Fate of Carbon Passing Through the Glucose Pool of Rumen Digesta

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Received for publication 13 July 1971

The metabolism of the free glucose pool in rumen digesta from sheep fed roughage rations was studied by adding an insignificant quantity of glucose as uniformly labeled 14C-glucose of high specific activity to in vitro incubation systems. In all experiments wherein only trace quantities of glucose were added to digesta, most of the 14C-glucose entered acetate. This was true whether label was presented either as a single dose or by continuous addition over a period of 2 hr. Digesta collected at all times after feeding either once daily or at hourly intervals gave similar glucose dissimilation patterns. If, however, a relatively large quantity of carrier glucose was added together with the tracer, the 14C-acetate:14C-propionate ratio was reduced by a factor of about 10. Physical removal of most of the protozoa from digesta generally had little effect on the dissimilation of 14C-glucose added in tracer amounts, but in one experiment there was a decreased turnover of the free glucose pool and a marked reduction in 14C entering butyrate. The paucity of 14C entering propionate when only trace amounts of glucose were added to digesta suggests that this acid was largely formed from substrates whose carbon did not equilibrate with that in free glucose or with that in intermediates of free glucose metabolism.

A number of studies have been published concerning the utilization of soluble carbohydrates by the mixed populations of organisms from the rumen (1, 2, 5, 11–16, 20). Without exception, however, massive amounts of carbohydrate were added to the system in comparison to the size of the carbohydrate pool which normally occurs within the rumen. Since, under these conditions, the fermentations become abnormal in that, for instance, lactic acid accumulation frequently occurs, it seems that the results of previous work may not reflect the fate of carbon arising from the degradation of complex plant polysaccharides and passing through the free carbohydrate pools within the rumen of normally fed animals. This report concerns the utilization of carbon passing through the free glucose pool in rumen contents from hay-fed sheep.

MATERIALS AND METHODS

Bacteriological. Incubations were done in vitro with samples of rumen digesta obtained from a mature sheep fed a daily ration of 750 g of wheaten hay chaff plus 250 g of lucerne hay chaff given as a single feed. In one experiment, digesta samples were obtained from a sheep fed the same ration, but provided to the animal in equal portions hourly throughout the day. Water was freely available at all times. Digesta sampling was as described by Walker and Forrest (18), and about 10 times the amount required for in vitro incubation was withdrawn from the rumen. This was thoroughly mixed and subsampled, and the excess was returned to the rumen.

To 15 g of digesta was added 1 μCi of glucose-U-14C (300 μCi/μmole) in 1 ml of solution. The air space above the reaction mixture was briefly flushed out with nitrogen, the vessel was sealed with a rubber stopper, and incubation was carried out at 39 C for 1 hr except where otherwise stated. Solids were removed from the digesta by squeezing through terylene voile, and 5 ml of the liquid was centrifuged at 20,000 × g for 10 min. The clear supernatant fluid was retained for determination of 14C in volatile fatty acids (VFA) and glucose and for determination of total and individual VFA. The packed cells were resuspended in 5 ml of water and 1 ml of 2 N H2SO4 was added. After centrifuging, the cell debris was washed twice with 0.5 N H2SO4 and finally suspended in 5 ml of water for determination of 14C in cell constituents and of deoxyribonucleic acid (DNA) concentration.

Chemical determinations. DNA was determined on duplicate 1-ml samples of cell suspension by the method of Burton (3). Concentrations of individual VFA species in rumen fluid were determined after separation of the acids on a column of Dowex AG50W × 12 (200 to 400 mesh, H form) by elution with water (6) and titration of the effluent with a Radiometer automatic titrator.

Glucose was determined by using glucose oxidase (Glucostat, Worthington Biochemical Corp.).
Radiochemical. All radioactivity determinations were done by liquid scintillation counting with a Triton-diphenyl oxazole-p-bis-[2-(5-phenyloxazolyl)] benzene phosphor (10). Uniformly labeled $^{14}$C-toluene was used as internal standard.

Glucose-$U^{-14}$C of high specific activity was obtained from the Radiochemical Centre, Amersham, England.

Glucose and VFA in 1 ml of rumen fluid were separated on ion-exchange resin as described above, collecting 1.3- to 1.4-ml fractions. A 1-ml portion of each fraction was used for determination of radioactivity.

$^{14}$C in cell debris was determined by mixing 1 ml of the washed debris suspension with 5 ml of Triton-phosphor. Virtually all of the solid material in the suspension was solubilized by the phosphor.

$^{14}$C in protein and polysaccharide was determined after hydrolysis of cells from 2 ml of debris suspension in 0.4 ml of 30% KOH for 3 hr at 100°C. After cooling, 0.4 ml water and 2.4 ml of absolute ethanol were added to precipitate polysaccharide which was removed by centrifuging. The precipitated polysaccharide was washed three times with 70% ethanol, dissolved in hot water, and quantitatively transferred to counting vials for $^{14}$C determination. A sample of the supernatant fluid remaining after precipitation of polysaccharide was neutralized with perchloric acid and allowed to stand for several days at 4°C before before removing the precipitated KClO$_4$ by centrifuging. By using the cysteine-sulfuric acid method of Dische (4), it was found that the amino acid-containing supernatant fluid contained carbohydrate. Therefore, the neutral supernatant fluid was placed on a column of Amberlite CG 120 exchange resin (Na$^+$ form, 350 to 400 mesh) measuring 10 cm long by 0.5 cm diameter and equilibrated with 0.2 N sodium citrate buffer (pH 2.0). The columns were then washed with three bed volumes of citrate buffer (pH 2.0) and the washings were dried, redissolved in 1 ml of water, and counted. Qualitative tests with ninhydrin showed a virtual absence of amino acids in this fraction and the radioactivity present was included with that for the precipitated polysaccharide. The amino acids were eluted from the ion-exchange resin with three bed volumes of 2 N NH$_4$OH and the eluates were dried, redissolved in 1 ml of water, neutralized with 0.4 N H$_2$SO$_4$, and counted. Radioactivity in this fraction was assumed to have arisen from cell protein.

RESULTS

Effect of glucose concentration on distribution of glucose carbon in VFA. Since preliminary results with $^{14}$C-glucose added in amounts such that the glucose added did not materially affect the size of the glucose pool had shown that most of the label recovered in VFA was present in acetate, whereas previous reports (2, 11, 20) indicated substantial conversion to propionate, the effect of normal and high glucose concentration was studied. Digesta (15 g) were incubated for 1 hr with 1 $\mu$Ci (0.6 $\mu$g) of glucose-$U^{-14}$C and with 1 $\mu$Ci of glucose-$U^{-14}$C plus 20 mg of $^{13}$C-glucose. The distribution of radioactivity between the individual VFA species is shown in Table 1.

In the presence of carrier glucose, the recovered $^{14}$C in acetate and propionate was 60.2 and 24.9% of label in the VFA, whereas in the absence of carrier the corresponding values were 94.6 and 3.9%.

Diurnal variation in distribution of glucose carbon in VFA and cell material. Walker and Nader (19) have shown considerable variation in intraruminal metabolism of energy-yielding substrates during the day in sheep fed once daily. Such variations might be expected to be mirrored in the metabolism of carbon passing through the glucose pool. Accordingly, digesta samples were removed at intervals of 2 hr from a sheep fed once daily and allowed 4 hr to consume its ration. In all incubations, glucose-$U^{-14}$C was added without carrier. Figure 1 shows the distribution of recovered label between the VFA, CO$_2$ (methane), and cell material. CO$_2$ (methane) was calculated as being half of the total label entering acetic and butyric acids since both must arise by decarboxylation of pyruvate formed from the uniformly labeled glucose pool. Mean recovery of label was 87.5% ± 6.4%, which was considered adequate since no attempt was made to assess radioactivity associated with the organisms left with the solids in the digesta after removal of the fluid. The proportion of $^{14}$C appearing in propionate rose, and that in acetate fell during feeding but readjusted rapidly after cessation of feeding. At all times, by far the largest portion of the $^{14}$C derived from the glucose pool appeared in acetate. The proportion of label appearing in butyrate did not alter appreciably. The molar proportions of VFA in the rumen contents used were 68:19:13 and 69:20:11 for acetate, propionate, butyrate at 2 and 20 hr after feeding. The labeling of cell material rose proportionately.

| TABLE 1. Effect of glucose concentration on distribution of glucose carbon in individual VFA |
|-----------------------------------------------|-----------------|
| Carbon source | Dpm recovered with various amounts of glucose added per 15 g of digesta |
|----------------|-----------------------------------------------|
|                | 20 mg$^a$ | 0.6 $\mu$g$^a$ |
| Glucose       | 645,400  | 14,600       |
| Acetate       | 191,600  | 1,034,100    |
| Propionate    | 79,200   | 42,500       |
| Butyrate      | 47,600   | 14,800       |

$^a$ Percentage distribution of acetate, propionate, and butyrate, respectively, at 20 mg of glucose: 60.2, 24.9, 14.9; at 0.6 $\mu$g of glucose, 94.6, 3.9, 1.5.
were removed just prior to delivery of a portion of feed to an animal fed hourly by an automatic dispensing device. Three such samples were taken followed by one 15 min after the consumption of a 1/24th portion of the daily feed, and all were incubated with glucose-\(\text{U}^{14}\text{C}\) without added carrier. Table 2 shows that a fairly constant proportion of the label appeared in the VFA and that, compared to the sheep fed once daily, rather less appeared in acetate and more in the cell material. Again, of the radioactivity incorporated into cell material, most was in the carbohydrate fraction (mean of 7.7% of recovered activity), a lesser amount being in protein (mean of 3.2% of recovered activity).

Effect of removal of protozoa. The conversion of the bulk of the glucose carbon in acetate was recognized as corresponding to the reported activities of the rumen protozoa metabolizing soluble sugars (1, 8). Consequently, experiments were done to assess the effect of removal of protozoa from rumen digesta. Protozoa were removed by squeezing digesta collected 20 hr after feeding through terylene voile and centrifuging the fluid at about \(500 \times g\) for a few seconds. The solids were gently washed twice with clarified rumen fluid from the same animal, and the washings were discarded. The supernatant fluid from the centrifuged fluid was then added back to the solids, and the reconstituted digesta were thoroughly mixed and divided into two equal portions. Half of the protozoa from the centrifuged fluid were then added to one portion and an equal volume of clarified rumen fluid was added to the other.

This procedure has been shown to remove about 90% of the protozoa and less than 10% of the bacteria in rumen fluid (unpublished data).

Three such treatments were done on separate occasions. Incubations were as described above except that a shorter incubation period was used in two of the experiments. Results are given in Table 3. In the first experiment, glucose pool turnover was unusually slow when protozoa had been removed and was accompanied by a decrease in \(14\text{C}\) appearing in butyrate. However, the second and third experiments showed no significant effect of protozoal removal on distribution of \(14\text{C}\). A parallel determination of rate of total VFA production, done in conjunction with experiment 3, disclosed an 8% fall due to removal of protozoa.

Two control experiments were done to compare untreated rumen contents with contents fully reconstituted after undergoing all steps of the protozoa removal treatment. There were no differences in distribution of \(14\text{C}\) between the VFA species, or in rate of total VFA production,
indicating that the treatment used to remove protozoa had no deleterious effect on any of the organisms present.

**Continuous infusion of glucose-U-14C in vitro.**

Since one particular organism or group of organisms may have contributed substantially to the metabolism of the glucose pool labeled by a single dose of 14C and since turnover of the pool appeared to be relatively rapid, there existed the possibility that the added glucose did not equilibrate with all of the free intracellular pools available. Therefore, an experiment was done with 200 g of digesta (collected 20 hr postfeeding) to which a priming dose of 2 μCi of carrier-free glucose-U-14C was added. Further 14C was infused at a rate of 12 μCi (in 12 ml) per hr while stirring the reaction mixture fairly rapidly. To assist in keeping pH relatively constant during incubation, sodium bicarbonate at a concentration of 0.2 mmol/ml was included in the glucose solution infused. Samples (5 ml) of rumen fluid were withdrawn at 30-min intervals for 2 hr and promptly acidified with 1 ml of 1 N perchloric acid. After 2 hr, a 20-ml sample of fluid was obtained; 10 ml was treated with 2 ml of 1 N HC104 and 10 ml was placed in an ice-cold tube and centrifuged at 0 C. This last sample was used to attempt the estimation of specific activities of the total free glucose pool and the extracellular pool.

Rate of total VFA production was linear over the 2-hr period, being 7.5 μmoles per hr per ml of rumen fluid. Radioactivity in the total glucose pool rose over the first 60 min and remained constant thereafter. Accumulation of radioactivity in individual VFA species was linear (Table 4), and all of these factors show that steady-state conditions were established. Distribution of 14C in the individual VFA species again showed that most of the glucose carbon was converted to acetate carbon. In addition, there was five times as much activity in butyrate as there was in propionate (Table 4). Specific activities of glucose in the total and extracellular pools appeared to be the same, being 2,370 and 2,450 dpm/μg, respectively. However, no significant difference in total and extracellular pool sizes was observed, so the question of equilibration of all available pools with the added glucose remains unresolved.

A repeat of the continuous infusion experiment gave substantially the same results (Table 4).

Since attempts to demonstrate equilibration between intracellular and extracellular glucose pools directly were not successful, it was decided to try to assess the rate at which glucose entered the microbial cells and was converted to products. Rumen fluid and 14C-glucose without added carrier were pumped into a mixing cell and magnetically stirred together, and the effluent was led through a length of plastic capillary tubing marked at points corresponding to known times.
after mixing. Effluent was collected first from the point most distant from the mixing chamber into dilute H$_2$SO$_4$ and, thereafter, the capillary was cut at points progressively closer in time to the time of mixing (P. R. Monk, unpublished method). By this means, it was established that glucose was metabolized very rapidly.

In one experiment, the label in acetate was 30% of that in glucose after 6.2 sec, rising to 106% in 123 sec; the disappearance of $^{14}$C-glucose was first order and yielded a rate constant for glucose pool turnover of 0.32 per min. A second experiment showed label in acetate after 1.2 sec to the extent of 8% of that remaining in glucose. In both experiments, label appeared in a compound, tentatively identified as lactate from the position of its elution from the ion-exchange resin column, in greater amount than in acetate at times close to mixing. At times further removed from the point of mixing, radioactivity in lactate decreased as that in acetate increased.

**DISCUSSION**

Previous investigators of the metabolism of carbohydrate by the mixed microbial population of the rumen have used very large quantities of carbohydrate substrate in comparison to the sizes of the free pools normally existing in rumen digesta (1, 2, 5, 11-14, 17, 19). Even when isotopically labeled substrates have been used, the concentrations of substrate were quite high. For instance, Pazur et al. (11) used $^{14}$C-xylase at a concentration of 100 µmoles per ml with washed rumen organisms and incubated for 12 hr; Wallnöfer et al. (20) used $^{14}$C-glucose at a final concentration of 1.4 µmoles per ml with a 2-hr incubation period. The $^{14}$C-glucose concentration used by Baldwin et al. (2) was 28 µmoles per ml. These concentrations are to be compared with values of 0.02 to 0.09 µmoles of glucose per ml of rumen fluid determined by specific enzyme assay in this laboratory and the highest reported value obtained for a brief period in the rumen of a sheep immediately after feeding of 0.44 µmoles per ml (15). The results reported in this paper clearly demonstrate a very large effect of substrate concentration on the distribution of glucose carbon among the VFA end products of fermentation. When only a trace amount of glucose was added to rumen contents (about 1/100th the pool size), glucose carbon appeared in acetate and propionate in the ratio 24:1. On the other hand, when glucose was added in sufficient quantity to increase the glucose pool size by about 300-fold, glucose carbon appeared in acetate and propionate in the ratio 2.4:1. No doubt, a high concentration of glucose leads to the rapid generation of large amounts of reducing power, the disposal of which is reflected in greater propionate production. In addition, relatively large concentrations of lactate transiently accumulate when high concentrations of glucose are used and this substrate may preferentially give rise to propionate (17, 20). The value of 2.4:1 for the ratio of radioactivity in acetate and propionate in the presence of excess glucose is close to other values obtained by using excess glucose-$U^{14}$C and organisms from roughage-fed cattle of 3.1:1 (2) and 5.2:1 (20).

In pure culture, *Selenomonas ruminantium* has been shown to yield differing ratios of fermentation end products over a range of glucose concentrations between 50 and 5,000 µg per ml (7). However, in this case, decreasing glucose concentration resulted in an increase in the proportion of propionate relative to acetate, and lactate was always found in large quantity.

The fate of carbon derived from the free glucose pool does not alter greatly during the day whether the animal is fed once or 24 times daily. During feeding in the animal fed once daily, the proportion of glucose carbon reaching acetate falls by some 10%, whereas that reaching propionate rises slightly. Presumably, the presence of soluble carbohydrates in the feed raised

| Time (min) | Expt 1 | Expt 2 | Expt 1 | Expt 2 | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
|-----------|--------|--------|--------|--------|--------|--------|--------|--------|
| 30        | 6.85   | 9.25   | 33.07  | 44.65  | 0.84   | 1.09   | 3.52   | 4.75   |
| 60        | 8.98   | 12.12  | 59.39  | 80.17  | 1.47   | 1.98   | 7.13   | 9.63   |
| 90        | 9.16   | 12.37  | 88.14  | 118.95 | 2.10   | 2.83   | 10.60  | 14.31  |
| 120       | 9.03   | 12.19  | 118.40 | 159.83 | 2.71   | 3.66   | 13.99  | 18.89  |

* Mean percentage distribution of $^{14}$C in volatile fatty acids: acetate, 87.6; propionate, 2.1; butyrate, 10.3.
the concentration in the free sugar pools in the rumen (15), and this is the nearest approach under natural conditions to the concentrations used in previous investigations on carbohydrate metabolism. It is apparent, however, that the small change in distribution of glucose carbon to individual VFA species occurring during feeding is quickly reversed upon cessation of feed intake.

A rather more marked change observed during feeding once daily is the greatly increased incorporation of glucose carbon into reserve and possibly structural carbohydrate within the microbial cells. This phenomenon has previously been studied in this laboratory (17, 19) by using chemical determinations and is confirmed by using isotopes.

Carbon derived from the glucose pool appearing in microbial protein accounted for about 3% of the glucose metabolized when feed was offered hourly. Accepting Hungate's (8) estimate of a cell yield of 10% of the substrate fermented, 65% of this being protein, it would seem that, of the microbial growth associated with glucose fermentation, about half of the carbon entering cell proteins is derived from glucose itself.

In one experiment where physical removal of protozoa showed a distinct effect, a relatively large amount of 14C was left in free glucose after 1 hr. In addition there was a lowered incorporation of 14C into butyrate when protozoa were removed, an observation in keeping with studies showing acetate and butyrate as the chief products of protozoal carbohydrate metabolism (1, 8) and consistent with the finding of lowered butyrate concentrations in the VFA species in the defaunated sheep rumen (9). Since, however, two other experiments demonstrated no difference in 14C distribution due to removal of protozoa, it is not yet resolved whether the protozoa play a major role in the metabolism of free glucose.

Intracellular glucose pool size appears to be too small to be measurable by taking the difference between extracellular and total pool sizes, so it has not been possible to determine whether equilibration of the added 14C-glucose with all available glucose pools was achieved. Nonetheless, the distribution of 14C among the VFA end products was the same whether label was added as a single dose or by continuous infusion over a 2-hr period, indicating that all pools available for equilibration with added glucose were being reached.

Appearance of 14C in acetate was very rapid, again indicating fast transport of extracellular glucose into the cells and prompt metabolism. Rapid appearance of label in a compound which is probably lactate as well as in acetate suggests that intracellular glucose concentration would be very low under the normal rumen conditions of low extracellular glucose pool size.

Overall, it seems reasonable to suppose that the metabolism of the added 14C-glucose was similar to that of glucose arising from feed component breakdown. The mean results of 15 experiments on distribution of 14C from the labeled glucose pool indicate the following equation for ruminal glucose utilization in roughgut-fed animals: 1.0 glucose → 1.76 acetate + 0.04 propionate + 0.10 butyrate + 1.95 CO2 + 3.48 [2H]. The major feature of this equation is the relative paucity of propionate compared to that proportion normally present in the rumen. This suggests that most of the propionate formed arises from sources whose carbon does not equilibrate with that in the free glucose pool or with that of intermediates of free glucose metabolism. A further apparent anomaly is the excess of reducing power available. However, in terms of the total rumen fermentation, the excess of reducing power would be quite small since unpublished preliminary estimates based upon pool size and turnover rates suggest that less than 10% of the VFA produced, in experiments so far completed, arises from the free glucose pool.

Further studies are in progress to evaluate the contribution of free glucose to the VFA produced at various times after feeding.

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