IN VITRO METABOLISM OF UNIFORMLY LABELED GLUCOSE-C^{14} BY BOVINE RUMEN MICROORGANISMS

by

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TABLE OF CONTENTS

FIGURES AND TABLES

INTRODUCTION

Fermentation in the rumen of cattle is of major significance to the nutrition and economy of man for its conversion of abundant cellulose and low quality nitrogen compounds to intermediates in the production of meat, milk, wool and leather. The fermentation is the result of complex interactions of an unknown number of kinds of microorganisms which can be modified by subtle changes of diet or rumen environment. Methods for the study of mixed culture fermentations are almost non-existant. The use of labeled tracers provides one of the most direct approaches to untangling the metabolic pathways through which products may pass in this dynamic system. To develop these methods, gravimetric procedures were employed in preliminary studies conducted in this laboratory by J. H. Newman (28). Newman used unlabeled glucose, casein and sodium acetate or sodium propionate to develop procedures for fractionation, drying and oxidation. In Newman's final studies, uniformly labeled glucose-C^{14} was fermented by rumen microbes in vitro for 1.5 hr. (Short incubation periods were used since it had been determined by Moore (25) that rumen fermentations become measurably abnormal in vitro both quantitatively and qualitatively beyond 1.5 hr). It was hoped that the use of radioactive glucose might help to locate the analytical losses of carbon, as well as demonstrate the metabolic

pathways of carbohydrate metabolism. The added glucose represented one-tenth of the normal rumen glucose level as measured by the notatin analysis (unpublished results of this laboratory). Both control and fermented samples were fractionated into major groups of compounds followed by wet oxidation of the carbon compounds to carbonate according to a modification of the method of Van Slyke et al. (34), and subsequent precipitation with $Ba(OH)_2$ to $BaCO_3$ for weighing and counting. The results of Newman's final studies, Trials 1 and 2, are summarized in Table 1. The "wash" fraction represented the aqueous supernatant from the second precipitation of the protein and particulate matter fraction; in later experiments this was combined with the first supernatant from the protein precipitation. The per cent carbon-14 recovery of the control subsamples compared favorably with that of the fermented subsamples plus gas, however, the low (77-88%) recovery indicated an analytical problem. Carbon-14 recovery was also low in the unfermented sugar fractions, although separation from the other fractions appeared to be complete. The labeled sugar in the fermented samples was apparently fully utilized during the 1.5 hr fermentation, since residual levels were comparable with the contamination levels found for the amino acids in the control samples. Although the method showed promise, lack of precision and low total carbon recoveries required

TABLE 1

(Summary of work by J. H. Newman)

Distribution of C^{14} in control and fermented fractions analyzed by wet combustion: Trials 1 and 2

Fraction	Trial 1[a] % Recovery C^{14} Aliquot 1	Trial 1[a] % Recovery C^{14} Aliquot 2	Trial 1[a] Gravimetric mgm Carbon Aliquot 1	Trial 1[a] Gravimetric mgm Carbon Aliquot 2	Trial 2[b] % Recovery C^{14} Aliquot 1	Trial 2[b] % Recovery C^{14} Aliquot 2	Trial 2[b] Gravimetric mgm Carbon Aliquot 1	Trial 2[b] Gravimetric mgm Carbon Aliquot 2
0 hr								
Subsample	80.82	88.16	330.75	344.25	76.98	77.38	346.5	419.4
"Protein"	3.99	3.12	105.75	169.05	3.01	3.71	106.5	94.8
Wash	10.43	9.16	17.55	15.93	6.50	8.52	11.3	11.4
Ether extract	0.21	0.38	99.90	245.00	0.83	0.65	104.9	246.3
"Amino acid"	0.36	3.67	11.90	8.58	4.05	2.73	6.6	6.4
"Sugar"	58.68	40.71	8.33	68.11	25.07	26.24	3.3	2.7
Total	73.67	57.04	243.43	506.67	39.46	41.85	232.6	361.6
1.5 hr								
Subsample	69.12	69.92	311.38	302.56	65.81	67.41	264.6	357.0
"Protein"	29.02	30.18	111.86	102.39	14.46	14.27	91.9	88.1
Wash	3.14	0.87	9.4	8.73	6.13	4.43	14.9	12.1
Ether extract	10.82	10.01	83.47	193.03	23.41	23.73	103.3	110.9
"Amino acid"	0.07	0.64	4.14	21.15	0.59	0.29	6.4	30.8
"Sugar"	0.43	0.78	6.39	44.65	1.11	2.47	3.0	4.5
CO_2	15.61		9.2		14.26		21.0	
CH_4	0.34		5.6		0.44		9.20	
Total	59.43	58.43	230.06	384.75	60.40	59.89	249.7	276.6

[a] Fistulated yearling Holstein steer, twin of animal used in Trial 2; sample taken 6 hr after first feeding on 2/18/58.

[b] Fistulated yearling Holstein steer, twin of animal used in Trial 1; sample taken 15 min after second feeding on 4/21/58.

correction before studies could be made to determine the many factors which control the rumen fermentation.

The present study was designed to increase the precision of Newman's analytical method and determine the distribution of C^{14} from fermented uniformly labeled glucose-C^{14} in various metabolic fractions of the major components, such as the individual fatty acids in the ether extractable fraction. Reduction of the analytical variation was essential so that statistical analysis of the fermentation process could be made. Comparison of the rumen fermentation products from various radioactive substrates, e.g. glucose, cellibiose and cellulose, between animals, between days within animals, between times within days, and between rations within and between animals, was contemplated. To this end, additional experiments were conducted using radioactive glucose and the wet oxidation procedure. Changes were made in the ether extraction procedure, the ion exchange column, and precipitation of the carbonate resulting from wet oxidation. The complete fractionation scheme and direct plating method, including an internal standard, which was developed in the Experimental Procedure is amenable to the study of the metabolism of labeled compounds which are important in rumen fermentation, since all of the principle classes of carbon compounds which occur in measurable quantities in the rumen are determined. Much

of the analytical variation was eliminated, however, liquid-liquid ether extraction and sample preparation of that fraction remains unreliable as a quantitative procedure. Newer methods using scintillation counting of unoxidized fractions in suitable solvents appear to be advisable.

REVIEW OF THE LITERATURE

The literature is replete with research reports concerning the diverse rumen organisms, the symbiotic relationship between these organisms contained within the rumen and their specific host, and the various facets of rumen fermentation. The rumen and its activities have aroused the curiosity and captured the fascination of scientists from the standpoint of both basic and applied research. It would seem that every conceivable aspect of the rumen and the activity of its specific microbes have been attacked, yet many questions remain unanswered. The many phases of ruminology are discussed in several excellent Reviews (5, 6, 9, 17, 19).

The complexity of the fermentation reactions and the dependence of the microbial metabolic pathways upon numerous environmental factors preclude most direct analyses of individual segments of the rumen process. The application of in vitro rumen fermentation (7, 10, 18, 21) and radioisotope tracer technique (2, 3, 4, 8, 11, 12, 14, 15, 20, 24, 29, 30, 31, 32) have offered the opportunity to determine rumen fermentation pathways carried out by the mixed flora and fauna under conditions like those which actually occur in the animal and upon which the animal is dependent.

It is well known that dietary carbohydrates are rapidly fermented to volatile fatty acids and gasses by rumen

microorganisms, and that these fatty acids provide a large portion of the ruminant's total energy requirement. Changes in the rumen environment and/or the host's diet are known to change the proportions of the volatile fatty acids produced, which result in changes in milk and animal fat, rate of gain, and productive performance, but the mechanisms of these changes are obscure.

Bovine rumen microorganisms obtained by supercentrifugation of rumen fluid from an alfalfa hay-fed steer were used for 30 and 48 hr in vitro fermentations by Hershberger et al. (16), to study the effect of low concentrations (1 to 2 mmoles) of glucose and metabolic intermediates on the cellulolytic activity of the microbes through the conversion of these compounds and cellulose to fatty acids. Alanine, α-ketoglutarate and lactate decreased the rate of cellulose digestion, whereas pyruvate, malate, succinate, aspartate, malonate, and glutamate had no effect on the rate of cellulose digestion. Approximately 40% of the cellulose carbon and 50% of the glucose carbon were recovered in acetic, propionic, and butyric and higher acids in the ratios 18:21:9 and 16:24:1 respectively. The remaining carbon was not accounted for. All of the metabolic intermediates tested affected the metabolism of glucose to fatty acids, and all, except alanine, were metabolized in part to fatty acids. Major metabolic pathways involved in

the conversion of glucose to fatty acids were affected by the added metabolic intermediates. The decarboxylation of succinic acid is the major pathway of propionic acid formation.

Leffel (24) studied volatile fatty acid production by washed rumen bacterial cells incubated with radioactive glucose and acetate. Radioactivity was determined in the volatile fatty acids produced, and results were reported in terms of the acetic, propionic, butyric, and higher fatty acid fraction as per cent of the activity recovered in the volatile fatty acids. It was demonstrated that different diets did affect the microbial population in such a way as to change the proportion of fatty acids produced in the washed cell suspensions prepared from the animals on each diet, and that washed cells did effectively convert acetate carboxyl carbon to butyrate. In the case of organisms obtained from animals on a high carbohydrate diet, acetate carboxyl carbon appeared in propionate and in higher fatty acid products. In the absence of a complete balance trial, differences in the magnitude of the total fatty acid production or other products of the substrates cannot be ascertained. Washed cell suspensions represent only a portion of the microbial population and cannot reflect the effect of rumen environment upon the individual species present. That individual rumen bacteria are sensitive to

environmental changes has been established with pure cultures by Lee and Moore (23).

Eusebio et al. (12) studied the dissimilation of uniformly labeled glucose-C^{14} in vitro using strained rumen fluid from Holstein heifers fed special diets. Strained rumen fluid and the 4 hr in vitro fermentation products were analyzed for volatile fatty acids at the end of each feeding period. The results demonstrated again that the proportions of volatile fatty acids change with changes in diet. A change from a roughage-concentrate diet to an all concentrate diet resulted in a change in the acetate:propionate ratio from almost 3:1 to about 1:1. The distribution ratio of C^{14} in acetate and propionate was similar to the acetate: propionate ratios found in vivo. In both cases, acetic and propionic acids accounted for 80-90% of the total volatile fatty acids. It was noted that diet affected certain changes in the proportions of butyric, valeric, and higher acids, and that there were some differences in the percentage of these acids between in vivo and in vitro experiments. No data were given to show what per cent of the added activity was recovered in the volatile fatty acids, or in what compounds the remaining C^{14}, if any, was found.

The metabolic fate of dietary uniformly labeled glucose-C^{14} in a lactating Holstein was studied by Otagaki et al. (29). Respired CO_2, samples of rumen and duodenal

contents, jugular and ruminal vein blood, and milk were analyzed for carbon-14. Maximum carbon-14 activity in respired CO_2 was reached by 45 min, in jugular and ruminal vein blood volatile fatty acids by 15 min, and in plasma glucose collected from the jugular and ruminal vein by 2 hr after the administration of glucose. The specific activity of the rumen volatile fatty acids was highest in the first sample taken 1 hr after the introduction of glucose (blood volatile fatty acid levels indicate maximum rumen volatile fatty acid activity had occurred much earlier), and no carbon-14 was detected in the rumen volatile fatty acids after 24 hr. Of the milk constituents, citric acid had the greatest specific activity followed by milk fat, lactose and casein. The ratio of specific activity of acetic, propionic, butyric and higher acids in the rumen volatile fatty acids was approximately 7.4:1.3:1.3. No appreciable amounts of C^{14} in the volatile fatty acids or glucose were found in duodenal contents. Most of the C^{14} which was found in the duodenal contents was in the fraction precipitated by alcohol and, although not identified chemically, was presumed to be primarily cellular protein. The per cent recovery of added C^{14} found in the volatile fatty acids or other constituents was not given. A comparison of these results with previous work in which glucose-C^{14} was injected intravenously into a lactating cow,

shows the maximum specific activity of respired CO_2 occurred about 10 min earlier and was one and one-half times greater. Lactose in the milk of this cow had the greatest specific activity and was about 40 times greater than the lactose recovered after glucose-C^{14} was added to the rumen; casein and citric acid also had greater specific activities and milk fat a lower specific activity than after addition of glucose-C^{14} to the rumen. The distribution of C^{14} in milk constituents after the intravenous injection of acetate-1-C^{14} and acetate-2-C^{14} more nearly resemble the results after addition of glucose-C^{14} to the rumen than after intravenous injection of glucose-C^{14}. The results demonstrate rapid conversion of glucose to short-chain fatty acids by rumen microbes, equally rapid absorption of the acids into the blood, and extensive metabolism of glucose-C^{14} before its utilization in the cow's tissues. The conclusion, that little, if any, glucose was absorbed directly from the gastro-intestinal tract is explained by the rapid conversion of glucose-C^{14} to fatty acids and rapid absorption of these acids with oxidation of some of the acids by the cow's tissues. This resulted in the early appearance of activity in respired CO_2. Lower specific activity of fatty acids from jugular plasma compared with fatty acids from ruminal vein plasma indicates additional fatty acid absorption by the liver. The conversion of fatty acids to glucose and

its release from the liver into the blood would account for the higher specific activity of glucose in jugular vein blood compared to ruminal vein blood.

Baldwin et al. (2) utilized strained rumen fluid from three cows, each fed a diet different in available carbohydrate for in vitro fermentations of 45 min duration with added glucose-1-C^{14}, glucose-2-C^{14}, or glucose-6-C^{14}. No attempt was made to account for all of the added radioactivity, or to identify the metabolic products containing labeled carbon except for that found in acetic and propionic acids. The results demonstrate that propionate is formed from the fermentation of glucose in the rumen via two pathways: propionate was labeled as though 70-100% was formed via the randomizing (succinate) route and 0-30% via the nonrandomizing (acrylate) route. As the available carbohydrate increased, the contribution of the acrylate pathway increased.

Bath and Head (3) obtained C^{14} labeled α-cellulose and hemicellulose from perennial rye grass grown in an atmosphere of $C^{14}O_2$. Separate fermentations of these carbohydrates were conducted in dialysis sacs bathed in physiological saline. Cheesecloth filtered bovine rumen fluid was added to the sacs initially. At 12 hr intervals samples were withdrawn from each sac for analysis (about 8% of the total sac volume) and replaced with synthetic rumen saliva and

food similar to the cow's from which the rumen fluid was obtained. After 36 hr cellulose or hemicellulose was added to the fermentation. The fermentations continued for an additional 48 hr after the addition of cellulose and 24 hr after the addition of hemicellulose. Samples were then taken and the volatile fatty acids analyzed for C^{14}. No data were given with respect to activity in the dialysate, fractions other than the fatty acids, or per cent recovery of total added activity. The results showed that acetic and propionic acids account for about 90% of the volatile fatty acids produced from cellulose and hemicellulose when fermented under these conditions. The fermentations produced acetate:propionate ratios of 1.7:1 for α-cellulose and 2.4:1 for hemicellulose. In each case some higher acids were produced, especially n-butyric and n-valeric acids. The authors conclude that their results and those reported by other workers show that the proportions of volatile fatty acids from any given carbohydrate substrate are largely dependent on the other constituents present in the diet.

In related studies, Gray et al. (15) used acetic acid-1-C^{14} and propionic acid-1-C^{14} in an in vitro fermentation carried on by rumen organisms from sheep on a hay diet. Labeled acetate carbon appeared in all the higher fatty acids, especially butyric, while labeled propionate

carbon was found in valeric but not in butyric acid. It was suggested that the higher fatty acids were synthesized by condensation of lower ones with a 2-C compound in equilibrium with acetic acid. In subsequent in vivo experiments with sheep, Gray et al. (14) found acetate-1-C^{14} was converted to butyric acid and to a lesser extent propionic acid. Propionate-1-C^{14} was partially converted to butyric acid and to a lesser extent acetic acid, while butyrate-1-C^{14} was partially converted to acetic and propionic acids. Sheppard et al. (32) reported similarly that acetate carbon-1 was partially converted to butyric and to lesser amounts of propionic and higher acid fractions. They estimated that acetate production in the rumen could account for 30% of the maintenance requirement of the sheep.

Otagaki et al. (30) reported in vitro studies with rumen microorganisms from strained sheep rumen ingesta using labeled casein-C^{14}, glutamic acid-1-C^{14}, leucine-3-C^{14}, and carbon-14 dioxide. Carbon distribution was determined among the steam distilled volatile fatty acids, the microbial cells after centrifugation, and the CO_2 after 48 hr incubations of rumen fluid diluted with buffer salts and nutrients. Very little radioactive carbon was found in the volatile fatty acid fraction after incubation with radioactive CO_2. Carbon from the CO_2 was fixed in both essential and nonessential amino acids. The principle

decomposition products from the metabolism of casein, glutamic acid, and leucine were volatile fatty acids and CO_2, indicating that proteolytic enzymes were present in rumen fluid, and that deaminases or transaminases were present for the decomposition of amino acids. Different ratios of activity were found in the products for each substrate. Total recovery of radioactivity ranged from 13% for the CO_2 trial to 61% for the leucine trial. No attempt was made to account for the remaining radioactivity or to identify the components of the fatty acid fraction. The authors postulated a pathway by which rumen microbes could degrade leucine to isovaleric acid. The active metabolism of leucine to volatile fatty acids (50.6% of the added C^{14}) was contrary to the results reported by Sirotnak et al. (33) with washed cells from the bovine rumen. Again, this indicates that in the absence of proper environmental conditions, including co-factors, the washed cell technique is inadequate. Hueter et al. (18) cautioned that the results of only one or two-step reactions were valid using the washed cell technique. Fulghum and Moore (13) demonstrated that a large number of proteolytic bacteria were present in the bovine rumen which were also capable of producing fatty acids from glucose, the kind and amount depending on the species.

Dehority et al. (8) obtained microflora by

supercentrifugation of steer rumen ingesta, which were then suspended in a phosphate buffer at pH 7.0, and supplied a basal medium to carry on in vitro fermentations of DL-valine-1-C^{14}, uniformly labeled L-proline-C^{14} and several metabolic intermediates of these amino acids, for periods of up to 30 hr. All of the added activity was not accounted for, and all C^{14} products were not identified. The results indicated that valine (the D-form was not metabolized) was converted to isobutyric acid and proline gave rise to valeric acid. The authors concluded that these amino acids, as well as the proposed intermediates in valine, proline, leucine, and isoleucine metabolism, which gave rise to these fatty acids accounted for the enhanced digestion of cellulose when these compounds were added to the basal medium. Certain of these amino acids have been shown to be required by cellulolytic rumen bacteria. The function of the fatty acids in increasing cellulose digestion is under study. In other experiments only 30% of added proline was converted to valeric acid at the end of a 24 hr fermentation, indicating that the conversion was not quantitative. It was also found that valeric acid increased cellulose digestion by a much greater extent than did proline. The basal medium contained valeric acid except when the amino acids or their metabolites were being tested. When the addition of valeric acid was delayed until the fermentation had proceeded for

12 hr, a marked decrease in cellulose digestion was observed. Apparently, cellulose digestion was limited in the early portion of the fermentation with proline because of insufficient "cellulolytic factor", presumably valeric acid or a metabolite thereof. This writer questions whether these amino acids would be metabolized via the proposed pathways when fatty acids are present in rumen concentrations.

Pazur et al. (31) reported the conversion of D-xylose-1-C^{14} and D-xylose-2-C^{14} into volatile organic acids by washed cell suspensions of rumen bacteria. Carbon dioxide production from xylose-1-C^{14}, xylose-2-C^{14}, pyruvate-1-C^{14}, pyruvate-3-C^{14}, lactate-1-C^{14}, succinate-1-C^{14} and the specific carbon labeling of acetic, propionic, and butyric acids were determined. The probable enzymatic routes of conversion of substrate carbon to fatty acid carbon were thereby indicated.

Jayasuriya and Hungate (20) investigated the fate of lactate-2-C^{14} in vitro and found that lactate in the hay-fed steer was not an important intermediate in the rumen fermentation and was not a precursor of the formed propionate. In grain-fed animals, lactate represented as much as one-sixth of the total converted substrate. The distribution of volatile fatty acids produced from lactate by both hay-fed and grain-fed steers, expressed as the

average recovery of the total volatile fatty acid activity, was acetic acid 81%, propionic acid 14%, and butyric acid 5%. Discrepancies between isotopic and manometric results of lactate fermentation apparently arose from the interaction of lactate intermediates with intermediates from substrates other than lactate. Bruno and Moore (4) used various labeled substrates to follow the formation and subsequent conversion of lactic acid to fermentation acids by bovine rumen ingesta in vitro. This work demonstrated that the distribution of fatty acids resulting from (a) glucose fermentation, shifted as fermentation time increased, and (b) the fermentation of commercial alfalfa meal, differed at various buffered pH levels after a 2 hr incubation. When uniformly labeled lactic acid-C^{14} was fermented at pH 6.3 (the pH at which rapid lactate metabolism occurs), 35% of the added activity was converted to acetic acid and 9% to propionic acid. Thirty-three per cent of the activity was recovered in the aqueous residue after ether extraction, with the remainder distributed in the fermentation gases and other fatty acids. Hence, at pH 6.3, carbohydrate converted to lactic acid as an intermediate was ultimately converted primarily to acetic acid, which agrees with the results of Jayasuriya and Hungate (20).

Allison et al. (1) found that ethanol was consistantly detected in rumen material from both cattle and sheep after

they had been fed large amounts of readily fermentable carbohydrate. This was especially pronounced in the rumen contents of sheep overfed with cracked wheat and suffering from acute indigestion. The authors also report, "Most samples of ruminal material from animals on hay rations contain very small quantities of ethanol or none at all." Moomaw and Hungate (26) reported that ethanol was produced by some common rumen bacteria in pure culture, but that ethanol did not accumulate in the rumen. Using manometric and tracer techniques, they showed that the rate of ethanol metabolism in the bovine rumen was slow and was not an important extracellular intermediate in the rumen of normal animals.

Although a variety of analytical methods have been used by different groups of workers, the results of experiments dealing with rumen metabolic pathways are in general agreement. Volatile fatty acids are the major end products of carbohydrate fermentation and supply the major source of energy for the host. The reports indicate much metabolic conversion of these volatile fatty acids occurs as a result of microbial activity. It is apparent that few of the rumen metabolic pathways, and the enzymes involved, are known. It is interesting to note, however, that none of the experiments reviewed attempted to account for the fermentation products of the test material among

each of the major groups of biologically important compounds.

EXPERIMENTAL PROCEDURE

Trials 3 and 4 were conducted with rumen fluid from a fistulated Holstein utilizing the fractionation scheme and wet oxidation procedure developed by Newman (28). In these experiments, however, the carbonate was precipitated with a mixture of $BaCl_2$ and $Ba(OH)_2$. This modification allowed a titrimetric and gravimetric determination of the amount of carbon in each fraction, as well as a count of the radioactivity.

Before Trial 4 was conducted, a check was made on the purity of the stock radioactive glucose solution. A sample of the solution was chromatographed by descending strip paper chromatography using a 2 hr equilibration period and overnight development in a 65% isopropyl alcohol - 25% water - 10% glacial acetic acid solvent system. When developed with para-amino hippuric acid reagent, the chromatogram revealed a single colored spot; when run through a strip Geiger counter, a single radioactive spot was observed coincident with the colored spot and corresponding to the R_F of glucose. As a result of the large variation between duplicate analyses and low recovery using the wet oxidation method in Trials 3 and 4, a new set of experiments was conducted using a similar fractionation scheme, but a 45 min fermentation and a direct sample plating procedure. This method was so much more convenient that two fermentations

and their controls could be run simultaneously. Contrary to preliminary experiments, total carbon was not determined; total added carbon-14 was accounted for, however.

The fistulated steers used in Trials 6 through 10 (Table 2) were confined to box stalls and fed a maintenance ration of good quality mixed grass hay and water ad libitum, and four pounds of 12% protein concentrate once a day at 8 AM. For each animal tested, rumen ingesta, from the middle of the rumen and about 8 inches below the surface of the ingesta, was removed and squeezed through two layers of cheesecloth to obtain approximately 1 liter of rumen fluid. Within 15 min of removing the ingesta from the rumen the experimental fermentation was started. No provision, other than the large sample size, was made for keeping the fluid at 39 C in transit. After a second filtration through two layers of cheesecloth and agitation to resuspend particulate matter, a 25 ml sample of rumen fluid was placed in a 60 ml polyethylene fermentation bottle (Figure 1) and gassed with synthetic rumen gas (65% CO_2, 34% CH_4, and 1% H_2). One ml of aqueous uniformly labeled glucose-C^{14} solution (approximately 28.5 million disintegrations per min per ml; 12.8 μc) was added to the bottle, which was then connected to a closed mercury manometer system and burette for the collection of gas. During incubation, the sample was agitated mechanically and maintained in a 39 C water bath.

TABLE 2

Experimental design

Trial	Day	Animal[a]	Time	Date	Hr after feeding
5 and 6	1	1 and 2	10 AM	4/7/60	2
7 and 8	2	1 and 2	10 AM	5/5/60	2
9 and 10	2	1 and 2	2 PM	5/5/60	6

[a] Animal 1: 3.5 year old Holstein steer; also used in Trials 2, 3, and 4. Animal 2: 1.5 year old Jersey steer.

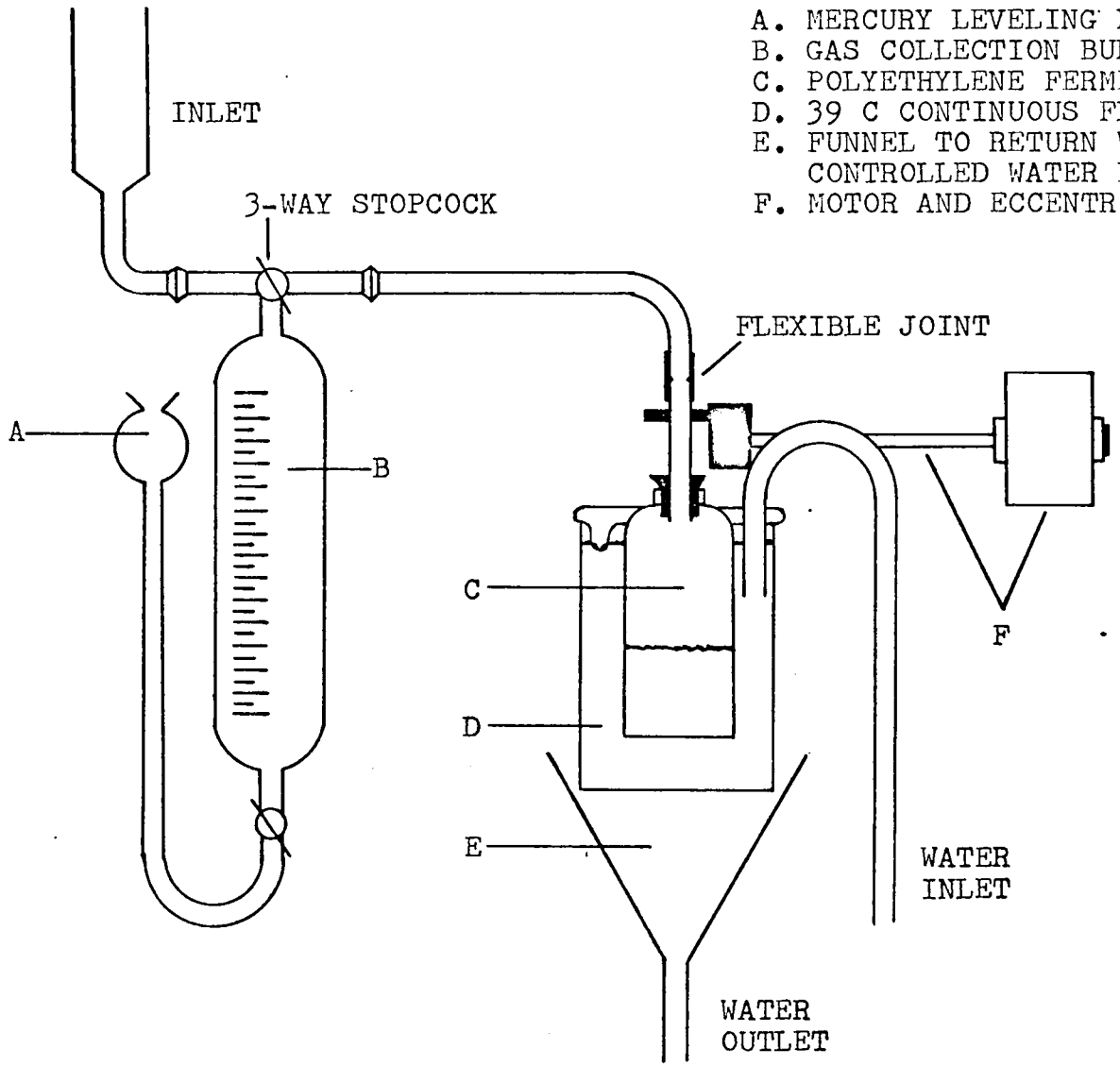

Fig. 1. Fermentation apparatus.

After 45 min, 0.5 ml of 18 N H_2SO_4 plus 2 drops of Nalco 71-D5 antifoam were rinsed into the fermentation bottle with distilled water through the three-way stopcock. The temperature of the water bath was reduced, and shaking was continued until the foam was broken. A partial vacuum was maintained during the acidification and defoaming period. The gas was extracted under negative pressure for two to three min and the bottle was then squeezed until the liquid contents reached the three-way stopcock where the gas was separated from the liquid. Distilled water was used to flush the capillary as the bottle was allowed to expand.

As soon as the fermentation was started, a second 25 ml rumen fluid sample (control) was acidified with 0.5 ml of 18 N H_2SO_4, and 1 ml of uniformly labeled glucose-C^{14} solution plus 2 drops of antifoam were added and mixed.

The volumes of the control sample and the fermented sample were determined after quantitative transfer to a graduated cylinder. Each sample was stirred to suspend the particulate matter and 2 ml of material from both the control and fermented samples were taken for analysis as subsamples. In Trials 9 and 10 one ml of 2 N NaOH was added to each subsample to hold the fatty acids. The remainder of each sample was stirred and divided into two measured aliquots, duplicates 1 and 2, and fractionated according

to the following scheme prior to determination of the radiocarbon in each fraction (Figures 2 and 3). Each duplicate was transferred to a 50 ml stainless steel centrifuge tube with the addition of 2 ml of 25% $ZnSO_4$ solution according to the method of Neish (27). The pH of the liquid was adjusted to 7.6-7.8 with 2 N NaOH using a pH meter. The tubes were stoppered and the precipitated protein and particulate matter separated from the liquid by centrifugation for 30 min at 20,000 x g in a refrigerated centrifuge. The supernatant was then decanted into the continuous liquid-liquid ether extraction apparatus, while the precipitate was resuspended in distilled water. Again, 2 ml of the $ZnSO_4$ solution was added and neutralized with NaOH and the precipitate separated by centrifugation. The second supernatant was pooled with the previous supernatant, acidified to pH 2 with 10 N H_2SO_4 and extracted with 100 ml anhydrous redistilled ether for 24 hr. The ether extract was trapped in 5 ml of 5 N NaOH in Trials 5 through 8. Visible "puddles" of water on the plated hygroscopic ether extract samples from Trials 7 and 8, coupled with low total recovery, suggested decreased counting efficiency was responsible for the "lost activity". In Trials 9 and 10, 2 N NaOH was used to trap the ether extract. After extraction, one ml of the NaOH from each of the fermented samples, but not the control samples, was pooled and used

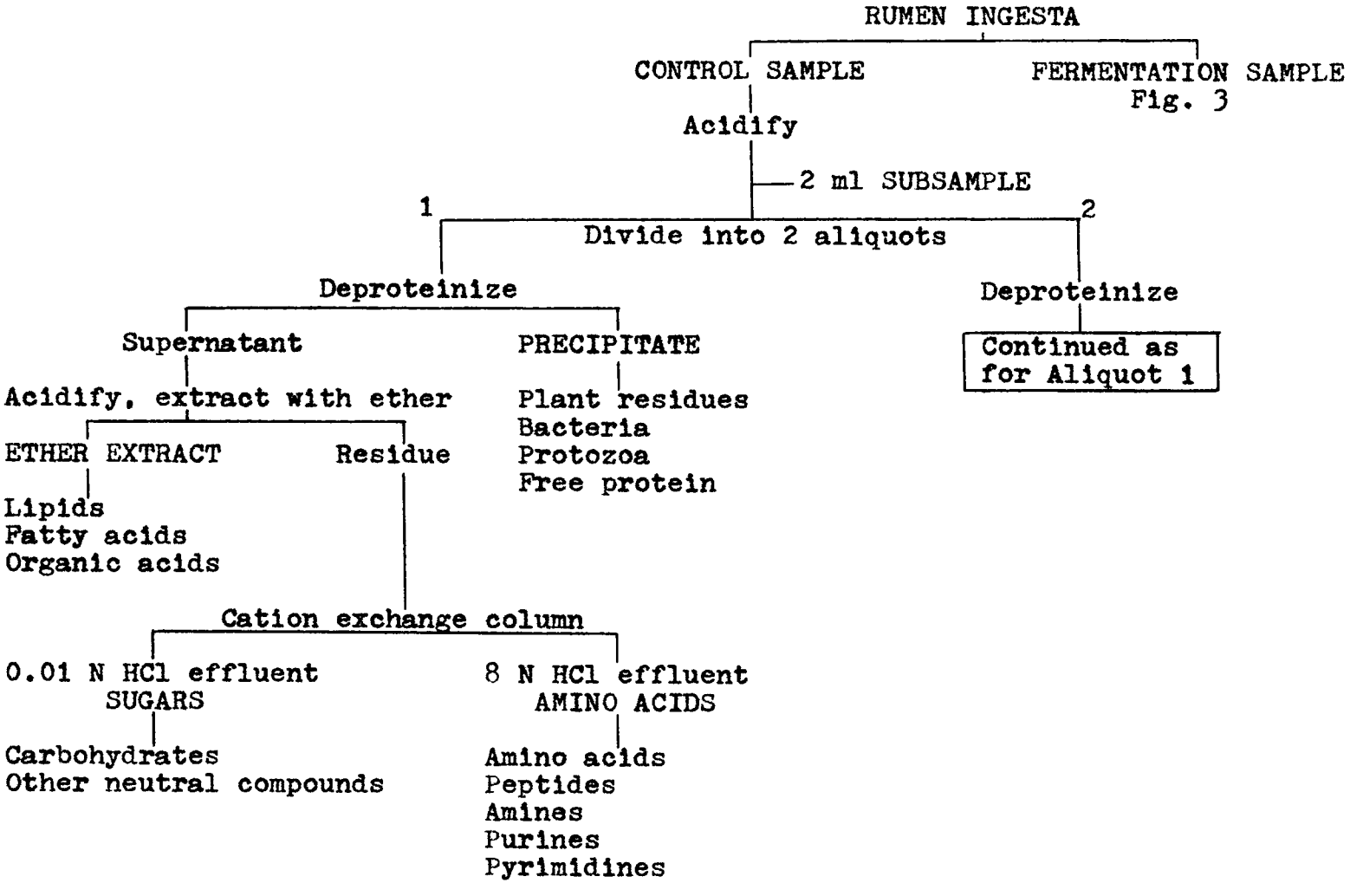

Fig. 2. Fractionation scheme for control rumen ingesta.

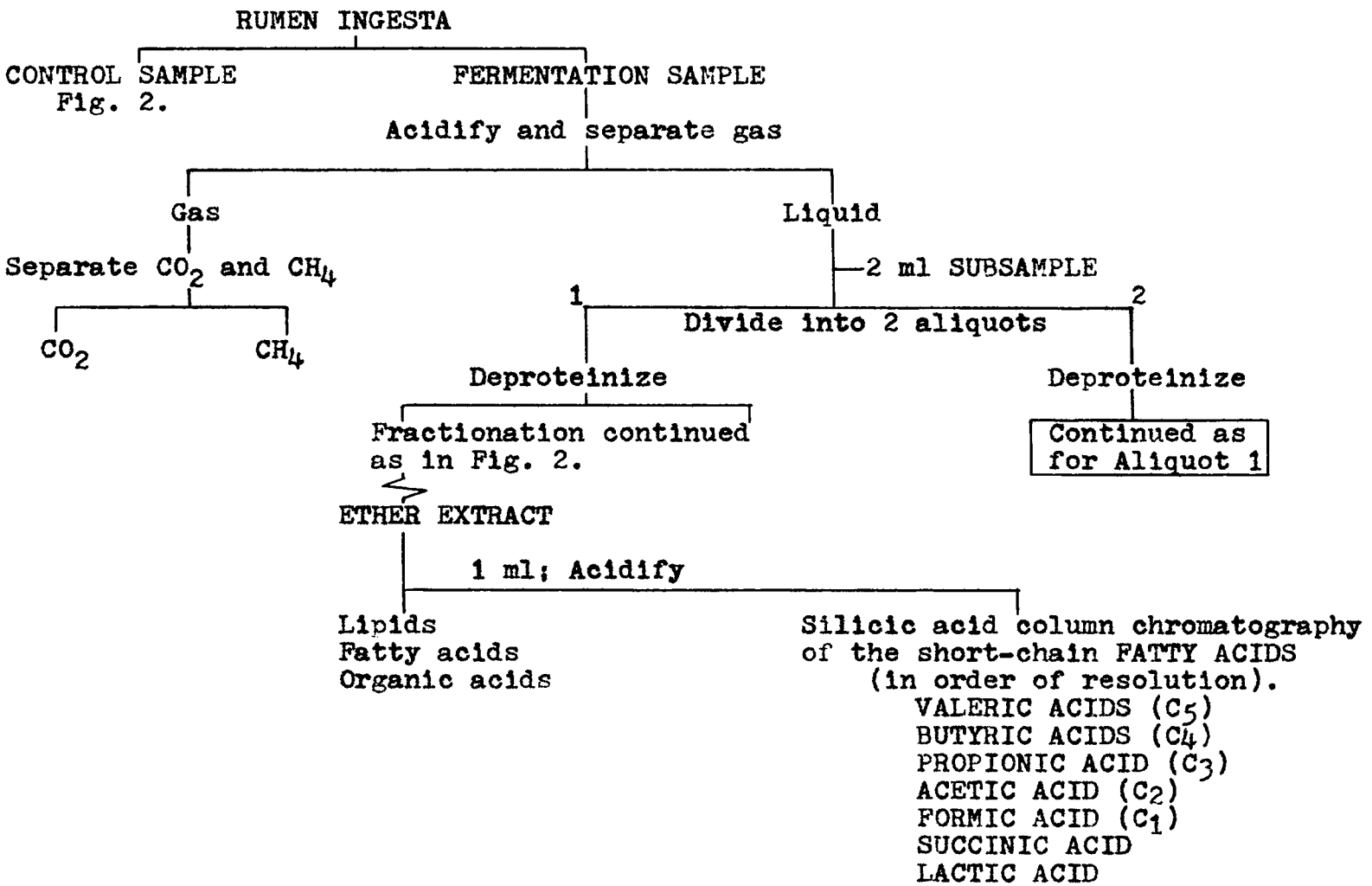

Fig. 3. Fractionation scheme for rumen ingesta after fermentation of uniformly labeled glucose-C^{14}.

for column chromatography of the short-chain fatty acids according to the method of Bruno and Moore (4). The fatty acids were transferred to 5 ml of 0.2 N NaOH in Trial 5, but to 5 ml of 2 N NaOH in Trials 8, 9, and 10, by evaporating the benzene-ether solvent away in a boiling water bath. The remainder of the NaOH was neutralized to pH 8 with 8 N HCl and counted as ether extract. The aqueous residue was neutralized to pH 7 with HCl and evaporated to approximately one ml. This material was adjusted to below pH 2 with 8 N HCl and quantitatively transferred to an ion exchange column (Figure 4). The column was prepared from Amberlite IR 120, Type 1 cation exchange resin. The sugars and uncharged molecules in the aqueous residue were eluted from the column with 40 ml of 0.01 N HCl. Elution of the amino acids and other charged molecules with 150 ml of 8 N HCl followed. The precipitate was suspended in distilled water and pH adjusted to approximately 8, then transferred to a graduated cylinder for measurement.

The fermentation gas was analyzed on a precision mine air analysis apparatus. The CO_2 was trapped in 190 ml 0.2 N NaOH and the CH_4 was oxidized over a hot platinum wire to CO_2 and trapped in a separate portion of NaOH.

Triplicate samples of 0.2 ml of each fraction were direct plated on stainless steel planchets (first cleaned

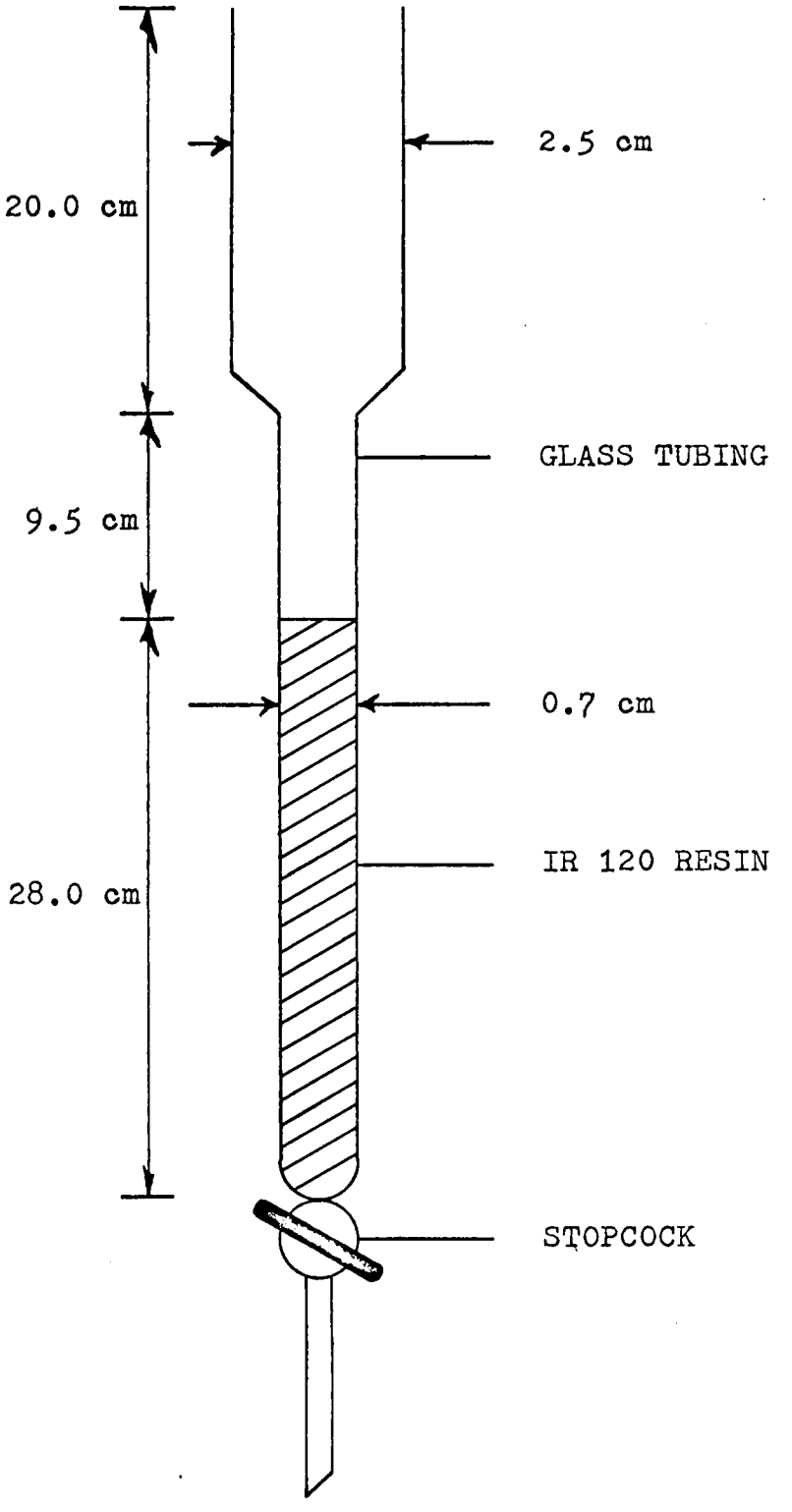

Fig. 4. Ion-exchange resin column.

with absolute ethanol and acetone) for counting in a Nuclear-Chicago D-47 thin window gas flow Geiger counter. The Q gas used in the counting system was a mixture of 98.7% helium and 1.3% isobutane (Nuclear-Chicago Corporation, Des Plaines, Illinois). Each sample was slowly applied with a micropipette while the planchet was rotating on a sample spinner, and dried slowly with a heat lamp. Those fractions which contained fatty acids or CO_2 were plated at a slightly alkaline pH, while the other fractions were plated at a slightly acid pH. The sugar fraction, for example, was made alkaline to brome phenol purple (purple) then slightly acid (yellow) and plated. To prevent the flow of the sample to the edge, the outer edge of each planchet was ringed with a thin layer of petroleum jelly. Two drops of 5% Sparkleen detergent solution were added to each sample to ensure uniform plating. Subsamples of each fraction containing an internal standard of 0.01 ml (0.128 μc) of a reference uniformly labeled glucose-C^{14} solution were also plated in triplicate to allow direct correction for geometry, self absorption, and efficiency. Because of the high amount of radioactivity in the 2 ml subsamples, they were neutralized to pH 8 and diluted with water to a calculated 50% rumen fluid before plating 0.2 ml. Dilution to 50% rumen fluid permitted slower, more accurate counting and uniformity among all of the subsamples. In Trials 9 and 10 the

subsamples were diluted to approximately 50% rumen fluid by the addition of 2 ml of 2 N NaOH to hold the fatty acids. The precipitate fraction was plated in the presence of 2 drops of a 1% solution of gelatin to stabilize the material on the planchet. Very alkaline samples, such as the ether extract and the fatty acids from the column chromatography, were found to be hygroscopic. To overcome the resulting counting absorption problem, all samples and their internal standards were desiccated over P_2O_5 overnight and exposed to the humidity of the counting room for at least one hr prior to counting. All samples were counted twice with a sufficient number of counts accumulated to ensure counting precision. The first count was made the day following plating. The amount of radioactivity to be found in each fraction, or subfraction in the case of the fatty acids, was determined through calculations outlined in the Appendix.

With the development of a direct plating procedure, experiments could be completed fast enough to allow an improved experimental design. Two samples were fermented simultaneously according to the schedule shown in Table 2. This design allowed an estimation of the variation between animals, between days within animals, and between times within days, which served to illustrate the practicability of the method.

RESULTS AND DISCUSSION

The results of Trials 3 and 4 in the present study are presented in Table 3, and are similar to those obtained by Newman (28) in Trials 1 and 2. Considerable difficulty was encountered in the oxidation of the ether extractable fraction and in determination of the titrimetric end points. The data in Table 1 and Table 3 illustrate the extreme variation in carbon recovery with this method. In every instance the recovery of radioactive carbon was low. The large variation in the ether extract and sugar fractions appeared to account for the major part of the variation in the over-all results.

The results of Trials 5 through 10 are summarized in Table 4. In general, the duplicate analyses for the control fractions agreed. The values for Trial 7 control ether extracts, indicated by footnote a, were higher than observed in any other trials. It was noted at the time of analysis that the aqueous phase in the liquid-liquid extraction apparatus contaminated the ether extract NaOH trap. Thus, the ether extract value was higher and the sugar and/or amino acid value correspondingly lower. The large error between sugar duplicates in Trial 5 and amino acid duplicates in Trial 9 could not be explained. Since labeled glucose was added to the rumen fluid after addition of H_2SO_4 and cessation of fermentation, all of the radioactive carbon

TABLE 3

Distribution of C^{14} in control and fermented fractions analyzed by wet combustion: Trials 3 and 4

Fraction	Trial 3[a] % Recovery C^{14} Aliquot 1	% Recovery C^{14} Aliquot 2	Gravimetric mgm Carbon Aliquot 1	Gravimetric mgm Carbon Aliquot 2	Titrimetric mgm Carbon Aliquot 1	Titrimetric mgm Carbon Aliquot 2
0 hr						
Subsample	93.75	85.94	227.71	337.41	206.16	338.44
"Protein"	2.38	1.53	68.61	80.46	72.80	87.91
Ether extract	15.93	-[b]	234.58	-[b]	305.16	-[b]
"Amino acid"	-[b]	0.56	-[b]	37.06	-[b]	-[c]
"Sugar"	18.89	11.13	315.35	255.72	212.66	149.06
Total	37.76	29.15	655.60	607.82	-	-
1.5 hr						
Subsample	64.38	67.90	253.24	282.08	262.01	310.28
"Protein"	19.67	12.54	141.62	115.70	146.89	111.63
Ether extract	51.22	40.04	379.41	171.21	382.84	151.35
"Amino acid"	0.18	0.23	9.29	15.58	-[c]	140.14[cd]
"Sugar"	1.27	3.15	6.20	4.01	0.84	2.33
CO_2	16.16		11.23		8.66	
CH_4	0.53		7.44		12.06	
Total	89.03	72.65	555.19	325.17	691.43	426.17

[a] Fistulated Holstein steer used in Trial 2; sample taken 5.5 hr after feeding on 4/22/59.

[b] Samples lost because of oxidation procedure.

[c] Poor titrimetric end points.

[d] Estimated.

TABLE 3 (Cont'd)

Trial 4[e]

Fraction	% Recovery C^{14} Aliquot 1	% Recovery C^{14} Aliquot 2	Gravimetric mgm Carbon Aliquot 1	Gravimetric mgm Carbon Aliquot 2	Titrimetric mgm Carbon Aliquot 1	Titrimetric mgm Carbon Aliquot 2
0 hr						
Subsample	88.97	93.28	256.20	258.29	421.35	310.43
"Protein"	5.20	7.91	156.16	161.82	161.47	174.08
Ether extract	48.45	40.24	169.54	169.05	265.18	270.58
"Amino acid"	1.11	0.27	8.07	12.73	-[f]	-[f]
"Sugar"	28.00	51.20	2.78	9.29	13.13	12.18
Total	82.76	99.62	336.55	352.89	439.78	456.84
1.5 hr						
Subsample	69.17	72.93	229.77	271.04	275.47	279.06
"Protein"	13.47	12.93	133.72	142.25	147.88	140.99
Ether extract	51.15	44.51	220.62	522.56	325.57	595.75
"Amino acid"	2.05	0.57	11.31	7.92	-[f]	-[f]
"Sugar"	1.08	2.30	2.87	6.86	5.88	7.27
CO_2[g]	-		-		-	
CH_4[g]	-		-		-	
Total	67.75	60.31	368.52	679.59	479.33	744.01

[e] Fistulated Holstein steer used in Trial 2; sample taken 5.5 hr after feeding on 7/7/59.

[f] Poor titrimetric end points.

[g] Gas sample lost prior to analysis.

TABLE 4

Distribution of C^{14} in control and fermented fractions analyzed by direct plating: Trials 5 through 10

Fraction	Trial 5 Animal 1, Day 1 10 AM % Recovery C^{14} Aliquot 1	Aliquot 2	Trial 6 Animal 2, Day 1 10 AM % Recovery C^{14} Aliquot 1	Aliquot 2	Trial 7 Animal 1, Day 2 10 AM % Recovery C^{14} Aliquot 1	Aliquot 2	Trial 8 Animal 2, Day 2 10 AM % Recovery C^{14} Aliquot 1	Aliquot 2
0 hr-Control								
Subsample	121.16		113.65		107.77		109.85	
Precipitate	9.24	5.81	6.98	9.03	4.02	6.46	5.50	7.18
Ether extract	1.53	1.71	2.93	1.55	9.81[a]	12.54	0.91	0.92
"Amino acid"	1.72	3.88	0.60	0.84	0.55	2.45	1.29	1.10
"Sugar"	77.05	20.72	74.68	51.30	87.59	84.06	88.67	87.74
Total	89.54	32.12	85.19	62.72	101.97	105.51	96.37	96.94
1.5 hr-Ferm.								
Subsample	33.50		54.38		84.44		80.44	
Precipitate	17.52	20.62	16.05	16.75	25.14	22.95	12.70	15.61
Ether extract	60.72	61.27 (25.26)[b]	61.97	60.47	35.38	40.12	48.28	51.03 (67.45)[b]
"Amino acid"	0.99	3.24	1.02	0.11	0.82	1.43	1.79	1.79
"Sugar"	3.51	3.53	3.48	4.60	5.47	5.18	6.35	4.72
CO_2	11.25		13.69		12.22		14.04	
CH_4	0.28		0.24		0.21		0.25	
Total	94.26 (58.80)[c]	100.19 (64.18)[c]	96.45	95.86	79.24	82.11	83.40 (102.58)[c]	87.43 (103.86)[c]

a, b, and c See text.

TABLE 4 (Cont'd)

Fraction	Trial 9 Animal 1, Day 2 2 PM % Recovery C^{14} Aliquot 1	Aliquot 2	Trial 10 Animal 2, Day 2 2 PM % Recovery C^{14} Aliquot 1	Aliquot 2
0 hr-Control				
Subsample	94.47		72.78	
Precipitate	6.03	4.07	2.17	1.24
Ether extract	2.31	1.77	3.03	3.90
"Amino acid"	9.93	0.79	0.42	1.55
"Sugar"	52.90	57.87	70.74	76.85
Total	71.17	64.50	76.36	83.54
1.5 hr-Ferm.				
Subsample	62.48		63.78	
Precipitate	14.33	15.29	14.50	16.91
Ether extract	30.00	3.45	6.09	33.64
	(15.82)[b]		(16.41)[b]	
"Amino acid"	0.36	0.36	0.35	0.39
"Sugar"	3.43	14.83	4.36	3.45
CO_2	16.11		17.04	
CH_4	0.14		0.28	
Total	64.36	50.18	42.61	71.71
	(50.18)[c]	(62.55)[c]	(52.93)[c]	(54.48)[c]

a, b, and c See text.

should have been recovered in the sugar fraction. However, incomplete fractionation apparently led to some contamination of each fraction, especially the precipitate fraction where 4-9% of the activity was recovered in 5 of the 6 trials. Although two washings were thought to be sufficient to remove such a soluble material, this contamination probably resulted from glucose being trapped within the precipitate and particulate material, due to incomplete washing and resuspension for the second centrifugation. This problem was recognized after Trials 5 and 6 and a separate check on sugar recovery from two ion-exchange resin columns was conducted. One-hundredth ml of glucose-C^{14} was placed on each column and eluted. Triplicate samples of the eluent from each column were plated in the acid condition while others were neutralized before plating. This test indicated that sugar was uniformly stripped from duplicate columns and there was no difference between the samples. Although internal standards were not prepared here to check quantitative recovery, samples of ion-exchange resin counted after elution of glucose showed no residual radioactivity. After Trials 7 and 8, a check was made to determine whether glucose was destroyed in alkaline solution. Two 5 ml solutions of 2 N NaOH plus 0.01 ml of radioactive glucose solution were prepared on day 1, and plated and counted at various intervals of days apart. Some variation occurred

due to absorption of water by NaOH, but no loss of glucose was indicated either in solution or on the planchet. The recovery of C^{14} in subsamples within and between Trials 6 through 9 was good, while recovery was too high in Trial 5 and too low in Trial 10. Duplicate control subsample analyses should have been made. The difference between average C^{14} recovery of the totals, excluding very low sugar recovery of Trial 5, Aliquot 2, and the recovery of the subsamples shows a range from -39.70% in Trial 6 to +7.17% in Trial 10. In general, however, there was agreement within each fraction for all trials, indicating that the fractionation scheme was basically sound.

The recoveries of C^{14} in fermentation sugar fractions were similar to those obtained in the 1.5 hr fermentations in the preliminary trials, indicating that glucose was rapidly and completely fermented within 45 min. High C^{14} recovery in the sugar fraction, Trial 9, Aliquot 2, appeared to be due to poor ether extraction.

The fermentation ether extract values, Table 4, footnote b, in Trials 5, 8, 9, and 10, were the per cent of total radioactivity added to the fermentation sample as determined by recovery from silicic acid column chromatography, and the corresponding change in total C^{14} recoveries (Table 4, footnote c) using these figures rather than the ether extract values determined by direct analysis.

Considerable variation existed in the C^{14} recovered from fermentation subsamples in each trial. Fermentation totals within each trial agreed except for the error created by very low ether extract recoveries obtained for Trial 9, Aliquot 2, and Trial 10, Aliquot 1. Although the ether extract value from the silicic acid column improved the total results in Trial 8, Trials 5, 9, and 10 were worsened. After Trial 8 the NaOH used to trap the ether extract was reduced from 5 N to 2 N. This was expected to reduce the "loss" of fatty acids by improving the counting characteristics of the samples. However, improved recovery of C^{14} in fatty acid fractions, hence total recoveries, in Trials 9 and 10 was not realized. A comparison between the per cent recovery of total radioactivity added in glucose and the per cent recovery of radioactivity in the averaged ether extract fraction data for Trials 8, 9, and 10, presented in Table 5, indicated that fatty acids were lost prior to total ether extract or chromatographic analysis. The similarity of data for Trials 9 and 10 (Tables 4 and 5) implied that reduction in normality of NaOH used to trap the ether extract reduced actual recovery of fatty acids from the ether extract much more than did the apparent loss due to counting efficiency. After Trial 5, the increase in normality of NaOH, from 0.2 to 2.0, used to trap fatty acids from the silicic acid column, resulted in increased per cent

TABLE 5

Distribution of C^{14} in fermentation ether extracts: Trials 5 through 10[a]

Acid fraction	Trial 5	Trial 8	Trial 9	Trial 10
C_5	0.25	2.88	0.30	0.22
C_4	11.32	9.48	6.32	4.03
C_3	7.86	2.60	7.21	8.39
C_2	79.17	51.50	85.44	86.27
C_1	0	0.47	0	0
Succinic	0.50	0.66	0	0
Lactic	0.90	3.41	0.72	1.10
Water bath	-	29.02	0	0
Total %	100.00	100.02	99.99	100.01
% recovery of total radioactivity added in glucose	25.26	67.45	15.82	16.41
% recovery of radioactivity in averaged ether extract fraction	41.54	134.20	94.59	82.60

[a] Ether extracts not chromatographed for Trials 6 and 7.

recovery of radioactivity in averaged ether extract fractions. Variation in ether extract and fatty acid analyses apparently accounted for the low total recoveries. Although total recoveries were not 100%, they were good in Trials 5 through 8, and consistant with subsample recoveries in Trials 7 through 10. The results of duplicate aliquot analyses for both control and fermented samples in Trials 7 and 8 were excellent.

The distribution of C^{14} in fatty acids of the ether extract fractions is recorded in Table 5. When the chromatographed fatty acids were transferred to NaOH by evaporation of the solvent layer, the appearance of phenolphthalein in the water bath was thought to indicate minor spillage on the outside of the containers. However, when water bath samples from Trial 8 were plated, 29% of the total fatty acid activity was recovered. No activity was detected in the water bath in Trials 9 and 10; this further illustrated the problem connected with the recovery of ether extracts and fatty acids. It appears possible that the 0.75 hr fermentation ether extract values were low because the fatty acids were not quantitatively absorbed by the alkali trap during the extraction period. Since the highest recovery of activity in the ether extract after chromatography occurred in Trial 8, the only trial where the extraction ether was stored over the alkali for one week

before it was removed by evaporation, and a large quantity of fatty acid activity moved from the chromatographic solvents after chromatography to the water bath during evaporation of these solvents, it appeared that the fatty acids were not effectively trapped from the solvents by the aqueous alkali and remained volatile. The errors of ether extraction were confirmed and removed in later work in this laboratory which omitted this procedure and chromatographed direct samples with excellent total recoveries (Bruno and Moore).

Overall, the data showed that uniformly labeled glucose-C^{14} was rapidly fermented with most of the radioactive carbon found in the ether extractable product as acetic acid (up to 86% in Trial 10), propionic acid (up to 8% in Trial 10) and butyric acid (up to 11% in Trial 5). These results are consistant with values reported by Bruno and Moore (4), Eusebio et al. (12), and Otagaki et al. (29). The observation that 51-86% of the glucose carbon in the ether extract product is in disagreement with the results obtained by Leffel et al. (24) in which only 30% of the activity was recovered in the fatty acid fractions. In those trials, no balance data were reported. It is possible that the differences in fermentation environment might account for the discrepancy between these data.

Thirteen to 25% of the glucose carbon was found in the

precipitate and 11-17% was found in CO_2. The small quantity of CH_4 and little or no appearance of formate is compatible with evidence that CH_4 is produced from the reduction of CO_2 by H_2 derived from formate dissimilation (19, 35). No more than contamination levels of C^{14} were found in the fermented amino acid or residual sugar fractions.

Although the data from these trials were variable, some general comparisons are suggested. The only major difference in the fermentation products between Animals 1 and 2 in Trials 5 and 6, 7 and 8, and 9 and 10, where the day and time of day were constant, occurred between Trials 7 and 8 where the C^{14} recovered in the precipitate fraction was 10% lower in Trial 8. The fatty acid distribution for Animals 1 and 2 could only be compared in Trials 9 and 10; no apparent differences occurred.

Where the time of sampling after feeding was constant, but the day varied, the C^{14} recovery for Animal 1, Trials 5 and 7, showed about 5% increase in the precipitate fraction, and 23% decrease in ether extract in Trial 7. Animal 2 showed almost the same fermentation product distribution on both days.

Where the time of sampling after feeding was varied within the same day, the C^{14} recovery for Animal 1, Trials 7 and 9, showed 4% increase in CO_2 production and 9% decrease in precipitate in Trial 9. These differences are

not significant, since total C^{14} recovery was about 23% lower than in Trial 7. Animal 2, Trials 8 and 10, showed 3% increase in CO_2 production. The values for the other fractions were comparable except for the marked decrease in ether extract in Trial 10, but again, this was probably due to low total C^{14} recovery. The fatty acid distribution for Trials 8 and 10 could not be compared because of the large "water bath" recovery and high recovery of added C^{14} in Trial 8.

The results of Trials 5 through 10 indicated that for Animals 1 and 2 there was little, or no, real difference in the distribution of products resulting from the fermentation of uniformly labeled glucose-C^{14} between animals, between days within animals, and between times within days. One possible exception may be the increased CO_2 production by each animal at the 2 PM sampling.

The extraction, preparation of the samples for counting, and counting of the samples from the ether extract fraction, and the preparation and counting of samples from the fatty acid fractions presented the greatest analytical problems. Complete recovery of the glucose in the sugar fraction of the control seemed less of a problem. Two possible approaches to correcting these faults were immediately evident. Greater uniformity would undoubtedly result from samples counted in an enclosure which provided a low,

constant relative humidity. The best humidity in which to count would have to be determined experimentally with samples from each fraction. Loss of the fatty acids and ether extractable material after fractionation might be reduced as done by Bruno and Moore, the ether extract step was omitted, or by the use of liquid scintillation counting where the sample is essentially sealed into the sample container (2, 29, 32). Improved results with all fractions might be achieved by using this counting technique. Certainly the use of liquid scintillation counting contains its own inherent problems with regard to sample preparation. The most pressing problems would probably be with the particulate matter in subsamples and precipitate fractions, and the ether extractable materials which are necessarily highly alkaline.

In spite of the problems, there is every reason to believe that the fractionation scheme and method of direct plating, or perhaps liquid scintillation counting, would be applicable to the study of many compounds and their fermentation pathways in rumen ingesta, and provide a much sought after method for the study of mixed cultures as they interact under their normal conditions for short periods of time.

SUMMARY

A procedure was developed for the quantitative separation of major fermentation products of uniformly labeled glucose-C^{14} produced by bovine rumen microorganisms in vitro. After 45 min, the fermentation mixture was fractionated into (a) one control subsample, and duplicate fractions of (b) solid matter "precipitate", (c) ether extract, (d) "amino acid", (e) "sugar", (f) CO_2, and (g) CH_4. Similar fractionation of an unfermented control sample was made. A portion of the fermentation ether extract was subjected to column chromatography to resolve (a) C_1, (b) C_2, (c) C_3, (d) C_4, and (e) C_5 fatty acids, (f) succinic, and (g) lactic acids. Each fraction was analyzed in triplicate for C^{14} by a direct plating technique. Corrections for geometry, self absorption, and efficiency were made by direct plating additional triplicate fraction subsamples, each containing a uniformly labeled glucose-C^{14} internal standard. The data were expressed as per cent recovery of added C^{14}. The results indicated that glucose was rapidly fermented with most of the C^{14} found in the ether extractable fraction as acetic acid. Significant levels of C^{14} were found in the "precipitate" fractions. The data were compatible with evidence that CH_4 was derived from CO_2. The results of 6 trials indicated that there was no significant difference in the distribution of products

resulting from the in vitro fermentation of uniformly labeled glucose-C^{14} between animals, between days within animals, or between times within days.

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APPENDIX I

The recovery of C^{14} from each fraction and the trial totals were calculated using the Model Formulae presented below. The coincidence loss for all calculations was determined by a scale furnished with the counter based on the manufacturer's guaranteed resolving time of 100 microseconds. The per cent recovery of internal standards gave a direct measure of the geometry, self absorption, and efficiency corrections. Depending upon the humidity and the amount of self absorption, the per cent recovery calculated for individual samples ranged from 3 to 38. The average per cent recovery for all fraction samples of a particular fraction type which were counted together, was used to calculate the disintegrations per min of the individual fraction samples. According to the fraction type, the average recoveries ranged from 5 to 25%.

Model Formulae

Abbreviations:

avg : average
bkg : background
coin: coincidence
c/m : counts per minute
d/m : disintegrations per minute
IS : internal standard
vol : volume

(1) Avg corrected c/m in the fraction sample:

Planchet #1 {count 1, c/m; count 2, c/m}
Planchet #2 {count 1, c/m; count 2, c/m}
Planchet #3 {count 1, c/m; count 2, c/m}
= avg c/m

avg c/m X coin % = coin count + avg c/m - bkg =

avg corrected c/m

(2) Avg corrected c/m in the fraction sample plus the IS:

Planchet #1 {count 1, c/m; count 2, c/m}
Planchet #2 {count 1, c/m; count 2, c/m}
Planchet #3 {count 1, c/m; count 2, c/m}
= avg c/m

avg c/m X coin % = coin count + avg c/m - bkg =

avg corrected c/m IS

(3) Actual count increase due to IS = (2) - (1)

(4) Theoretical increase due to IS = (d/m added as IS) =

$$\frac{0.2 \text{ ml (vol of fraction + IS mixture plated)}}{\text{vol of fraction sample + 0.01 ml glucose-C}^{14}\text{ standard}} \times 285{,}000 \text{ d/m (activity of 0.01 ml glucose-C}^{14}\text{)}$$

(5) % recovery for individual fraction sample =

$$\frac{\text{actual count increase due to IS (c/m)}}{\text{theoretical increase due to IS (d/m)}} \times 100 = \frac{(3)}{(4)} \times 100$$

(6) Avg % recovery for all fraction samples of a given fraction type counted together from paired experiments =

$$\sum \frac{\text{actual count increase due to IS (c/m)}}{\text{theoretical increase due to IS (d/m)}} \times 100 =$$

$$\sum (5)$$

(7) d/m in individual fraction sample =

$$\frac{\text{c/m for sample}}{\text{avg \% recovery}} = \frac{(1)}{(6)}$$

(8) Aliquot factor =

$$\frac{\text{aliquot vol}}{\text{total vol of fermentation or control fraction}}$$

(9) Aliquot fraction dilution and plating factor =

$$\frac{\text{0.2 ml (vol plated)}}{\text{diluted aliquot fraction vol (due to the addition of rinse water, alkali, etc.)}}$$

(10) Total d/m represented by the whole fraction in the original 25 ml fermentation or control vol =

$$\frac{\text{d/m in individual fraction sample}}{\text{aliquot factor X aliquot fraction dilution and plating factor}} = \frac{(7)}{(8) \times (9)}$$

(11) % recovery in whole fraction =

$$\frac{\text{total d/m in whole fraction}}{\text{total d/m added (28,500,000)}} \times 100 = \frac{(10)}{28,500,000} \times 100$$

(12) Trial grand total recovery =

$$\sum \text{individual fraction d/m} = \sum (10)$$

(13) Trial grand total % recovery =

$$\sum \text{fraction \% recoveries} = \sum (11)$$

Example

The following example shows the application of the Model Formulae to the calculation of the C^{14} recovery in the control sugar fraction, Trial 8, Aliquot 1:

(1) Avg corrected c/m in the fraction sample:

Planchet #1, 2 count avg: 9366 c/m
Planchet #2, 2 count avg: 9062 c/m
Planchet #3, 2 count avg: 9170 c/m
} avg = 9199 c/m

9199 c/m X 1.53% = 141 c/m + 9199 c/m - 26 c/m = 9314 c/m

(2) Avg corrected c/m in the fraction sample plus the IS:

Planchet #1, 2 count avg: 10449 c/m
Planchet #2, 2 count avg: 10704 c/m
Planchet #3, 2 count avg: 10704 c/m
} avg = 10619 c/m

10619 c/m X 1.78% = 189 c/m + 10619 c/m - 26 c/m =

10782 c/m

(3) Actual count increase due to IS =

10782 c/m - 9314 c/m = 1468 c/m

(4) Theoretical increase due to IS =

$$\frac{0.2 \text{ ml}}{10 \text{ ml} + 0.01 \text{ ml}} \times 285{,}000 \text{ d/m} = 5694 \text{ d/m}$$

(5) % recovery for individual fraction sample =

$$\frac{1468 \text{ c/m}}{5694 \text{ d/m}} \times 100 = 25.78\%$$

(6) Avg % recovery for all fraction samples of a given fraction type counted together from paired experiments =

24.05%

(7) d/m in individual fraction sample =

$$\frac{9314 \text{ c/m}}{0.2405} = 38728 \text{ d/m}$$

(8) Aliquot factor =

$$\frac{12.6 \text{ ml (vol of Aliquot 1)}}{27.0 \text{ ml (total vol of Trial 8, control)}} = 0.46667$$

(9) Aliquot fraction dilution and plating factor =

$$\frac{0.2 \text{ ml}}{60.9 \text{ ml (total vol eluted from ion exchange column + rinse)}} = 0.00328$$

(10) Total d/m in the sugar portion of the total vol of Trial 8, control =

$$\frac{38728 \text{ d/m}}{0.46667 \times 0.00328} = 25,270,630 \text{ d/m}$$

(11) % recovery of C^{14} in the sugar fraction =

$$\frac{25,270,630 \text{ d/m}}{28,500,000 \text{ d/m}} \times 100 = 88.67\%$$

These formulae were applied to all fractions and fraction samples in each trial. The subsample, although not divided into aliquots, required the use of the "aliquot" factor since 2 ml were removed from the total fermentation or control volume for analysis. The correction for the dilution of the subsample to 50% rumen fluid is made with the aliquot fraction dilution and plating factor. The gas fraction was not divided into aliquots either, but

required the "aliquot" factor because only 10 ml of the 190 ml of NaOH used to trap the CO_2, or 10 ml of the 190 ml of NaOH used to trap the CO_2 produced from CH_4 oxidation, was neutralized for plating 0.2 ml counting samples. Because of the large volume of eluent containing the amino acids, only a portion of the eluent was neutralized for plating 0.2 ml samples for counting, hence a second "aliquot" factor calculation was made for this fraction.

ABSTRACT

IN VITRO METABOLISM OF UNIFORMLY LABELED GLUCOSE-C^{14} BY BOVINE RUMEN MICROORGANISMS

by

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Twenty-five ml samples of filtered steer rumen ingesta were incubated at 39 C with uniformly labeled glucose-C^{14} solution for 45 minutes in a closed system. After the fermentation was stopped, the fermentation mixture was fractionated into (a) one 2 ml subsample, and duplicate frcations of (b) solid matter "precipitate", (c) ether extract, (d) "amino acid", (e) "sugar", (f) CO_2, and (g) CH_4. Similar fractionation of an unfermented control sample was made. A portion of the fermentation ether extract was subjected to column chromatography to resolve (a) C_1, (b) C_2, (c) C_3, (d) C_4, and (e) C_5 fatty acids, (f) succinic, and (g) lactic acids on a silicic acid column using a benzene-ether integrated solvent system. Each fraction was analyzed in triplicate for C^{14} by direct plating of 0.2 ml fractions on stainless steel planchets for counting with a thin window gas flow Geiger counter. Corrections for geometry, self absorption, and efficiency were made by direct plating additional triplicate 0.2 ml fraction subsamples, each containing a uniformly labeled glucose-C^{14} internal standard. Unlike work reported by other laboratories, an attempt was made to account for all of the added C^{14} and the data were expressed as per cent recovery of total added C^{14}. The results indicated that glucose was rapidly fermented with most of the C^{14} found in the ether extractable fraction; up to 86% of this was found in the acetic acid portion.

These results support those of other workers. Thirteen to 25% of the glucose carbon was found in the "precipitate" and 11-17% was found in the CO_2. The small quantity of CH_4 was compatible with evidence that CH_4 is derived from CO_2. No more than contamination levels of C^{14} were found in the fermented "amino acid" or "sugar" fractions. The results of 6 trials indicated that there was no significant difference in the distribution of products resulting from the fermentation of uniformly labeled glucose-C^{14} between animals, between days within animals, or between times within days.