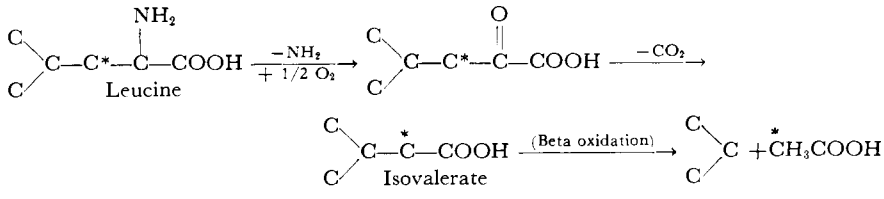
Results with Leucine-3-Carbon-14. The experiments with carbon-14–labeled leucine were carried out in the same way as with glutamate (Table III).

About 50% of the administered isotope was recovered in the volatile fatty acid fraction; another 10% was found in the cellular material, and almost none in the carbon dioxide fraction.

The low isotope level in the carbon dioxide fraction shows that the 3-carbon atom of leucine was not appreciably oxidized. Such a result indicates that the volatile fatty acids formed in the rumen are not oxidized to any significant extent.

These data show that, contrary to the findings of Sirotnak and others (*18*), there was active metabolism of the leucine in the rumen system. The degree of destruction of any amino acid in the rumen would depend on the nutritional environment and under other conditions might be less severe than that indicated by the data presented. Nevertheless the results demonstrate the presence of enzymes for degrading amino acids and, in the case of leucine-3-carbon-14, the main end products are volatile fatty acids.

From known pathways for leucine degradation, the following results might be expected:



Thus, the volatile fatty acid fraction may contain isovalerate-2-carbon-14, acetate-2-carbon-14, or even butyrate-2,4-carbon-14 by condensation of two acetate molecules.

Results with Carbon-14 Carbonate. There is considerable evidence that carbon dioxide is not an inert end product of metabolism but actually participates in several intermediary reactions, appearing as fixed carbon in many organic compounds (*1*, *9*, *10*, *12*, *14–17*). To increase the sensitivity of the study of the role of carbonate in rumen metabolism, a very large dose (222 microcuries), of carbon-14 dioxide was released into the system. When this quantity of isotope was used, even those compounds containing very little fixed carbon might be expected to be within the range of sensitivity of Geiger counting.

Heavy-walled 500-ml. filter flasks were used as reaction flasks for the study of carbon dioxide fixation. The flask was closed with a two-holed rubber stopper carrying a glass-stoppered separatory funnel and a gas-delivery glass tube extending almost to the bottom of the flask. On the short stem of the separatory funnel was suspended a small test tube in which the charge of radioactive barium carbonate ($BaC^{14}O_3$) was placed. The flask was charged with twice the usual amount of medium and rumen fluid, and then a slow stream of nitrogen was led into the delivery tube until an oxygen-free atmosphere was obtained. The flask was then closed and partially evacuated to approximately 550 mm. of mercury. Ten milliliters of 20% lactic acid were placed in the

Table III. Distribution of Radioisotope among Fractions Isolated after in Vitro Incubation with Leucine-3-C^{14}

Fraction	Total Activity Introduced, μc.	Specific Activity, μc. C^{14}/Gr. at ° C			Activity Recovered, μc.	Recovery, %
		Flask 1	Flask 2	Av.		
Leucine-3-C^{14}	16.56	...	...	...	...	...
V.F.A.[a]	...	(574)	(520)	547	8.38	50.60
Protein[b]	...	(130)	(131)	131	1.79	10.83
CO_2	...	(1.6)	(2.4)	2	0.01	0.06

[a] Volatile fatty acids.
[b] Microbial cells obtained after high speed centrifugation.

Table IV. Distribution of Radioisotope among Fractions Isolated after in Vitro Incubation with $NaHC^{14}O_3$

Fraction	Total Activity Introduced, μc.	Specific Activity, μc. C^{14}/Gr. at ° C			Activity Recovered, μc.	Recovery, %
		Flask 1	Flask 2	Av.		
$NaHC^{14}O_3$	222	...	...	...	...	...
V.F.A.[a]	...	(77)	(77)	77	1.23	0.55
Protein[b]	...	(2475)	(3125)	2800	21.14	9.52
CO_2	...	(3028)	(2818)	2923	5.89	2.65

[a] Volatile fatty acids.
[b] Microbial cells obtained after high speed centrifugation.

Table V. Specific Activities of Amino Acids Recovered from in Vitro and in Vivo Studies with Carbon-14 Bicarbonate

	μc. C^{14} per Gram at ° C	Rumen in Vitro	Cow in Vivo
Glutamic acid	1002	100	100
Aspartic acid	1490	150	280
Alanine	555	56	58
Glycine	635	64	45
Proline	118	12	10
Arginine	1364	136	33
Leucine-isoleucine	118	12	0
Lysine	820	82	0
Histidine	178	18	0.5
Valine	306	31	0
Methionine	680	68	2.5

Results are expressed in relation to glutamic acid as 100. In vitro work was with rumen microorganisms; in vivo work reflects primarily synthetic ability of tissues of cow, as the bicarbonate was injected intravenously.

separatory flask and allowed to drop slowly into the test tube containing the carbon-14. The reaction flask was then placed in a rocking machine in the incubator and slowly rocked to and fro for 48 hours.

At the end of this period, the reaction was stopped by addition of 10 ml. of 20% sulfuric acid through the dropping funnel. The carbon dioxide set free was recovered by connecting a gas-washing bottle containing sodium hydroxide to the side tube of the filter flask and outgassing the whole system with a stream of nitrogen led in through the gas delivery tube. The recovered carbonate was later converted to barium carbonate for carbon-14 assay.

Protein material in the digests was precipitated by adding 1 volume of 10% sodium tungstate and 1 volume of 0.67*N* sulfuric acid to 8 volumes of the digests. The protein precipitate was removed by centrifuging and washing several times with small volumes of tungstic acid solution prepared as above. Carbon-14 was determined after combustion in the usual manner. Total volatile fatty acids were quantitatively determined by steam distillation of the supernatant liquid and recovered for radioactive assay. The results of the carbon fixation trials are given in Table IV.

Only slightly more than 10% of the added isotope was recovered in the three fractions collected and most of that was in the cellular material. Only a fraction of 1% was recovered in the volatile fatty acids, demonstrating that they contain little carbon from carbon dioxide fixation. When these results are considered together with those obtained from glutamate-1-carbon-14, it is apparent that the relatively large transfer of isotope from glutamate to the volatile fatty acids could not have gone via carbon dioxide fixation.

The protein fraction which contained most of the recovered carbon-14 was hydrolyzed and the amino acids were separated on ion exchange columns, as described elsewhere (*4*). The specific activities of the amino acids are listed in Table V.

Most of the carbon from carbon dioxide fixation was in aspartic acid, arginine, and glutamic acid, but there was considerable labeling of all amino acids recovered. In order to compare the results with those obtained in vivo with cows, the specific activities are expressed relative to the specific activity of glutamic acid as 100. The contrast between the two groups of results is most apparent in the essential amino acids. While the cow transferred almost no carbon from carbonate to the essential amino acids, the rumen microflora labeled all amino acids. Lysine contained more carbon from carbon dioxide fixation than did alanine.

Summary

In vitro studies carried out with suspensions of rumen microoganisms, incubated together with carbon-14–labeled casein, glutamic-1-carbon-14 acid, leucine-3-carbon-14, and carbon-14 dioxide demonstrate that proteolytic enzymes are active in the rumen fluid and that deaminases or transaminases are present for the decomposition of the amino acids. The principal decomposition products detected were volatile fatty acids and carbon dioxide.

Carbon from carbon dioxide fixation was found principally in the cellular material and very little was detected in the volatile fatty acids.

The radioactivity assay of the amino acids recovered after the carbon dioxide fixation studies demonstrated that rumen microorganisms fix carbon from carbon dioxide in both essential and nonessential amino acids, while the tissues of the cow fix carbon only in the nonessential amino acids.

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