APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1986, p. 700–705
0099-2240/86/100700-06$02.00/0

Vol. 52, No. 4

Substrate Utilization by *Campylobacter jejuni* and *Campylobacter coli*

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Received 20 November 1985/Accepted 15 July 1986

An attempt was made to elucidate in *Campylobacter* spp. some of the physiologic characteristics that are reflected in the kinetics of CO_2 formation from four ^{14}C-labeled substrates. *Campylobacter jejuni* and *C. coli* were grown in a biphasic medium, and highly motile spiral cells were harvested at 12 h. Of the media evaluated for use in the metabolic tests, minimal essential medium without glutamine, diluted with an equal volume of potassium sodium phosphate buffer (pH 7.2), provided the greatest stability and least competition with the substrates to be tested. The cells were incubated with 0.02 M glutamate, glutamine, α-ketoglutarate, or formate, or with concentrations of these substrates ranging from 0.0032 to 0.125 M. All four substrates were metabolized very rapidly by both species. A feature of many of these reactions, particularly obvious with α-ketoglutarate, was an immediate burst of CO_2 production followed by CO_2 evolution at a more moderate rate. These diphasic kinetics of substrate utilization were not seen in comparable experiments with *Escherichia coli* grown and tested under identical conditions. With *C. jejuni*, CO_2 production from formate proceeded rapidly for the entire period of incubation. The rate of metabolism of glutamate, glutamine, and α-ketoglutarate by both species was greatly enhanced by increased substrate concentration. The approach to the study of the metabolism of campylobacters here described may be useful in detecting subtle changes in the physiology of cells as they are maintained past their logarithmic growth phase.

The nutritional and biochemical characteristics of campylobacters have been studied in great detail (8, 9, 19–22), but their isolation from the environment has not always been successful, even when epidemiological evidence strongly suggested that campylobacters were present (17). Smibert (21, 22) reviewed evidence that, while campylobacter cells in logarithmic growth phase are highly motile spirally curved rods, in old cultures they tend to become spherical or coccoid. These forms for the most part are not culturable. This evidence has recently been confirmed by Merrell et al. (12) and Ng et al. (13). This phenomenon resembles one described by Colwell et al. (4) for *Vibrio cholerae* and other pathogens, which, when collected from the environment, often are not culturable by standard techniques, although by certain tests they still appear to be viable.

To better understand the physiology of viable but not culturable campylobacters (17), we need to develop additional information on the kinetics of utilization of some of their more rapidly metabolized substrates. In this study, we examined CO_2 formation from four such substrates by a strain of *Campylobacter jejuni* and a strain of *C. coli*. For both strains, there appeared to be, with some substrates, an initial burst of activity followed by a more moderate rate of metabolism. Furthermore, the kinetics of some of these reactions were greatly influenced by the concentration of the substrate. This work was done with highly motile cells known to be in logarithmic growth phase. These reactions may change as the cells are tested past this growth phase.

MATERIALS AND METHODS

Bacterial strains. *C. jejuni* ATCC 29428 was obtained from the American Type Culture Collection, Rockville, Md. *C. coli* WR, a human diarrheal isolate, was received from the Walter Reed Army Hospital, Washington, D.C. The wild-type strain of *Escherichia coli* K-12 was obtained from our colleague, Michael E. Dobson.

Preparation of cells for metabolic experiments. Cells to be used in metabolic experiments were cultivated from frozen stock cultures. Following a passage on 5% sheep blood agar in an atmosphere of 5% O_2–10% CO_2–85% N_2, the cells were passaged twice at 37°C in biphasic medium (18). The incubation time was 12 h unless otherwise indicated. Cultures from the second passage were checked for morphology and motility, and only monomorphic cultures with highly motile spiral cells were used. The cells were harvested by centrifugation (17,500 × *g* for 15 min) and suspended in phosphate buffer or other diluent, as described below, to an optical density at 625 nm of 0.5 to 1.0 and diluted to the desired cell concentration for metabolic tests. The buffer consisted of 0.08 M monopotassium phosphate adjusted to pH 7.2 with NaOH. $MgSO_4$ was also added to a final concentration of 0.005 M. In most cases, the buffer was diluted prior to use with an equal volume of minimal essential medium (MEM) (Flow Laboratories, Inc., McLean, Va.) without glutamine.

The protein content of the cell preparations was determined by the Bio-Rad protein assay procedure (2), as described previously (23).

Metabolic experiments. L-[U-^{14}C]glutamic acid, L-[U-^{14}C]glutamine, L-[U-^{14}C]α-ketoglutaric acid, and L-[^{14}C]formic acid were obtained from New England Nuclear Corp., Boston, Mass. These compounds were diluted with their unlabeled counterparts to provide 0.05 μCi per tube and a 0.02 M concentration unless otherwise indicated. The formation of CO_2 was determined, as previously described (23), with tubes (16 by 100 mm) containing final volumes of 0.4 ml. The tubes were closed with rubber stoppers from which were suspended plastic cups containing filter paper wicks. The tubes were shaken in a water bath at 37°C. The reactions were stopped by injecting 0.15 ml of Hyamine hydroxide (New England Nuclear) into each cup and 0.1 ml

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TABLE 1. Influence of the suspending medium on CO_2 production from 0.02 M glutamate by *C. jejuni*

Suspending medium	Amt of CO_2 (μmol)/mg of protein[a] after incubation at 37°C for:		
	0.5 h	1.0 h	2.0 h
Phosphate buffer	6.2	9.4	16.2
Brain heart infusion broth containing 1% yeast extract[b]	2.1	4.7	17.0
Phosphate buffer + MEM vitamins	7.4	12.5	22.8
Phosphate buffer + MEM vitamins and essential and nonessential amino acids[c]	6.7	12.6	32.4
MEM[c] diluted 1:2 with phosphate buffer	9.8	18.2	32.4

[a] Protein was used at 16 μg per tube.
[b] The medium was diluted 1:8 with phosphate buffer.
[c] No glutamine.

of 25% trichloroacetic acid or acetic acid into each reaction mixture as described in Results. After an additional 0.5 to 1.0 h of incubation to allow the Hyamine to absorb the CO_2, the cups and the wicks were transferred to liquid scintillation vials, to which 1.5 ml of methanol and 7.5 ml of Econofluor (New England Nuclear) were added. Radioactivity was measured with a liquid scintillation spectrometer. The amount of CO_2 produced was calculated from the counts per minute for these vials minus the counts per minute for control tubes without cells, the final specific activity of the substrate, and the number of labeled carbons. The amount was expressed as the mean of triplicate specimens per milligram of bacterial protein. The variation among triplicate determinations was usually <10%.

RESULTS

Table 1 illustrates some of the experiments designed to determine which diluent was the most appropriate for kinetic studies of substrate utilization by campylobacters. These tests were done with *C. jejuni* cells and with glutamate as the substrate. When the cells were suspended in unsupplemented phosphate buffer, activity was relatively low and the rate decreased with time. When a rich medium was used as the diluent (brain heart infusion broth with 1% yeast extract [Difco Laboratories, Detroit, Mich.]), activity was very low, most probably because of competing constituents in the medium, even though the medium was diluted 1:8 with phosphate buffer. There was an increase in activity between 1 and 2 h, possibly because of competing metabolite depletion or campylobacter multiplication. Since niacin is required for the growth of campylobacters and other vitamins are stimulatory (21), the phosphate buffer was enriched with the vitamin constituents of MEM to determine whether the vitamins would stabilize the cells without competing with glutamate metabolism. The rate of CO_2 formation was improved over that obtained with unsupplemented phosphate buffer, and variation among replicate experiments was reduced. On the other hand, when phosphate buffer was enriched with MEM vitamins plus MEM essential amino acids without glutamine and the nonessential amino acids, there was evidence of competition at 0.5 and 1 h but increased activity at 2 h. Somewhat similar results were obtained when nonessential amino acids were omitted (data not shown). When MEM without glutamine was diluted with phosphate buffer, the most satisfactory results in terms of activity and linearity were obtained with a 1:2 dilution (Table 1). In the experiments described below, MEM diluted 1:2 with phosphate buffer was used.

The experiment illustrated in Table 1 was done with cells harvested from biphasic medium at 12 h. The influence of the time of harvest on the kinetics of glutamate catabolism by *C. jejuni* is shown in Fig. 1. For this experiment, the cells were grown in shaken flasks containing brain heart infusion broth supplemented with 1% yeast extract, rather than in a stationary biphasic medium. Moderate differences in kinetics were obtained for cells harvested at 8, 12, and 24 h, but specific activity was greatly reduced by 36 h and was almost negligible by 48 h. Plate counts (Fig. 1, inset) indicated that, by 48 h, the number of culturable bacteria in the suspension had greatly declined. Since the highest activity was obtained with the 12-h harvest, this time interval was used for cell harvests in subsequent experiments.

The optimal cell density and the effect of washing the cells with phosphate buffer or MEM (1:2) prior to testing were investigated next. The best results were obtained when the density of the cell suspensions was adjusted spectrophotometrically to provide no more than 0.08 mg of cell protein per tube. Comparable results were obtained with washed and unwashed cells, suggesting that chemical contamination from medium constituents was not great enough in

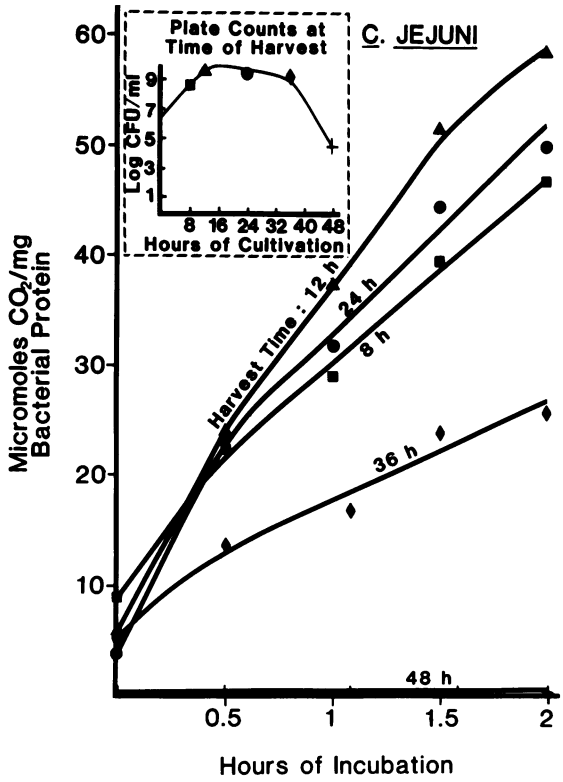

FIG. 1. CO_2 formation from glutamate by *C. jejuni*. The cells were grown at 37°C in shaken flasks containing brain heart infusion broth with 1% yeast extract, harvested at the times indicated, and suspended in MEM diluted 1:2 with phosphate buffer, as described in the text. For the metabolic tests, 0.02 M glutamate with 0.05 μCi of [^{14}C]glutamate and approximately 50 to 80 μg of bacterial cell protein in a total volume of 0.4 ml were added to each tube. The tubes were incubated at 37°C in a water bath with moderate shaking for the time intervals indicated, following a 2-min period of thermoequilibration. Each point represents the mean of triplicate determinations. The values obtained after thermoequilibration (0 h) were not subtracted from the other values. The relative numbers of culturable cells at the various time intervals of the test are shown in the inset.

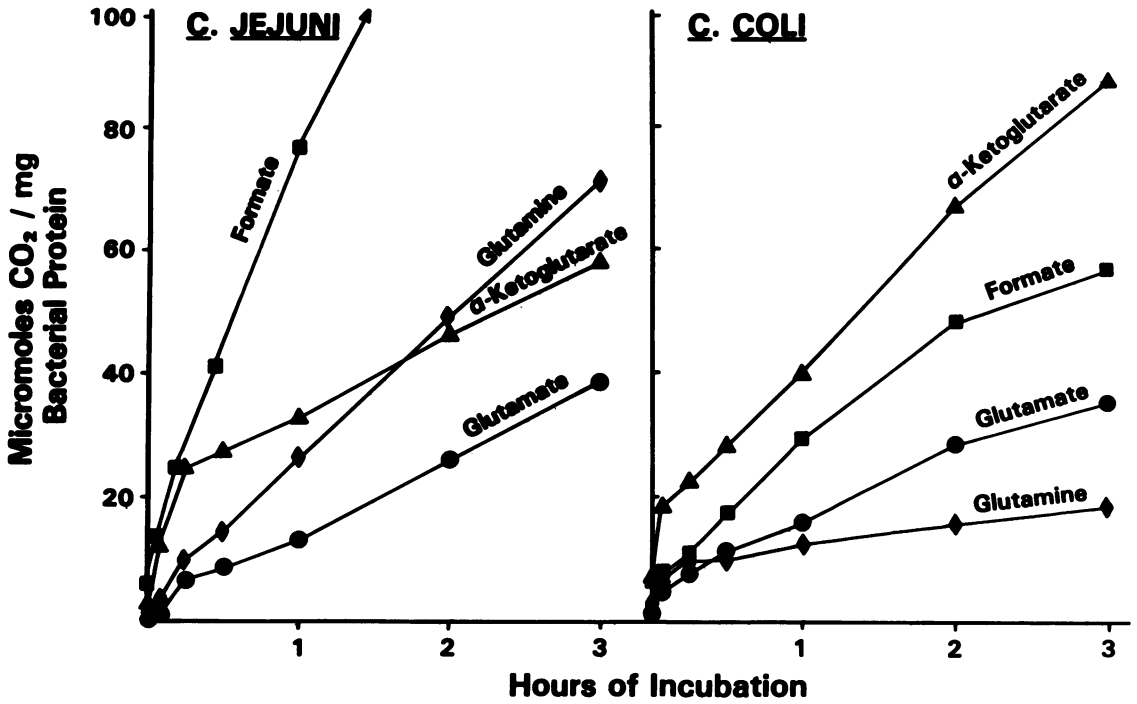

FIG. 2. CO_2 formation from four substrates by *C. jejuni* and *C. coli*. The cells were grown in biphasic medium and harvested at 12 h. The suspending medium and the procedures were as described for Fig. 1 and in the text. For the determination of CO_2 production from formate by *C. jejuni*, the bacterial cell protein concentration was reduced to 24 μg per tube because, with larger numbers of cells, residual substrate concentration became rate limiting. With the lower cell concentration, the reaction with formate remained approximately linear (139 and 199 μmol/mg of bacterial protein at 2 and 3 h, respectively).

unwashed cells to compete with the substrates tested. Unwashed cells were used in most of the subsequent experiments. Attempts were also made to use cells that had been subjected to controlled-rate freezing and stored at −100°C in a liquid-nitrogen chest. The cells were harvested from logarithmic growth phase and suspended in small volumes of 0.2 or 0.4 M sucrose, 15% glycerol, or a combination of sucrose and glycerol prior to being frozen. Although this procedure was entirely satisfactory for *Legionella* spp. and greatly facilitated comparative studies (23), it proved to be unsuitable for campylobacters. Only a small percentage of the activity was retained by cells that had been frozen.

It was also necessary to determine the most satisfactory method for terminating the reaction of cells incubated with formate. Both 25% trichloroacetic acid and undiluted acetic acid added to stop the reaction caused the chemical release of some CO_2 from formate. However, when due precautions were taken not to exceed 0.1 ml as the volume of acid injected into each tube and not to exceed 30 min for secondary incubation (to trap CO_2 to the Hyamine), chemical release of CO_2 was not excessive. With 25% trichloroacetic acid or undiluted acetic acid, it was estimated that about 0.7 and 0.1%, respectively, of the total CO_2 from formate was released chemically when the concentration of formate was 0.02 M. With the appropriate corrections, the results obtained by either method were the same. At lower concentrations of formate, proportionally larger corrections were required. Undiluted acetic acid was used to stop the reaction with formate in subsequent experiments.

Figure 2 illustrates the kinetics of CO_2 production by *C. jejuni* and *C. coli* from four substrates. The substrate concentration was 0.02 M, and the conditions for the experiments were as described above. A feature common to many of these reactions was the very rapid release of CO_2 during the first few minutes. In fact, a significant amount of CO_2 was released by the time defined as zero. Time zero, by our definition, was preceded by the time required to fill and stopper the tubes in ice-water plus a 2-min period of thermal equilibration at 37°C. The values obtained at time zero were not subtracted from the other values in Fig. 2, to better illustrate the initial burst of activity. The possibility that this activity was due to free enzymes released from the cells was checked by quickly removing the cells from the diluent by centrifugation and filtration. No activity was detected in the cell-free diluent. The initial burst of activity was usually followed, at 5 to 15 min, by a second, less rapid, rate of activity. The two-rate kinetics were particularly obvious with α-ketoglutarate as the substrate. Considerable variation was encountered in repeated experiments with α-ketoglutarate, although the two-rate kinetics usually prevailed (data not shown). This variability was possibly due to the instability of this compound or to the presence of trace amounts of ^{14}C-labeled impurities or both. With *C. jejuni* cells incubated with formate, the initial burst of activity continued at a virtually undiminished rate for all 3 h of incubation. This was demonstrated only in an experiment with a low cell concentration that did not exhaust the substrate.

Figure 2 also illustrates differences in the kinetics of substrate utilization between the two species. *C. jejuni* utilized glutamine more rapidly than glutamate and formate more rapidly than α-ketoglutarate. The reverse was true of *C. coli*. Whether these results reflected species or strain differences is not known.

It appeared of interest to determine whether another bacterium unrelated to *Campylobacter* would show the same diphasic kinetics and high rate of CO_2 production if grown

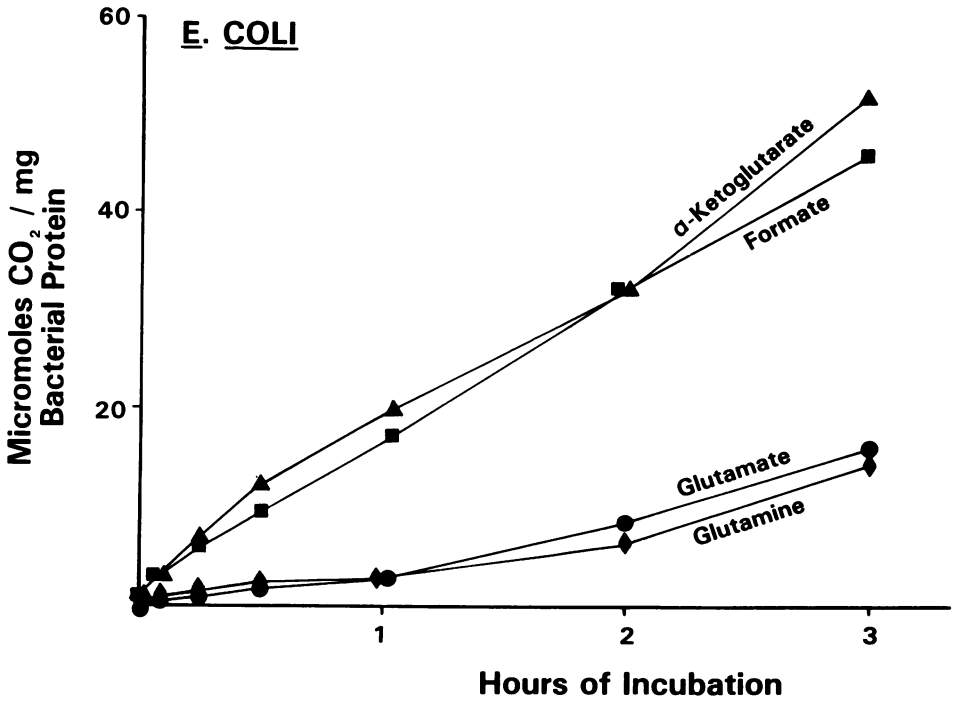

FIG. 3. Experiments comparable to those shown in Fig. 2 carried out with *E. coli* K-12. *E. coli* was grown in the medium used for campylobacters, except that, because of more rapid growth, the cells were harvested after 6 instead of 12 h of incubation. Bacterial protein content was approximately 30 to 40 μg per tube.

and tested under identical conditions. Such an experiment was done with the K-12 strain of *E. coli* (Fig. 3). *E. coli*, in contrast to campylobacters, metabolizes glucose, a constituent of MEM, and is capable of replication in such a medium. The kinetics of CO_2 production from the four substrates were quite different from those of the campylobacters. CO_2 production from glutamate and glutamine was low for 1 h and increased during the next 2 h. This increase can be attributed to exhaustion of glucose, which undoubtedly was utilized preferentially, or to cell replication, or both. α-Ketoglutarate and formate were metabolized more rapidly than the other two substrates, but even for these substrates, the rates were generally lower than those obtained with the campylobacters. Although it cannot be excluded that the rates during the first 30 min were somewhat higher than during the following 30 min, the obvious diphasic kinetics of campylobacter metabolism were not seen.

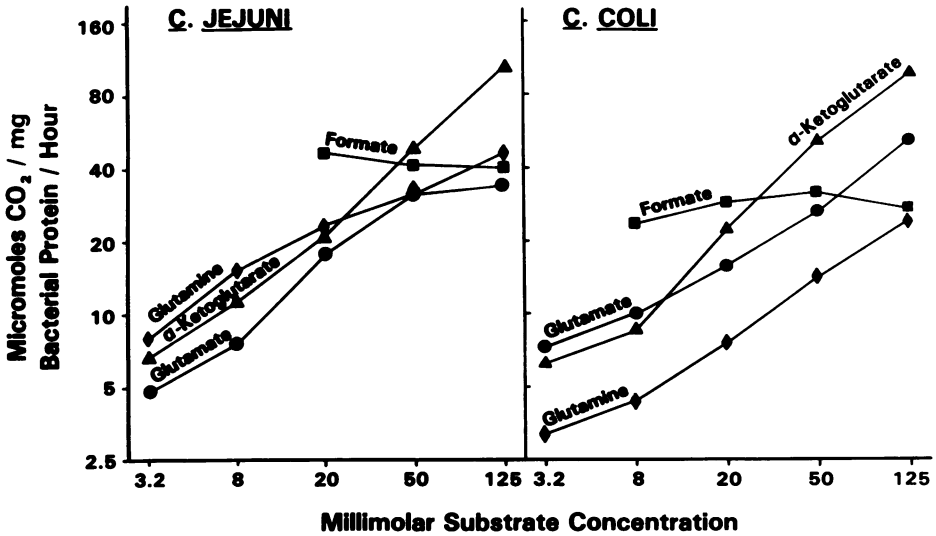

FIG. 4. CO_2 formation by *C. jejuni* and *C. coli* from four substrates used at different concentrations as shown. Each symbol represents CO_2 evolution measured after 1 h of incubation. The values obtained after thermoequilibration (0 h) were subtracted. The procedures were otherwise identical to those described for Fig. 1 and 2. Note that both abscissa and ordinate depict geometric progressions.

Figure 4 illustrates CO_2 production by the two species of *Campylobacter* when the concentrations of substrate varied over a wide range (from 0.0032 to 0.125 M). The values shown represent the activities during the first hour, following subtraction of the time zero values. Somewhat similar trends were seen when the activities were measured after 2 h of incubation (not shown). Three of the substrates (glutamate, glutamine, and α-ketoglutarate) were utilized at substantially higher rates as the concentrations were increased. This suggests that these substrates were metabolized through multiple pathways and that saturation of all the enzymes involved was not achieved under the conditions of these experiments. This was not true of formate, which was utilized at approximately the same rate irrespective of concentration. As indicated above, when formate was tested at the lower concentrations, large corrections were required, and some of the results were not sufficiently accurate to be shown. Even with these limitations, the difference between the metabolism of formate and that of the other three substrates examined under these conditions is quite obvious.

DISCUSSION

The buffer used to suspend cells in our metabolic experiments was in part based on the results of Hänninen (7) and Doyle and Roman (5), which indicated that most *Campylobacter* strains are stabilized by a medium of moderate electrolyte content. To further stabilize the cells, it was necessary to select a medium that would not unduly compete with the substrates to be tested. MEM without glutamine, diluted 1:2 with 0.08 M phosphate buffer, appeared to fulfill this requirement.

The four substrates used in this study are rapidly metabolized by campylobacters. Our results are widely supported by oxygen uptake, electron transport, enzymatic, and nutritional studies reported by other investigators. Alexander (1) found that glutamate and α-ketoglutarate serve as excellent energy sources when added to a basal medium for the growth of *V. fetus*. Kiggins and Plastridge (10) demonstrated in manometric experiments that glutamic acid and glutamine are actively oxidized, glutamate more rapidly than glutamine, by whole cells and crude cell extracts of *V. fetus*. α-Ketoglutarate is oxidized well by cell extracts, but only slightly or not at all by whole cells. Lecce (11) studied electron transport by a large number of *V. fetus* strains and found that formate and α-ketoglutarate are excellent electron donors. However, growth of the vibrios is inhibited when formate concentrations of 0.5% are added to the medium.

Recent work has focused on the importance of formate as a substrate for *C. sputorum* (14–16) and for *C. jejuni* and other species (3, 6, 9). Hoffman and Goodman (9) studied the respiratory activities of membrane vesicles of *C. jejuni* and observed that the activities with formate and hydrogen were 50- to 100-fold higher than with some of the other substrates, such as succinate, lactate, malate, or NADH. These results were consistent with their observations that the dehydrogenases of hydrogen and formate were located on the periplasmic face of the cytoplasmic membrane, while the other enzymes were inside the cytoplasmic membrane. These authors concluded that formate and hydrogen probably serve as major energy sources for growth. Goodman and Hoffman (6) developed a rapid test for hydrogenase activity by using the redox dye benzyl viologen and found that by this test, *C. jejuni* and *C. coli* were positive, while the other *Campylobacter* spp. were negative. When formate was tested with the same dye, the test was positive with all species.

The advantages of studying the metabolic activities of campylobacters by the method described in this paper are flexibility and sensitivity. A large number of substrates can be used singly or in combination with unlabeled substrates (23). With due caution, this method can be applied to formate as the substrate.

The rapid metabolism of formate, especially by *C. jejuni*, can best be interpreted by assuming that this compound is utilized in the periplasmic spaces, as observed by Hoffman and Goodman (9), and that transport across the cytoplasmic membrane is not required. The same may be true of the initial metabolic burst of activity found with α-ketoglutarate. This reaction presumably produced succinate, which then required transport for further utilization, and this is reflected in the more moderate, secondary rate of activity.

It is difficult to interpret the significance of the differences in the rate of substrate utilization by the two species or the differences encountered with both species in rates of metabolism as the substrate concentrations were increased. Such an interpretation would require the extension of this work to other strains and the elucidation of the carbon flux between pathways involved in the reactions described. It is tempting to speculate, however, that this approach to the study of the metabolism of campylobacters may provide sufficient detail to permit us to evaluate subtle changes in metabolism as the organisms are maintained beyond their logarithmic growth phase or under natural conditions that are encountered in the environment.

ACKNOWLEDGMENTS

We are greatly indebted to Paul S. Hoffman for suggesting the inclusion of formate in this investigation, Hermoise K. Mamay for participating in some of these experiments, and Toni Bredice for her excellent secretarial assistance.

This work was supported by the Naval Medical Research and Development Command Research Work Unit no. 61102 3M161102BS10. AB 422 and, in part, by the Office of Naval Research contract no. N00014-81-K-0638.

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