Path of Glucose Breakdown and Cell Yields of a Facultative Anaerobe, *Actinomyces naeslundii*

By B. B. BUCHANAN*

*Department of Microbiology, Duke University Medical School,
Durham, North Carolina, U.S.A.*

AND L. PINE

*Communicable Disease Center, Public Health Service,
U.S. Department of Health, Education, and Welfare,
Atlanta, Georgia, 30333, U.S.A.*

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SUMMARY

Actinomyces naeslundii fermented glucose primarily by the Embden–Meyerhof pathway, as based on ^{14}C -glucose fermentation data and enzyme studies. Enzymes of the oxidative pentose phosphate cycle were also present, but functioned only to a minor extent. Growth on glucose was increased 2- to 4-fold in the presence of substrate amounts of CO_2 or O_2 . This increase was attributed to the additional energy (ATP) made available from the breakdown of pyruvate to acetyl coenzyme A. In the absence of CO_2 or O_2 , pyruvate was reduced to lactate. The weight of organism produced/mole ATP (Y_{ATP}) was 15–18 g. units under anaerobic conditions with CO_2 , dependent on the growth medium, and 20 under aerobic growth conditions.

INTRODUCTION

Actinomyces naeslundii is a pathogenic organism which ferments sugars and requires substrate amounts of CO_2 for maximal growth. Pine & Howell (1956) and Buchanan & Pine (1963) investigated the glucose fermentation of *A. naeslundii* and found that fermentation products depended on the conditions of culture. Without added CO_2 (gaseous or bicarbonate), growth was limited and glucose was fermented to lactate

When substrate amounts of CO_2 were present, lactate formation was decreased, and, for each mole of CO_2 fixed, an equimolar amount of acetate, formate, and succinate was produced (Pine & Howell, 1956):

The ratio of products with added CO_2 was not constant, and dependent upon the specific fermentation a combination of equations (1) and (2) was observed. Based on the average results of three fermentations (Buchanan & Pine, 1963, table 1, no. 3, 5, and 7), a general equation was derived:

* Present address: Department of Cell Physiology, University of California, Berkeley, California.

CO₂ was fixed into the carboxyl groups of succinate (Pine, 1956, 1960). Presumably CO₂ functioned in the formation of oxaloacetate and malate, both of which acted as electron acceptors and permitted the formation of acetate and formate (Buchanan & Pine, 1963) from pyruvate. CO₂ could be partially replaced by either malate or fumarate, with a simultaneous increase in the formation of acetate and formate. Recently, Buchanan & Pine (1965) found that CO₂ was also required for the biosynthesis of aspartate for which the organisms lacked a permease.

When *Actinomyces naeslundii* grew aerobically, O₂ served as an electron (hydrogen) acceptor and CO₂ was required only to initiate growth (Pine & Howell, 1956). Buchanan & Pine (1963) found that glucose was degraded quantitatively to acetate and CO₂ in accordance with the equation :

As based on the above equations, glucose breakdown by *A. naeslundii* might occur by several pathways, and there was until now no evidence concerning which pathway was involved. The present paper presents evidence that *A. naeslundii* ferments glucose principally by way of the Embden-Meyerhof pathway, although the possible operation of other pathways to a minor degree is indicated from isotope data. Previous conclusions about the functions of CO₂ and O₂ in *A. naeslundii* were confirmed in the present work, and CO₂ and O₂ are related to the energy which is available for the growth of this organism.

METHODS

Actinomyces naeslundii (*A. bovis*, ATCC 10049), described previously (Buchanan & Pine, 1963), and *Actinomyces propionicus* (Buchanan & Pine, 1962) were grown at 37° in the casitone medium of Pine & Watson (1959) or in the casein hydrolysate medium of Pine & Howell (1956). Fermentation products were analysed as described previously (Buchanan & Pine, 1963). Lactate and succinate were isolated by Dowex-1 column chromatography (Busch, Hurlbert & Potter, 1952) as modified by Buchanan & Pine (1963). Purity was established by co-chromatography on paper of a sample of the isolated radioactive acids with authentic lactate or succinate. Chromatograms were developed descendingly by using ethanol + water + concentrated ammonium hydroxide (80 + 15 + 5, by vol.) as the solvent (Cheftel, Munier & Machebœuf, 1953). Acids were located as yellow spots against a blue background by spraying the chromatograms with bromocresol green (0.01% (w/v) in 95% (v/v) ethanol in water neutralized to pH 12 with 0.1 N-NaOH) and by radioautography. In all cases, yellow spots coincided exactly with the darkened areas of the X-ray film. Acetate and formate were isolated by steam distillation and were identified by their Duclaux constants.

For radioactivity determinations, compounds were oxidized to CO₂ and counted as BaCO₃. Oxidations were done in a combustion chamber equipped with a train of three collection tubes, one of which served as a KMnO₄ scrubber (5% (w/v) KMnO₄ in 0.3 N-H₂SO₄), leading from the chamber to two tubes containing a saturated solution of Ba(OH)₂. Helium was used to flush the system continuously. The BaCO₃ was separated by centrifugation in stoppered tubes, washed three times with boiling water, and suspended in 95% (v/v) ethanol in water. Suitable samples were pipetted into lightly greased planchets and dried under heat-lamps.

Radioactivity was measured with a continuous flow counter equipped with a 'micro-mil' window. 'Q-gas' (98.4% (v/v) He + 1.6% (v/v) butane) was used as the ionizable gas. Samples were corrected for background and self-absorption by reference to a standard curve obtained with known amounts of BaCO_3 .

Succinate, lactate and acetate were wet-ashed by the method of Van Slyke, Plazin & Weisiger (1951), and the specific activity of the BaCO_3 (c.p.m./ $\mu\text{mole BaCO}_3$) was multiplied by the number of carbon atoms in the molecule to give the average total specific activity. Formate was converted to CO_2 in the presence of acetate by oxidation with mercuric sulphate by the method of Friedemann (1938) as modified by Rabinowitz & Barker (1956). The residual acetate was collected by steam distillation and its purity verified by its Duclaux constants. Acetate was decarboxylated with the Schmidt reaction (Phares, 1951). After decarboxylation, the solution was made alkaline with KOH, and the methylamine was isolated by distilling into diluted HCl; methylamine was oxidized to CO_2 with alkaline permanganate (Katz, Abraham & Chaikoff, 1955). Lactate was decarboxylated with chromic acid (Calvin *et al.* 1949); the acetate formed was isolated and degraded as described above. Succinate was decarboxylated by the method of Phares & Long (1955). The specific activity of the methylene carbons of succinate was determined by subtracting the specific activity of the carboxyl carbons from the total specific activity obtained by wet-ashing. The validity of this method for determining the activity of the methylene carbons was established by decarboxylating a sample of synthetic succinate 2,3- ^{14}C . No radio-activity was released with decarboxylation and when the residual ethylenediamine was isolated on paper chromatograms (Buchanan, 1962), its specific activity was in excellent agreement with the specific activity of the original succinate.

For enzyme studies, the organism was grown in the Casitone medium containing 25 $\mu\text{moles NaHCO}_3/\text{ml.}$, under Na_2CO_3 + pyrogallol seals. A volumetric flask of either 1 or 2 l. capacity filled to the base of the neck was inoculated with 10 ml. of an actively growing culture and incubated for 3–4 days. Organisms were harvested by centrifugation, washed three times with 0.01 M-potassium phosphate buffer (pH 7.0) containing 0.01% (w/v) neutralized cysteine HCl. The packed cells were resuspended in this buffer to give a 50% (v/v) suspension.

Like corynebacteria and lactobacilli (Gunsalus, 1955) the actinomyces organisms were difficult to break. Treatment with lysozyme, grinding with glass beads, sonic oscillation with a 12 kc. apparatus (50 W.), and treatment in a French pressure cell were ineffective in releasing soluble protein. The only procedures found to break the organisms were grinding with alumina or subjection to a 20 kc. (75 W.) sonic oscillator (Heat Systems Co., Great Neck, New York).

For alumina-broken preparations, 8–10 g. alumina was added per g. wet wt. organism, and the suspension was ground at 4° in a Sorvall Omnimixer (Ivan Sorvall, Norwalk, Connecticut) for 10 min. in 30 sec. bursts at 1 min. intervals to prevent heating. For sonic preparations, suspensions of organism were subjected to sonic treatment at 4° for 10 min. in 30 sec. bursts at 1 min. intervals to prevent heating. Treated suspensions were centrifuged for 15 min. at 23,500 g at 4° to remove debris and unbroken organisms. The precipitate was discarded; the supernatant fluid was dialysed for 4 hr against 0.01 M-potassium phosphate buffer (pH 7.0) containing 0.01% (w/v) neutralized cysteine HCl. When the oxidation or reduction of nicotinamide nucleotides (NAD) was to be measured, the dialysed extract was centrifuged for

60 min. at 100,000 *g* in a preparative ultracentrifuge. The supernatant fraction, which contained the soluble enzymes, was saved; the black precipitate, which contained a very active NADPH₂ oxidase system, was discarded.

Enzymes were assayed at room temperature ($25^{\circ} \pm 2^{\circ}$). Oxidation and reduction of NAD were measured by the change in extinction at 340 m μ with a Beckman DU spectrophotometer. Fructosediphosphate aldolase (Enzyme Commission, subsequently referred to as E.C., no. 4.1.2.13) was assayed according to Sibley & Lehninger (1949) as modified by Bard & Gunsalus (1950), glucosephosphate isomerase (E.C. 5.3.1.9) according to Slein (1955), phosphate acetyltransferase (E.C. 2.3.1.8) according to Stadtman (1955) based on the reaction of acetyl phosphate with hydroxylamine (Lipmann & Tuttle, 1945), isocitrate dehydrogenase (E.C. 1.1.1.42) and malate dehydrogenase (E.C. 1.1.1.37) according to Ochoa (1955*a, b*), aconitate hydratase (E.C. 4.2.1.3) according to Anfinsen (1955), fumarate hydratase (E.C. 4.2.1.2) according to Massey (1955), and glutamate dehydrogenase (E.C. 1.4.1.3) according to Strecker (1955). Protein was estimated by the phenol method of Sutherland, Cori, Haynes & Olsen (1949) with bovine serum albumin as a standard. For each enzyme, the initial rate was directly proportional to the protein concentration. The rates varied little in different preparations.

The chemicals used were of reagent grade and were obtained from commercial sources.

RESULTS

One of the most useful techniques in elucidating metabolic pathways in micro-organisms is the use of ¹⁴C-labelled substrates (Wood, 1961). For heterotrophic organisms, an organic substrate labelled in a specific position is supplied to growing cultures and the fermentation products isolated and degraded to determine the positions of the label. For saccharolytic organisms such as *Lactobacillus casei* and *Streptococcus faecalis* (Gibbs, Dumrose, Bennet & Bubeck, 1950), glucose 1-¹⁴C and glucose 6-¹⁴C have been used most frequently. In the Embden-Meyerhof pathway, 1-¹⁴C and 6-¹⁴C glucose are both converted to fermentation products, such as lactate, which show identical labelling patterns. When glucose is fermented by other mechanisms (for example, the oxidative pentose phosphate pathway) the lactate formed in glucose 1-¹⁴C and glucose 6-¹⁴C fermentations shows quite different labelling patterns.

Table 1 shows the results of the fermentation of glucose 1-¹⁴C and glucose 6-¹⁴C by *Actinomyces naeslundii* grown anaerobically in substrate amounts (25 μ m./ml.) of CO₂ added to the medium as bicarbonate. The ratio of the fermentation products was essentially as is shown in equation (3). The radioactivity recovered in the formate, CO₂ and carboxyl groups of lactate and succinate was low. This eliminated the oxidative pentose phosphate cycle, Entner-Doudoroff, and hexose monophosphate pathways (see review by Wood, 1961) as major mechanisms for glucose dissimilation. Based on radioactivity recovered in the formate, CO₂ and carboxyl groups of acetate, formate and succinate, not more than 6% of the total glucose fermented was channelled through any one of these pathways.

In the Embden-Meyerhof pathway, the glucose 1-carbon and the glucose 6-carbon atoms are precursors of the methyl-carbon atoms of lactate, of acetate, and of a methylene carbon of succinate (assuming succinate synthesis from a C₃ unit + CO₂). Chemically the two methylene carbons of succinate are indistinguishable, and therefore

the total activity in both carbons must be considered. In general, the degradation data in Table 1 fit these requirements for formation by the Embden–Meyerhof pathway. The specific activities of acetate, lactate and succinate were approximately 50% of the original ^{14}C -glucose molecules. For unknown reasons, the specific activity of acetate isolated from the glucose 6- ^{14}C fermentation was low and was only 33% of the original ^{14}C -glucose. Of the total radioactivity in acetate and lactate, at least two-thirds resided in the methyl carbon atoms. Of the succinate, at least 90% of the total activity resided in the two methylene carbons.

Table 1. *Fermentation of glucose 1- ^{14}C and glucose 6- ^{14}C by Actinomyces naeslundii*

Organisms were grown anaerobically in 50 ml. Casitone medium (0.5%, w/v) containing either glucose 1- ^{14}C or glucose 6- ^{14}C with 25 μmoles NaHCO_3/ml . under Na_2CO_3 + pyrogallol seals. The following specific activities and total c.p.m., respectively, were used: glucose 1- ^{14}C 1012 c.p.m./ μmole and 12.75×10^6 c.p.m.; glucose 6- ^{14}C , 601 c.p.m./ μmole and 7.25×10^6 c.p.m. Assimilated glucose was determined by counting samples of washed organisms at infinite thinness. Other experimental details are given in the text.

	$\mu\text{moles}/100 \mu\text{moles}$ glucose fermented		Specific activity of ^{14}C -products in % of the original ^{14}C -glucose	
	Glucose 1- ^{14}C	Glucose 6- ^{14}C	Glucose 1- ^{14}C	Glucose 6- ^{14}C
CO_2 remaining	—	—	5.0	0.2
CO_2 fixed	52.5	54.9	—	—
$\text{CH}_3\text{—COOH}$	47.1	49.7	45.2	33.6
$\text{CH}_3\text{—}$	—	—	40.0	21.4
—COOH	—	—	7.0	9.3
HCOOH	48.2	52.9	5.8	0.8
$\text{CH}_3\text{—CHOH—COOH}$	21.4	23.6	52.7	48.0
$\text{CH}_3\text{—}$	—	—	37.8	37.8
—CHOH—	—	—	6.6	3.8
—COOH	—	—	4.0	6.5
$\text{HOOC—CH}_2\text{—CH}_2\text{COOH}$	54.9	54.5	53.1	47.5
HOOC—	—	—	2.0	0.4
$\text{—CH}_2\text{—}$	—	—	23.6	23.5
Redox index	0.98	0.98	—	—
Glucose assimilated	14.0	10.2	—	—
Carbon recovery (%)	77.9	76.7	—	—
^{14}C Carbon recovery (%)	78.0	79.4	—	—

Pertinent to demonstrating a particular metabolic pathway in an organism is the demonstration of its constituent enzymes. Aldolase is considered indicative of the Embden–Meyerhof pathway, and so far it has not been shown to function in other saccharolytic pathways (Buyze, van den Hamer & De Haan, 1957). Table 2 shows that aldolase was present in cell-free extracts of *Actinomyces naeslundii* and that its capacity was relatively high (cf. McDonald, Cheldelin & King, 1960). Extracts of a related organism, *Actinomyces propionicus* (Buchanan & Pine, 1962), also showed aldolase activity, with a rate about two-thirds that observed in *A. naeslundii*, thus indicating the Embden–Meyerhof pathway is possibly widespread in this group of organisms. Glucosephosphate isomerase, an enzyme of the Embden–Meyerhof pathway, and phosphate acetyltransferase, which often occurs in anaerobic bacteria (Stadtman, 1955), were also present in *A. naeslundii* (Table 2).

In a previous study, *Actinomyces naeslundii* was grown anaerobically in $^{14}\text{CO}_2$ and

glutamate and aspartate were isolated and degraded (Buchanan & Pine, 1965). The labelling pattern observed in glutamate was consistent with its biosynthesis from α -ketoglutarate, formed by the tricarboxylic acid cycle. This conclusion is supported by results of the present investigation which show that isocitric dehydrogenase, aconitase, and NADP-linked glutamic dehydrogenase were present in relatively high amounts in *A. naeslundii* (Table 2).

Table 2. *Demonstration of certain enzymes in cell-free extracts of Actinomyces naeslundii*

	μ moles substrate used/mg. protein/hr
Fructosediphosphate aldolase	1.6
Glucosephosphate isomerase	0.5
Phosphate acetyltransferase	18.0
Aconitate hydratase	1.7
Isocitrate dehydrogenase	8.5
Glutamate dehydrogenase (NADP linked)	7.5
Malate dehydrogenase	59.0
Fumarate hydratase	3.8
Glucose-6-phosphate dehydrogenase	0.5
Phosphogluconate dehydrogenase	2.6
Other enzymes of oxidative pentose phosphate pathway	0.1*

* This value does not represent the activity of any single enzyme, but instead is the over-all rate of conversion of ribose 5-phosphate to glucose 6-phosphate.

The mechanism by which CO_2 was incorporated into the carboxyl carbons of succinate by *Actinomyces naeslundii* was not determined unequivocally. However, a low but significant exchange reaction between $^{14}\text{CO}_2$ and the β -carboxyl carbon of oxaloacetate was demonstrated (Buchanan, 1962), and this provided evidence that the primary carboxylation reaction in *A. naeslundii*, leading to succinate, is the phosphopyruvate carboxylase (E.C. 4.1.1.32) reaction originally studied by Utter & Kurahashi (1954). Oxaloacetate would then be reduced to succinate by a reversal of the malic dehydrogenase, fumarase and succinic dehydrogenase (E.C. 1.3.99.1) reactions. The present demonstration (Table 2) of fumarase and malic dehydrogenase activities in *A. naeslundii* extracts supports this conclusion.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) and phosphogluconate dehydrogenase (E.C. 1.1.1.44) were demonstrated in extracts of *Actinomyces naeslundii* (Table 2). As based on the reduction of NADP with ribose 5-phosphate as substrate (Table 2), the other enzymes of the pentose cycle which convert ribose 5-phosphate to glucose 6-phosphate were also present. These data therefore indicate that *A. naeslundii* has a complete oxidative pentose phosphate cycle but, as pointed out above, its operation as a major mechanism of glucose breakdown in *A. naeslundii* was excluded on the basis of the glucose 1- ^{14}C fermentation data (Table 1). These data indicated that, at most, 6% if the total glucose fermented could go by this pentose pathway. Similar results were obtained in independent glucose 1- ^{14}C fermentations carried out under aerobic conditions (Buchanan, 1962), thus eliminating the possibility of its function as a major pathway in aerobic growth.

Because of its relation to *Actinomyces naeslundii*, *A. propionicus* was tested for certain enzymes found in *A. naeslundii*. Other than the aldolase mentioned above, 6-

phosphogluconic dehydrogenase and isocitric dehydrogenase were found in extracts of *A. propionicus*, at essentially the same concentration as in *A. naeslundii* (Buchanan, 1962).

With the recognition that glycolysis was the major pathway used for the fermentation of glucose by *Actinomyces naeslundii*, and that the fermentation products depended on the availability and nature of the added electron acceptors, we tried to correlate the relative yields of organism to the energy theoretically released in a particular type of

Fig. 1. Dependence of the yields of *Actinomyces naeslundii* on the conditions of culture. I, Aerobic growth in Casitone medium with CO₂ = ●; without CO₂ = ○. II, Anaerobic growth in Casitone medium with CO₂. III, Anaerobic growth in casein hydrolysate medium with CO₂. IV, Anaerobic growth in casein hydrolysate medium minus CO₂ plus malate. V, Anaerobic growth in casein hydrolysate medium minus CO₂. Cells were grown on a rotary shaker in test-tubes containing 5 ml. Casitone medium under KH₂PO₄ + Na₂CO₃ seals to supply gaseous CO₂ (I); in side-arm fermentation tubes (Pine, 1956) containing 50 ml. Casitone medium with 25 μmoles NaHCO₃/ml. under Na₂CO₃ + pyrogallol seals (II); in 50 ml. volumetric flasks containing 48 ml. casein hydrolysate medium with 25 μmoles NaHCO₃/ml. under either Na₂CO₃ + pyrogallol seals (III) or without NaHCO₃ under NaOH + pyrogallol seals (IV, V). Sodium malate was added to IV to 0.5% (w/v). Above values represent the average growth observed in duplicate vessels. Aerobic tubes were inoculated with 1 drop of a homogenized 72 hr culture diluted with water to an extinction of 0.5; anaerobic vessels were inoculated with the equivalent of 1 mg. dry wt. organisms. Growth was measured by the extinction at 600 mμ in a Spectronic-20 colorimeter. Extinction was related to dry weight by reference to a standard curve. The abscissa represents the initial concentration of glucose in the medium. All cultures were incubated 96 hr; maximum growth was usually attained in 48 hr; the ordinate represents the maximum value attained for each culture.

fermentation. The yields of organism obtained under various anaerobic or aerobic conditions of growth are given in Fig. 1. Except for anaerobic growth without CO₂, the extent of growth in all cases was proportional to the initial glucose concentration, at least for the first two points. As observed previously (Pine & Howell, 1956; Buchanan & Pine, 1963), growth was better in the presence of CO₂ or air, presumably owing in part to the additional energy released in pyruvate breakdown. In anaerobic growth without CO₂, growth was not proportional to the initial glucose concentration, although it was approximately linear up to 9.4 μ moles glucose utilized/ml. The non-linearity in the growth response was, in part, due to lack of aspartic acid, which could not be synthesized *de novo* without CO₂ and which could not be assimilated when added to the culture medium because of the absence of a permease (Buchanan & Pine, 1965).

Table 3. *Yields of Actinomyces naeshundii under various conditions of growth*

Curve of Fig. 1	Medium and substrate	Conditions	Glucose concentration (μ mole/ml.)	Y*	Equation no. (see text)	Net ATP/mole glucose*	Y _{ATP} †
I	Casitone, glucose	Air or air+CO ₂	4.7	87.2	4	4	21.8
			9.4	86.7	4	4	21.7
II	Casitone, glucose	Anaerobic+CO ₂	4.7	48.9	3	2.8	17.5
			9.4	51.0	3	2.8	18.2
			14.1	48.2	3	2.8	17.2
III	Casein hydrolysate, glucose	Anaerobic+CO ₂	4.7	42.5	3	2.8	15.2
			9.4	41.5	3	2.8	14.8
			14.1	41.1	3	2.8	14.7
IV	Casein hydrolysate, glucose+malate	Anaerobic, no CO ₂	9.4	40.4	5	4	10.1
V	Casein hydrolysate, glucose	Anaerobic, no CO ₂	4.7	24.7	1	2	12.3

* μ g. dry wt. organism/ μ mole glucose fermented (Bauchop & Elsdén, 1960).

† Based on the fermentation equations shown in the text, in which the formation of 1 mole of acetate represents a net gain of 2 moles of ATP and 1 mole of lactate or succinate/mole ATP.

‡ μ g. dry wt. organisms/ μ mole ATP (Bauchop & Elsdén, 1960).

Anaerobic growth in the absence of CO₂ but with malate (curve IV, Fig. 1) showed that malate partially replaced CO₂ by functioning as a source of aspartate and as an electron acceptor to become succinate, pyruvate being converted to acetate and formate. Analysis of fermentations done in absence of CO₂ but with added malate showed that *Actinomyces naeshundii* fermented glucose (at initial values not exceeding 10 μ moles/ml.) in accordance with the equation:

At this low concentration of glucose, malate effectively substituted for CO₂ with a yield of organism 80% of that observed for anaerobic growth with added CO₂. At higher glucose concentrations, growth was no longer proportional to the glucose concentration and large amounts of lactate were formed. Analysis of the products showed the fermentation occurred according to the equation:

Table 3 shows the yields of *Actinomyces naeshundii* calculated from data presented in Fig. 1, where growth was proportional to the added glucose. Growth is expressed in

the Y and Y_{ATP} units as defined by Bauchop & Elsdén (1960); the net moles ATP formed/mole glucose, as calculated from the appropriate fermentation equation given above, is shown also. For these calculations, we assumed that one net ATP is released in the formation of one mole of lactate or succinate; 2 ATP are formed in the production of acetate (one additional ATP being due to the breakdown of pyruvate to acetyl coenzyme A + CO_2 in air, or to acetyl coenzyme A + formate anaerobically). Bauchop & Elsdén (1960) defined the Y value as the g. dry wt. organism formed/mole glucose fermented; the value found for most organisms is 22 (Senez, 1962). The Y values observed with *A. naeslundii* grown anaerobically with substrate concentrations of CO_2 were 41.1–51.0, depending on the growth medium. These values are at least twice those reported for most other organisms. However, Gunsalus & Shuster (1961) reported a Y value of 37.5 of *Propionibacterium pentosaceum*, an organism which in many ways is closely related to the actinomyces species (Stanier & Van Niel, 1941; Buchanan & Pine, 1962). As a result of the high Y values observed with *A. naeslundii*, the Y_{ATP} values (g. dry wt. organism formed/theoretical mole ATP; Bauchop & Elsdén, 1960) are correspondingly 1.5–2 times higher than the Y_{ATP} value of 10.5 observed for most organisms (Senez, 1962). Similar but somewhat higher values were obtained for aerobically grown *A. naeslundii* (Table 3). However, when the fermentation was homolactic (curve V, Fig. 1) or when malate was substituted for CO_2 (curve IV, fig. 1), the Y_{ATP} values were 10.1 and 12.3, respectively. If the Y_{ATP} value of 10.5 be accepted as a universal constant (Senez, 1962), the data in Table 3 imply that *A. naeslundii* obtains additional energy in its glucose fermentation in the presence of CO_2 , perhaps at some point in the formation of succinate. However, this suggestion is complicated by the observation that the aspartate necessary for growth must be synthesized from a C_4 -dicarboxylic acid (Buchanan & Pine, 1965).

DISCUSSION

This communication shows that *Actinomyces naeslundii* ferments glucose primarily by the Embden–Meyerhof pathway. Enzymes of the oxidative pentose phosphate cycle are present, but isotopic data show that this is a minor pathway and does not account for more than 6% of the glucose fermented. The operation of phosphoketolase, although we have no direct evidence for its presence, could explain in part the low activity of ^{14}C in the methyl carbons of acetate when glucose 6- ^{14}C was the substrate. It is possible that the pentose pathway is essential for providing certain intermediates, such as pentoses, which are used for growth and that it does not function in energy production. Such a role would restrict the function of the pentose pathway in *A. naeslundii* to the interconversion of sugars needed for biosynthesis.

The dependence of the fermentation products on the conditions of growth shows that the pyruvate (or phosphoenolpyruvate) formed from glucose can take alternative routes. In the absence of CO_2 or O_2 , pyruvate is reduced to lactate. When substrate amounts of CO_2 are present, lactate formation is relatively minor and CO_2 is used in the synthesis of oxaloacetate and malate which function as electron acceptors to form succinate. CO_2 is believed to be fixed into phosphoenolpyruvate to yield oxaloacetate, which is reduced to succinate by a reversal of the malic dehydrogenase, fumarase and succinic dehydrogenase reactions. The reduction of oxaloacetate to succinate requires four electrons, and four electrons are released in the conversion of a glucose molecule

to two molecules of phosphoenolpyruvate. Therefore, when CO_2 is present, a second molecule of phosphoenolpyruvate is free and is converted to acetate and formate, by way of pyruvate. This over-all process establishes a redox index of unity in the hetero-lactic fermentation as shown in equation (3). In aerobic growth, oxygen serves as an electron acceptor and glucose is oxidized to acetate, CO_2 and H_2O ; the mechanism by which the electrons react with oxygen has not been determined. The over-all mechanisms of glucose fermentation for *Actinomyces naeslundii* are depicted in Fig. 2, as determined from this and preceding reports (Buchanan & Pine, 1963, 1965).

Fig. 2. Proposed mechanism of glucose breakdown by *Actinomyces naeslundii*. Major anaerobic fermentation products are singly underlined; major aerobic fermentation products are doubly underlined. Reactions believed to be involved principally in energy production are shown with solid arrows; reactions believed to be involved principally in biosynthesis are shown with broken arrows. The enzymes found in extracts are shown.

The breakdown of pyruvate to acetyl coenzyme A or acetylphosphate releases additional energy for growth. Under conditions promoting pyruvate breakdown, the yields of *Actinomyces naeslundii*/glucose molecule are higher than for most other organisms. Whether or not this is peculiar to the actinomyces (and the propionibacteria; Gunsalus & Shuster, 1961) is an interesting point that may be decided only by further experiments.

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Note added in Press

Recent cell wall analyses of strain ATCC 10049 showed the major amino acids to be glutamic acid, alanine, lysine, and glycine. The major sugars were galactose and mannose. Large quantities of hexosamine were also present. On the basis of cell wall analysis this strain is more correctly classified as *Actinomyces israelii* than *A. naeslundii*.