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Pathways of Anaerobic Acetate Utilization in *Escherichia coli* and *Aerobacter cloacae*

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Acetate-*1*-^{14}C was added to anaerobic glucose-fermenting cultures of *Escherichia coli* and *Aerobacter cloacae*. In the *E. coli* culture, lactate formation occurred late in the fermentation, when the rate of production of formate and acetate had decreased. The occurrence of acetate label in the lactate indicated formation of pyruvate from acetyl-coenzyme A (CoA) and formate. In the *A. cloacae* cultures, substantial amounts of acetate label were found in the 2,3-butanediol formed. Evidence is presented that the label could have entered the diol only by conversion of formate and acetyl-CoA into pyruvate. The observed levels of radioactivity in the diol indicated that during diol formation the reaction yielding formate and acetyl-CoA from pyruvate CoA was operating close to equilibrium. The shift in metabolism from formation of acetate, ethyl alcohol, and formate to the formation of butanediol or lactate appears to be due basically to an approach to equilibrium of the pyruvate-splitting reaction, whatever the induction mechanism by which the shift is implemented.

When *Escherichia coli* is grown anaerobically on glucose, the fermentation products are largely acetate, ethyl alcohol, formate, and lactate. Lactate formation has been observed to be greater at low *p*H values (24, 27) and occurs rapidly in the later stages of the fermentation (4). The mechanism of this apparent effect of extracellular *p*H on metabolic pathways has not been studied. It is reasonable, however, that lactic acid [yielding 2 moles of adenosine triphosphate (ATP) per mole of glucose] becomes the chief end product only when the rate of acetate and ethyl alcohol production (yielding 3 moles of ATP per mole of glucose) decreases.

In anaerobic glucose fermentation by *Aerobacter*, an analogous situation exists. At high pH values, the fermentation products are acetate, ethyl alcohol, and formate, as in the *E. coli* fermentation. At lower *p*H values, butanediol, ethyl alcohol, formate, and carbon dioxide are formed (16). As in *E. coli*, the metabolic pathway shifts from one giving 3 moles of ATP per mole of glucose to one giving 2 moles of ATP per mole of glucose.

In 1943, Slade and Werkman (22) added ^{13}C-labeled acetate to *Aerobacter* fermentations and found labeled carbon in butanediol. They interpreted this result as evidence that acetoin could be formed from acetaldehyde. Utter, Lipmann, and Werkman (28), however, suggested that the carbon-labeled butanediol may have been the result of reversal of the reaction in which pyruvate is converted to acetyl phosphate, probably via acetyl coenzyme A (acetyl-CoA). Both *E. coli* and *Aerobacter*, at high *p*H values, utilize pyruvate (from glucose) largely by this reaction, which will be called the pyruvate-splitting reaction. To determine the extent to which this reaction is reversed in growing cultures and to determine whether the equilibrium point of the reaction may be approached closely enough so that its reduced net rate would affect the course of glucose dissimilation, we have studied the uptake of added acetate-*1*-^{14}C by cultures of *E. coli* and *A. cloacae* growing anaerobically on glucose.

MATERIALS AND METHODS

Microbiological methods. *A. cloacae* UW-C83 obtained from M. C. Mahl (12) and *E. coli* K-12 W3110 obtained from Julius Adler were maintained on *p*H 7.3 agar slants of the same medium used in the fermentations, except that the $(NH_4)_2SO_4$, $MgSO_4$, $CaCl_2$, and trace minerals were increased fourfold and anhydrous sodium acetate (2.5 g per liter) was added.

Unless otherwise stated, the fermentation medium contained (per liter): 10 g of glucose, 1.0 g of $(NH_4)_2SO_4$, 0.025 g of anhydrous $MgSO_4$, 0.005 g of $CaCl_2 \cdot 2H_2O$, and 1.25 ml of a trace mineral solution. The solution of trace minerals contained (per liter): 0.2 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of $ZnSO_4 \cdot 7H_2O$, 0.05 g of $CuSO_4 \cdot 5H_2O$, 0.05 g of $MnSO_4 \cdot H_2O$, and 0.05 g of $CoSO_4 \cdot 7H_2O$. A mixture of KH_2PO_4 and K_2HPO_4

was added to the medium to give 0.065 M phosphate at the desired *p*H. For growth of *E. coli*, 0.0006 M sodium citrate was added to the medium. When the glucose concentration was increased to 20 g per liter, all components of the medium except phosphate and citrate were doubled.

The fermentation vessel consisted of a 1-liter Erlenmeyer flask, containing 900 ml of medium, with agitation provided by a magnetic stirrer. A rubber stopper held *p*H electrodes, an inoculation port, a sampling tube, a gas exit, and an inlet for base addition. The fermentor and medium were mounted in a water bath at 30 C and were flushed with N_2 before inoculation.

The inoculum was prepared by growing cells in a cotton-stoppered test tube or flask filled with the same medium used in the fermentation. Sufficient inoculum with an absorbance of 0.5 (600 nm, 1 cm, Beckman DB spectrophotometer) was added to the sterile medium to give an initial absorbance of 0.01 to 0.05. With *A. cloacae*, 1 g of dry cells per liter gave an absorbance of 3.31. With *E. coli*, the figure was 3.84.

For *p*H control, a glass electrode activated an automatic base addition system. The base used with *E. coli* was 7.7 N NaOH, and that used with *A. cloacae* was 3.8 N NH_4OH. The *p*H was maintained within 0.1 unit of the desired value. The fermentation was considered to be complete when base addition ceased for a period of 2 to 5 hr.

At the conclusion of the fermentation, the culture was centrifuged, and the fluid was stored frozen, if necessary, before analysis.

For determination of cell dry weights, washed cells from a known culture volume were weighed after drying either at 100 C and atmospheric pressure or at 60 C under vacuum. A cell carbon content of 47% was assumed in calculating cell carbon.

Analytical methods. Gases produced in *A. cloacae* fermentations were passed through NaOH. The gas not absorbed was collected by displacement of water.

At the completion of the fermentation, CO_2 and H_2 in the space above the liquid in the fermentor were displaced through the NaOH solution to the water displacement reservoir by adding 20 ml of 85% H_3PO_4 and CO_2-free water to fill the fermentor. The CO_2 was determined by titrating excess NaOH. The H_2 was determined from the volume of water displaced. Centrifuged culture fluid (100 ml) was fractionated by *p*H 7 distillation (10), collecting the first 50 ml and the third 25 ml. The residue was extracted with ether, first at *p*H 7 and then *p*H 2. The resulting four fractions contained, respectively, ethyl alcohol, acetoin, 2,3-butanediol, and organic acids.

The organic acids were steam-distilled. The distillate contained volatile acids. The residue was extracted with ether at *p*H 2; the ether extract contained nonvolatile acids.

The volatile acid fraction was steam-distilled after removing formic acid by HgO oxidation (18). The distillate contained acetic acid.

A portion of the nonvolatile acid fraction was oxidized with acid $KMnO_4$ (6), the volume was reduced by 50%, and the solution was extracted with ether at *p*H 2. The first step oxidized lactic acid and the second step removed the oxidation products. The ether extract contained succinic acid.

Product fractions were analyzed as follows. Ethyl alcohol was determined by dichromate oxidation (6). Acetoin was determined by the iodoform reaction (10). 2,3-Butanediol was determined by periodate oxidation (8). Acetic acid was titrated with base. Formic acid was determined by difference between the acetic acid titration and the total volatile acid titration. Lactic acid was determined by permanganate oxidation of the nonvolatile acids (3). Succinic acid was determined by titration of silver succinate with KCNS (6).

Product radioactivity was determined as follows. Samples (2 ml) for liquid scintillation counting were mixed with 13 ml of liquid scintillator of the following composition (per liter of dioxane): 100 g of naphthalene, 10 g of 2,5-diphenyloxazole, 0.25 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene. For each sample, at least 10,000 counts were accumulated unless the activity was very low.

Formate radioactivity was determined by subtracting acetate radioactivity from the volatile acid radioactivity. Lactate radioactivity was determined by subtracting succinate radioactivity from the nonvolatile acid radioactivity. Radioactivity incorporated into cell material was determined by subtracting the radioactivity of the centrifuged culture liquid from that of the whole culture.

Ethyl alcohol analyses and radioactivity were corrected for the small amounts of acetoin and 2,3-butanediol present in the ethyl alcohol fraction. 2,3-Butanediol radioactivity and analyses were corrected for the acetoin present and for the 2,3-butanediol in the *p*H 7 distillate.

Serial analyses of organic acids were made by partition chromatography. Acetoin was determined in enzyme reaction mixtures by the method described above (10) after incubating with acid to decarboxylate any acetolactate (1). Diacetyl was determined in distillates of the enzyme reaction mixtures by reaction of the diacetyl with *o*-phenylenediamine in HCl solution (7).

Chemicals. Acetate-*1*-^{14}C (Calbiochem, Los Angeles, Calif.) was subjected to a *p*H 7 steam distillation and then to a *p*H 2 steam distillation. The *p*H 2 steam distillate was used. The acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (17).

RESULTS AND DISCUSSION

Reversal of pyruvate splitting in E. coli. Figure 1 shows the results of serial analyses of an *E. coli* fermentation at *p*H 6.5. It will be noted that lactate accumulated late in the fermentation. The data of Fig. 1 may be used to obtain Fig. 2, which shows that the rate of acetate production (per gram of cells) continually decreased, whereas the rate of lactate production was increasing toward a maximum.

Table 1 shows the amounts formed of each of

the products of the fermentation of Fig. 1 and the distribution of the radioactivity (from the added radioactive acetate) among the products. Radioactivity from acetate was found to the greatest extent in ethyl alcohol and lactate. The radioactive pyruvate from which the radioactive lactate was formed could have received label from acetate only by reverse operation of the pyruvate-splitting reaction, presumably by condensation of acetyl-CoA with formate. Radioactive ethyl alcohol was formed from radioactive acetyl-CoA by reduction and had the same specific activity (0.86) as the average specific activity of the acetyl-CoA from which it was formed. The average specific activity of the pyruvate from which the lactate was formed was 0.30, or about 35% of that of the acetyl-CoA. Very substantial reverse operation of the pyruvate-splitting reaction is indicated by the observed level of lactate activity.

The continuous decrease in the rate of the pyruvate-splitting reaction apparent in Fig. 2 was not due to *p*H change; *p*H was held constant. It was not due to competition for pyruvate by lactic dehydrogenase; lactate formation did not become rapid until much of the decrease had already occurred. Because substantial reversal of pyruvate splitting occurred during lactate formation, it is reasonable to suppose that the observed decrease in the rate of pyruvate splitting was due to the approaching reaction equilibrium, that is, to the accumulation of formate and acetate.

Radioactivity from acetate carboxyl can hardly enter pyruvate by reactions other than reversal of pyruvate splitting. Anaerobically, the citric acid cycle cannot operate because succinate cannot be oxidized. If radioactive acetyl-CoA condensed with oxalacetate, and the resulting citrate was split to oxalacetate and acetate by a citritase, the resulting oxalacetate, depending on the steric specificity of the citritase, would either be without radioactivity or would be radioactive in the carboxyl that would be lost on its conversion to pyruvate. If it is postulated that carboxyl-labeled acetyl-CoA is oxidized to glyoxylate, which then condenses with acetyl-CoA to form malate, with subsequent reverse operation of the malic enzyme to form pyruvate, the resulting pyruvate label could be only in its carboxyl group. Carboxyl-labeled pyruvate could not be converted into radioactive butanediol. Substantial radioactivity was found in butanediol in the *A. cloacae* fermentations described in the next section of this report.

Reversal of the pyruvate-splitting enzyme, on the other hand, is well authenticated. Utter, Lipmann, and Werkman (28) demonstrated in-

TABLE 1. *Distribution of radioactivity from acetate carboxyl in the fermentation of glucose by E. coli*[a]

Product	(A) Amt (moles per 100 moles of glucose)	(B) Radioactivity (% of added radioactivity)	Relative specific activity (B/A)	Analytical control sample[b] (% of added radioactivity)
Ethyl alcohol	51.2	44.2	0.86	0.4
Cell carbon	33.2	1.1	0.033	
Acetoin	0.2	0.8	4.0	2.9
Acetate	57.9	34.9	0.60	94.1
Formate	97.3	2.0	0.02	0.11
Succinate	9.2	3.5	0.38	0.06
Lactate	53.6	16.2	0.30	0.08
Residue from ether extraction		1.0		0.07

[a] Products were determined on the culture of Fig. 1 at 43.5 hr. Recovery of added ^{14}C was 104%; carbon recovery was 91%.

[b] As a check on the radioactive purity of the acetate used and on the validity of the fractionation procedures used to prepare fractions for counting, a mixture was prepared that contained, in addition to the mineral salts of the medium, 0.05 M acetate, 0.05 M ethyl alcohol, 0.07 M lactate, 0.07 M succinate, 0.08 M glucose, and 4.56×10^7 counts/min of acetate-*1*-^{14}C per liter. The mixture was carried through the same procedures as the fermentation samples. The observed radioactivity in the acetoin fraction could have been due to a radioactive impurity in the acetate used or to an unrecognized manipulative error. It should be noted that the observed apparent specific activities of acetoin in Tables 2 and 3 are higher than those of any other product.

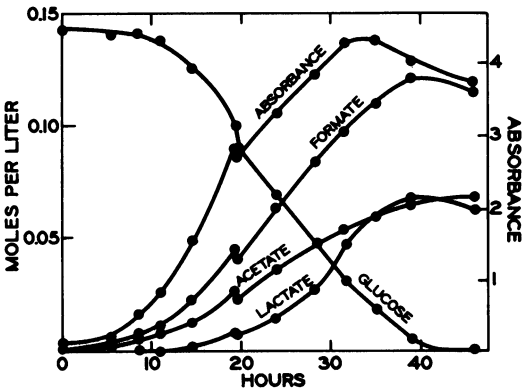

FIG. 1. *Cell growth and acid production by E. coli, growing anaerobically in a 2% glucose-mineral salts medium. The pH was controlled at 6.5. At 19.25 hr, acetate-1-^{14}C (6.05×10^7 counts per min per liter, 0.01 mole of acetate per 100 moles of initial glucose) was added.*

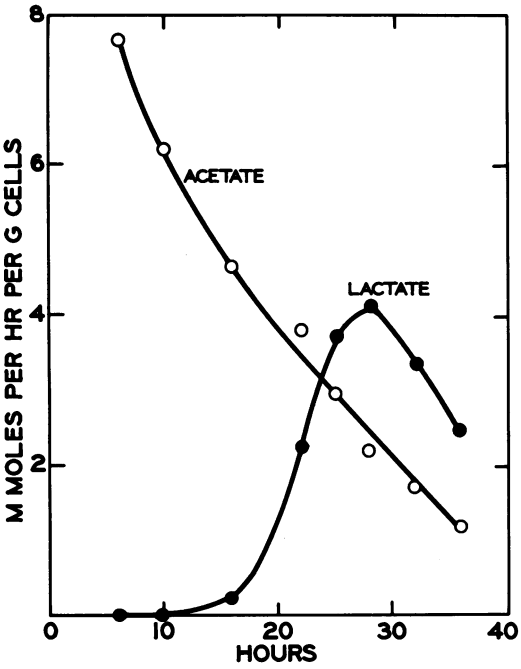

FIG. 2. *Rate of production of acetic and lactic acids per unit of cell mass, by the fermentation of Fig. 1. Cell weight was calculated from absorbance, with the aid of the known cell weight (1.00 g/liter) at the end of the fermentation.*

corporation of ^{13}C from acetate-*1*-^{13}C into pyruvate by such reversal. The incorporation of radioactive formate into pyruvate is often used as an assay for the pyruvate-splitting enzyme. Also, as pointed out later in this paper, the reversal is thermodynamically feasible.

Reversal of pyruvate splitting in A. cloacae. In anaerobic *Aerobacter* fermentations, pyruvate not converted to formate and acetyl-CoA is largely converted to acetoin, which is then reduced to 2,3-butanediol. Typically, acetate, ethyl alcohol, and formate are produced early in the fermentation, diol formation not becoming rapid until considerable amounts of the early products have accumulated (20). The onset of diol production has usually been assumed to result from decreasing *p*H (11, 13, 16). During the present investigation, we observed that, in fermentations operated at constant *p*H early in the fermentation, the molar concentration of ethyl alcohol was more that twice that of diol, whereas at the end of the fermentation the compounds were present in roughly equal amounts. Here, as in *E. coli* fermentations, the metabolic shift cannot be attributed solely to *p*H change.

Table 2 shows analyses for an *A. cloacae* fermentation carried out at *p*H 6.1, to which radioactive acetate was added. It is apparent from the high specific activity of the 2,3-butanediol that part of the diol originated from this added radioactive acetate. This presumably occurred through reverse operation of the pyruvate-splitting reaction. It does not seem likely that other pathways could have been involved. It has been shown (5, 21) that *Aerobacter* preparations converting pyruvate to acetoin will not accept acetaldehyde as a substrate. During the present investigation, we found that acetolactate-forming enzyme, prepared from *A. cloacae* cells essentially by the method of Störmer (25; carrying the preparation only through the heat-treatment stage), had high activity on pyruvate but the addition of acetaldehyde did not increase the amount of acetoin formed. Crude broken *A. cloacae* cells also did not give an increased yield of acetoin from pyruvate when acetaldehyde was added. We also considered the possibility that diol could be formed from acetate via diacetyl, which has been reported (23) to be formed from pyruvate and acetyl-CoA by lactic acid bacteria. Neither broken cells nor acetolactate-forming enzyme from *A. cloacae* gave diacetyl when incubated with pyruvate and acetyl-CoA.

There is no reason to believe that the radioactivity found in butanediol in the experiment of Table 2 originated in any other way than by the reversal of the pyruvate-splitting enzyme. Because ethyl alcohol is produced by reduction of acetyl-CoA, the intracellular acetyl-CoA of the experi-

TABLE 2. *Distribution of radioactivity from acetate carboxyl in the fermentation of glucose by Acetobacter cloacae*[a]

Product	(A) Amt (moles per 100 moles of glucose)	(B) Radioactivity (% of added radioactivity)	Relative specific activity (B/A)
Ethyl alcohol	57.4	51.8	0.90
2,3-Butanediol	40.1	14.5	0.36
Cell carbon	53.4	19.0	0.36
Acetoin	1.9	2.6	1.4
Acetate	5.8	0.5	0.09
Formate	33.8	<0.1	<0.003
Nonvolatile acid		3.7	
Succinate	4.6		
Lactate	3.2		
CO_2	128	0.1	0.001
H_2	39.8		

[a] The medium was 1% glucose-mineral salts plus acetate-*1*-^{14}C (4.66×10^7 counts per min per liter, 0.54 mole of acetate per 100 moles of glucose). The *p*H was controlled at 6.1. Recovery of added ^{14}C was 92; carbon recovery was 90%.

ment of Table 2 can be assumed to have the same average specific activity as that observed for ethyl alcohol. Since one molecule of butanediol originated from two molecules of pyruvate, the pyruvate can be assumed to have half the average specific activity observed for butanediol. It can be further assumed (somewhat erroneously) that the observed specific activities are valid average specific activities. Since the inferred pyruvate specific activity (0.18) is one-fifth of the inferred acetyl-CoA specific activity, for every four molecules of pyruvate formed from glucose, one must have been formed from acetyl-CoA. From the product distribution in Table 2, it is possible to obtain the figures given in Fig. 3A. In this figure, it is seen that the reverse rate of the pyruvate-splitting reaction was 38% of the forward rate.

The specific activity of the acetyl-CoA decreased during the fermentation as the added radioactivity was incorporated into products. Since the average butanediol molecule is formed later in the fermentation than the average ethyl alcohol molecule, the assumed average specific activity for pyruvate is too low, and the assumed specific activity for acetyl-CoA is too high. Therefore, the amount of reverse reaction shown in Fig. 3A is somewhat too low.

In the diol-forming phase of the fermentation, two-thirds of the reduced nicotinamide adenine dinucleotide (NADH) produced when pyruvate is formed from glucose must be reoxidized by reduction of acetyl-CoA to ethyl alcohol. The acetyl-CoA is supplied mainly by continued forward operation of the pyruvate-splitting reaction; it is also supplied, in part, by utilization of acetate accumulated early in the fermentation. Decrease in acetate concentration during diol formation is routinely observed (13, 14, 19, 20, 22). Utilization of preformed acetate is an indication that the net forward rate of acetyl-CoA formation from pyruvate is not rapid enough to supply the demand for hydrogen acceptor for NADH reduction. We have repeatedly observed that net acetate utilization occurs when acetate is added to *A. cloacae* fermentations conducted at *p*H 6.1. Such net utilization reduces the amount of pyruvate splitting required to provide hydrogen acceptor. Reversal of the splitting reaction should thus be more evident.

Table 3 gives analyses for a fermentation to which 0.0125 M labeled acetate was added at the time of inoculation. Other conditions were as in Table 2. Net acetate utilization occurred. Comparison of Table 3 with Table 2 shows that more butanediol was formed with added acetate, and that its specific activity was higher. In the experiment of Table 3, almost as much acetate carbon entered butanediol as entered ethyl alcohol. Figure 3B is calculated from the data of Table 3. It should be noted that the amount of reversal of pyruvate splitting necessary to obtain the observed level of radioactivity in diol is such that the reaction appears to be operating fairly close to equilibrium. Also, in Fig. 3B, as in Fig. 3A, the indicated degree of reversal would be greater if it were possible to correct the data for the decreasing specific activity of acetyl-CoA during the fermentation.

General discussion. It is probable that the cell is permeable to undissociated formic and acetic acids but not to the acid anions (2, 15). It is also probable that the intracellular *p*H is at least partially independent of the *p*H of the medium. Lowering the *p*H of a medium containing a given level of acetate or formate will thus increase the intracellular concentration of both the undissociated acid and the acid anion. The often-observed effect of low *p*H in inhibiting acid production and stimulating butanediol formation in *Aerobacter* cultures is to be expected if these increased intracellular levels can cause close ap-

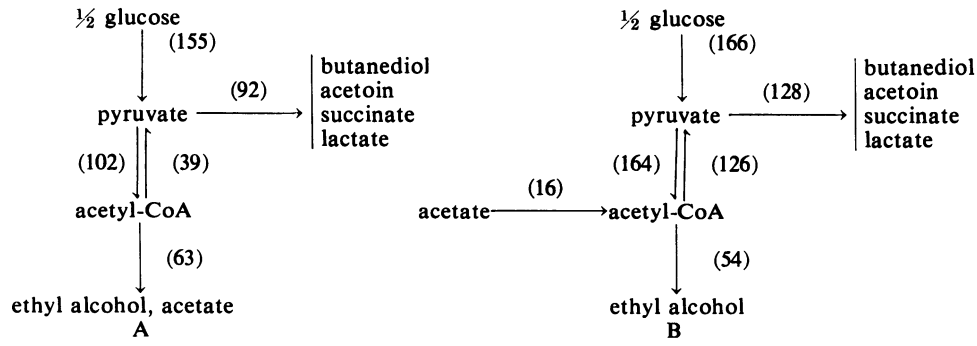

FIG. 3. *Rough quantitation of pathways for Aerobacter fermentations. A, fermentation of Table 2; B, fermentation of Table 3. Figures in parentheses are moles of metabolite (per 100 moles of added glucose) undergoing the indicated reactions. See the text for assumptions made in the calculations.*

TABLE 3. *Distribution of radioactivity from acetate carboxyl in fermentation of glucose plus acetate by Acetobacter cloacae*[a]

Product	(A) Amt (moles per 100 moles of glucose)	(B) Radioactivity (% of added radioactivity)	Relative specific activity (B/A)
Ethyl alcohol	54.0	39.4	0.73
2,3-Butanediol	57.4	36.0	0.63
Cell carbon	53.5	8.0	0.15
Acetoin	2.8	4.8	1.71
Acetate	7.1[b]	3.1	0.44
Formate	4.8	0.5	0.10
Succinate	3.4	1.2	0.35
Lactate	4.2	2.3	0.55
CO_2	152	0.1	0.001
H_2	24.7		

[a] The medium was 1% glucose-mineral salts plus 0.0125 M acetate (22.7 moles of acetate per 100 moles of glucose), containing 4.53×10^7 counts/min of acetate-*1*-^{14}C per liter. The *p*H was controlled at 6.1, and 99% of the added glucose was used. Recovery of added ^{14}C was 95%; carbon recovery was 91.5%.

[b] This figure represents acetate remaining in the medium. Net acetate production was 7.1 to 22.7, or minus 15.6 moles per 100 moles of glucose.

proach of the pyruvate-splitting reaction to equilibrium. A close approach to equilibrium under physiological conditions appears to be thermodynamically feasible. Lack of knowledge of intracellular concentrations of various reactants allows only order-of-magnitude calculations. Because the accumulating end product of the reaction is acetate rather than acetyl-CoA or acetyl phosphate, it is convenient to include in the calculation of an equilibrium constant the synthesis of ATP. The total reaction then becomes pyruvate + adenosine diphosphate (ADP) + inorganic orthophosphate (P_i) → acetate + formate + ATP. The equilibrium constant for this reaction (9) is about 0.5 at *p*H 6 and 2 at *p*H 7. Therefore, at equilibrium, the reaction is, roughly, (ATP)/(ADP) (P_i) = (pyruvate)/(acetate) (formate). It is apparent that approach to equilibrium may readily occur.

If glucose conversion to acetate, ethyl alcohol, and formate becomes slow because of approaching equilibrium, it is important that there be a suitable mechanism for induction of the alternative butanediol-producing enzyme system. Induction may appropriately be triggered by high intracellular acetate, formate, or pyruvate concentrations. Störmer (26) reported that the diol-producing enzyme system is induced by acetate.

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