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Truncated Glycolytic System in *Veillonella*

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ABSTRACT

ROGOSA, M., (National Institutes of Health, Bethesda, Md.), M. I. KRICHEVSKY, AND F. S. BISHOP. Truncated glycolytic system in *Veillonella*. J. Bacteriol. **90**:164–171. 1965.—Intact *Veillonella* cells do not utilize carbohydrates for growth, nor are carbohydrates fermented. In cell extracts, there is no detectable glucokinase or fructokinase. Cell extracts do not degrade glucose or fructose unless supplemented with yeast hexokinase. Under these conditions, triose phosphates are formed in the presence of a hydrazine trap. When glucose-C^{14} plus added hexokinase or fructose-1,6-diphosphate-C^{14} was incubated with cell extracts, the production of CO_2, acetate, pyruvate, propionate, and lactate was detected. It is concluded that, except for a hexokinase, all the activities required for a glycolytic system are present.

Members of the genus *Veillonella* are ecologically significant because of their relative abundance in the mouth of man and certain rodent species, and in the rumen of cattle and sheep (Lewkowicz, 1901; Hall and Howitt, 1925; Douglas, 1950; Langford, Faber, and Pelczar, 1950; Johns, 1951*a*; Rogosa, 1964). These organisms are metabolically active on such substrates as pyruvate, oxaloacetate, malate, fumarate, succinate, and lactate (Foubert and Douglas, 1948; Johns, 1951*b*; Rogosa, 1964).

Descriptions of *V. parvula* [Prévot (1933) and 5th, 6th, and 7th editions of *Bergey's Manual*] state that this organism ferments glucose. However, a large number of investigators [previously cited by Rogosa (1964)] have been unable to isolate any *Veillonella* capable of fermenting carbohydrates, and Rogosa (1964) demonstrated that glucose was not fermented by *V. parvula*, or indeed by any of 107 strains of *Veillonella* tested, regardless of specific epithet. Moreover, in addition to glucose, none of 27 carbohydrates was fermented by any member of the genus. It was also shown that growth occurred with pyruvate, lactate, fumarate, or malate. In subsequent unpublished experiments, glucose and fructose were not utilized even when they were incorporated into the medium of Rogosa and Bishop (1964) in combination with pyruvate, lactate, fumarate, or malate. There was also no growth with D-glucose-6 - phosphate (G6P), D - fructose - 6 - phosphate (F6P), D-fructose-1,6-diphosphate (HDP), 3-phospho-D-glycerate (3PPG), or 2-phospho-D-glycerate (2PPG).

Johns (1951*a*) discussed the nonutilization of carbohydrates in these pertinent terms: "The anabolic (biosynthetic) process from pyruvate to carbohydrate has usually been conceived as the reverse of the catabolic process. This involves the production of phosphorylated derivatives of glucose and the question arises, why is it that glucose is not fermented by some bacteria such as *Veillonella*? It seems that the enzyme necessary to carry out the primary phosphorylation of the glucose may be missing."

This paper will present evidence that *Veillonella* extracts lack hexokinase, but that the activity of other glycolytic enzymes is demonstrable.

MATERIALS AND METHODS

Glucose and fructose were chemically pure special products (Pfanstiehl Chemical Corp., Waukegan, Ill.) with specific rotations of +52.7° and −92.4°, respectively. The sodium salts of G6P and HDP, and the barium salts of F6P, 2PPG, and 3PPG, were Boehringer products obtained from Calbiochem. From Sigma Chemical Co., St. Louis, Mo., we obtained nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), adenosine-5′-(pyro)-triphosphate (ATP), adenosine-5′(pyro)-diphosphate (ADP), sodium pyruvate, crystalline hexokinase having an activity of 1,200,000 to 1,500,000 KM units per g (Kunitz and McDonald, 1946), glucose-6-phosphate dehydrogenase with an activity of 60 to 100 units per mg of protein (1 unit reduces 1 μmole of NADP per min at *p*H 7.4 at 25 C), and crystalline lactic dehydrogenase from beef heart muscle [1 unit equals a change in optical density at 340 mμ of 0.001/min at 25 C at *p*H 7.5 (1-cm light path), or an equivalent of the oxidation of 4.82×10^{-4} μmoles of NADH]. The

isotopically labeled compounds used were pyruvate-*2*-C^{14}, fumaric acid-*1,4*-C^{14}, succinic acid-*1,4*-C^{14} (Volk Radiochemical Co., Skokie, Ill.); uniformly labeled glucose-C^{14} and uniformly labeled HDP-C^{14} (Schwarz Bioresearch, Inc., Orangeburg, N.Y.); and malic acid-*3*-C^{14} (Calbiochem). Certain of these C^{14} compounds were used only as chromatographic standards.

For the preparation of cell extracts, cells were grown from 20% (v/v) inoculum at 36 C for 18 to 24 hr in a medium (V25) consisting of 0.5% Trypticase (BBL), 0.3% yeast extract (BBL), 1.5% (v/v) sodium lactate (60%), 0.075% sodium thioglycolate, 0.01% Tween 80, and 0.01% glucose; adjusted to *p*H 7.5 with solid K_2CO_3. The cells were harvested by centrifugation at 13,000 × *g* for 20 min at 2 C, or by continuous-flow centrifugation, and were washed once with distilled water. The washed cell crop was taken up in 1% or less of the original culture volume, and the cells were disintegrated by four passages through a French pressure cell (model 12-10 hydraulic press; American Instrument Co., Silver Spring, Md.). The cell extract was separated from debris by refrigerated centrifugation for 10 min at 27,000 × *g*. Protein in the crude cell extract was determined immediately in a suitable dilution by the biuret reaction (Gornall, Bardawill, and David, 1949). A part of the crude extract was used in experiments on the day of preparation, and any excess was stored at either −20 or −78 C.

A modification of a spectrophotometric method of Kornberg and Horecker (1955) to detect G6P dehydrogenase activity was used in hexokinase assays. The 1-cm cuvettes contained, in a total volume of 1.51 ml, 15 μmoles of glycylglycine (*p*H 7.5); 10 μmoles each of ATP and $MgCl_2 \cdot 6H_2O$, both at *p*H 7.0; 12.5 μmoles of glucose; 0.5 μmoles of NADP; 1.3 units of G6P dehydrogenase; and *Veillonella* extract, 5.2 or 10.4 mg protein. Readings were made at 340 mμ with an Optica monochromator equipped with a Gilford multiple sample absorbance recorder. The total chart span was 0.1 absorbance (optical density unit).

Triose phosphate was detected by a slight modification of the color development stage of the method of Sibley and Lehninger (1949) for the estimation of aldolase activity; i.e., 3 ml of 2.5 N NaOH were substituted for the final 7 ml of 0.75 N NaOH, the solution was incubated the usual 10 min at room temperature, and finally 4 ml of 95% ethyl alcohol were added to the tubes (20 by 150 mm). The tube contents were then thoroughly mixed with the aid of a Cyclo-mixer (Clay-Adams, Inc., New York, N.Y.), and the color was read in matched tubes in a Spectronic-20 colorimeter at 540 mμ. Spectral curves of the triose chromogen, made with a Bausch & Lomb Spectronic-505 spectrophotometer, were obtained for each reaction system to establish the identity of the chromogen.

Fructose and fructose esters were estimated according to Roe, Epstein, and Goldstein (1949). Glucose was detected by the glucose oxidase method with the use of the micro method with the Glucostat kit of Worthington Biochemical Corp., Freehold, N.J. Carbohydrates in certain fractions were also determined by the anthrone technique (Dreywood, 1946), and pyruvate was measured by the method of Friedmann and Haugen (1943). Total and inorganic phosphate were determined by the method of Lowry (1957), and lactic acid, according to Barker and Summerson (1941). Sugar phosphates were chromatographed on cellulose thin-layer plates with the following solvent systems (Bandurski and Axelrod, 1951): (i) methanol–formic acid–water (80:15:5) and (ii) methanol–ammonium hydroxide–water (60:10:30). Spots were revealed by spraying with the ferric chloride and sulfosalicylic acid reagents of Wade and Morgan (1953).

Sample preparation and packing of Celite columns were done as described by Swim and Utter (1957). However, the pretreatment of the Celite and the solvent elution program were modified according to a procedure developed by C. L. Wittenberger of this Institute. The Celite was pretreated as follows. About 5 lb (2.3 kg) of Celite 535 (Johns-Mansville Co., New York, N.Y.) were put on a layer of Whatman no. 2 filter paper in a large Büchner funnel to permit washing by suction with the following solvents: (i) three times with enough water to cover; (ii) two times with 3.78 liters of acetone each time; (iii) six times with 2.37 liters of analytical reagent chloroform each time (Malinckrodt Chemical Works, New York, N.Y.); (iv) two times with 3.78 liters of acetone each time. The Celite was spread out on aluminum foil and dried for 2 days at room temperature and further dried in an oven at 100 C for 2 hr.

The solvents employed for chromatographic elution were all saturated with 0.2 N H_2SO_4 by shaking 1 liter with 10 ml of the acid in a separatory funnel and filtering the separated solvent through Whatman 24-cm fluted no. 12 paper contained in a 15-cm funnel. The filtration served to remove excess water. The organic acids were eluted in 10-ml fractions from the 6-g Celite columns (1-cm internal diameter) by the following sequence of solvents: butyric or propionic acid, or both, were eluted by 50 ml of chloroform with a peak in tubes 3 to 4; acetic acid, by 60 ml of 3% butanol plus 97% chloroform with a peak in tubes 8 to 9; pyruvic or formic acid, or both, by 60 ml of 8% butanol plus 92% chloroform with a peak in tubes 14 to 15; and lactic or succinic acid, or both, by 90 ml of 20% butanol plus chloroform, usually peaking in tubes 20 to 22.

Ion-exchange column chromatography on Dowex 1-formate, with formic acid and ammonium formate as eluants, was performed as described by Bush, Hurlbert, and Potter (1952) and Bergmann, Towne, and Burris (1958).

The C^{14} content of samples was determined with a Tri-Carb liquid scintillation spectrometer, series 314E (Packard Instrument Co., La Grange, Ill.). Samples (1 ml) of the fractions from columns were dispensed into counting vials containing 15 ml of 0.4% 2,5-diphenyloxazole in reagent-grade

toluene, and 3 ml of absolute ethyl alcohol (U.S. Industrial Chemicals Co., New York, N.Y.). All other samples (up to 1 ml) were counted in 20 ml of a second counting fluid described by Kinard (1957), except that α-naphthylphenyloxazole was omitted. When a gel was required for dispersion of particulate samples, 4% Cab-O-Sil (Godfrey L. Cabot, Inc., Boston, Mass.) was also included (Gordon and Wolfe, 1960). Samples were corrected for quenching and overall counting efficiency by internal standardization. Results were calculated as disintegrations per minute (dpm).

RESULTS

In an assay for hexokinase, which catalyzes the phosphorylation of glucose to G6P, optical density at 340 mμ was balanced against a cuvette containing the reaction system except for the omission of glucose. No hexokinase activity was detected in an extract from *V. alcalescens* VH-11. A parallel determination with an extract of *Lactobacillus arabinosus* ATCC 8014, run as a positive control, showed an increase in optical density from 0.09 to 0.96 during 5 min. Although the protein content of the crude *Veillonella* extract in the reaction mixture (5.2 mg) was unusually high for this type of assay, it was still possible that weak activity might be detectable if more extract was used. Therefore, a second type of experiment was performed with a reaction mixture containing twice as much cell protein (10.4 mg). With *Veillonella* extracts alone, there was a slow negative drift of similar magnitude to controls without substrate (presumably due to the settling of solids). To test whether the reaction system was still capable of supporting hexokinase activity (if the enzyme were present), an active extract of *L. arabinosus* was added, and an immediate increase in optical density was recorded (0.0625 per min). Thus, under conditions where added *Lactobacillus* extract exhibited marked hexokinase activity, the *Veillonella* extract had no detectable hexokinase.

The next series of experiments were designed to test whether triose phosphates were formed in reaction mixtures containing various six-carbon glycolytic intermediates in the presence of a hydrazine trap. Table 1 summarizes the results obtained with *V. alcalescens* VH-11. It should be noted that the optical density of each sample was determined by use of a blank consisting of an identical reaction mixture in which the trichloroacetic acid was added prior to the substrate. Independent determinations indicated that none of the substrates reacted to give interfering colors under the test conditions. With glucose as the substrate, and no added hexokinase, triose phosphate formation was not detected. Direct assay of the reaction mixture demonstrated that none of the glucose had been degraded. When yeast hexokinase was added to such a reaction mixture, triose phosphate formation was evident. To assess the contribution of the hexokinase preparation to the overall sequence of reactions, a reaction mixture was prepared in which the bacterial extract was omitted. In this case, triose phosphate formation was only 17% as great as in the complete system. Furthermore, with G6P, F6P, or HDP as the substrates (without added hexokinase), triose phosphate was detected in increasing amounts correlated with the respective positions of the substrates in a classical glycolytic system. Since yeast hexokinase can utilize the furanose form of fructose as a substrate (Gottschalk, 1947), an analogous experiment, with fructose instead of glucose, indicated that the extract possessed no detectable activity capable of phosphorylating the keto sugar. The

TABLE 1. *Results of triose phosphate determinations with extracts of Veillonella alcalescens VH-11**

Substrate	Triose phosphate
	μmoles
Glucose	0
Glucose + hexokinase	9.2
Glucose + hexokinase (without cell extract)	1.6
G6P	1.4
F6P	16.8
HDP	>23.0
Fructose	0
Fructose + hexokinase	13.6

* The cell reaction mixtures for the detection of fructose ester formation from glucose or G6P, or triose phosphate formation from the hexose substrates were as follows: glucose, fructose, G6P, F6P, or HDP, 20 μmoles; tris(hydroxymethyl)aminomethane buffer (*p*H 8.6), 100 μmoles; hydrazine sulfate (*p*H 8.6), 140 μmoles; (except when HDP was the substrate) 20 μmoles of ATP (*p*H 7.0) and 5 μmole of $MgCl_2 \cdot 6H_2O$ (*p*H 7.0); 33 mg of cell extract protein for all but HDP where 3.3 mg of protein were used; 320 KM units of crystalline yeast hexokinase as indicated; total volume, 3.62 ml. Reaction mixtures were incubated for 1 hr at 37 C in tubes (15 x 125 mm) under stationary conditions. The reactions were stopped by adding 2 ml of 10% trichloroacetic acid. After centrifugation, supernatant samples (usually 1 ml) were analyzed. Controls included (i) reaction mixtures in which substrates were added after the addition of trichloroacetic acid; (ii) reaction mixtures to which substrate was never added; and (iii) reaction mixtures in which cell extracts were omitted. All triose phosphate values are corrected for endogenous activity.

addition of yeast hexokinase, however, resulted in the formation of triose phosphates.

The results with *V. alcalescens* VH-11 prompted studies with cell extracts of other strains and species. These were *V. alcalescens* FIS, *V. parvula* BYR-2, *Veillonella* sp. HV-19, and *Veillonella* sp. RV-12X, all representative of distinct serological groups. The results were qualitatively the same as shown in Table 1, thus suggesting a general similarity of behavior in the genus *Veillonella*.

The dependence of phosphofructokinase activity on ATP was tested by the aldolase assay. With a crude extract of *V. alcalescens* VH-11, ATP exerted a marked stimulatory effect on the production of triose phosphates from F6P. Magnesium ion was without effect under these conditions.

TABLE 2. *C^{14} balance from glucose**

Compound	Disintegrations/min × 10^{-4}	Per cent
Initial glucose	489.4	100.00
Residual glucose	45.3	9.26
Products		
Fructose	13.3	2.72
Sugar monophosphates	425.8	87.00
HDP	23.6	4.82
Lactate	1.8	0.37
Acetate	7.0	1.43
Propionate	0.3	0.06
Pyruvate	0.5	0.10
CO_2	2.0	0.41
Total residual glucose + products	519.6	106.17

* Except for hexokinase, all ingredients of the reaction mixture were adjusted to *p*H 7.0. The main compartment of vented double side-arm Warburg flasks contained 66.6 mg of extract protein, 320 KM units of hexokinase, and the following number of μmoles of each of the following: potassium phosphate (100); ATP (40); $MgCl_2 \cdot 6H_2O$ (4.8); and NAD (0.15). After gassing with N_2 and equilibration, 40 μmoles of glucose containing a C^{14} activity of 489 × 10^4 dpm were tipped in from one side bulb to make a total volume of 3.05 ml. The center well contained 0.05 ml of 4 N NaOH. After incubation at 37 C for 4 hr, the reaction was stopped by tipping in 0.10 ml of 4 N H_2SO_4 from the second side bulb. The C^{14} activities of 45.3 × 10^4 and 13.3 × 10^4 dpm for residual glucose and fructose formed, respectively, were calculated from the chemical analyses. This is in excellent agreement with the actual 58.7 × 10^4 dpm for peak 1 from Dowex formate columns in which these carbohydrates were found. The C^{14} activities of the carboxylic acids were derived from eluate fractions from Celite columns. The radioactivities of sugar monophosphates and HDP were derived from peaks 3 and 4, respectively, from Dowex formate columns.

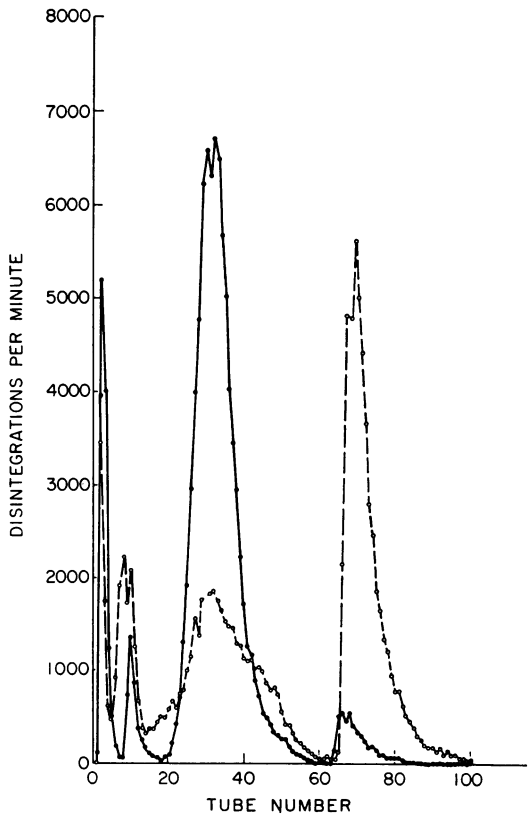

FIG. 1. *C^{14} distribution of eluate fractions from Dowex 1 formate. Eluate fractions from glucose + hexokinase (solid line) and HDP (broken line) fermentations with Veillonella alcalescens VH-11. Samples (0.3 ml from glucose + hexokinase and 0.4 ml from HDP reaction mixtures) were placed on the respective columns. Disintegrations per minute (dpm) represent the radioactivity detected in 1 ml from each 10-ml eluate fraction. Peak 1 occurred in the range of tubes 1 to 6 with maximal dpm in 2 to 3; peak 2 was from tubes 8 to 20 with maximal dpm in 9 to 10; peak 3 was from 20 to 60 with maximal dpm in 30 to 34; peak 4 was from 60 to 100 with maximal dpm in tubes 65 to 70. Experimental conditions were as in Tables 2 and 3.*

The foregoing experiments demonstrated that the addition of crystalline yeast hexokinase to reaction mixtures containing glucose enables cell extracts to convert the resultant G6P to fructose esters and triose phosphates. However, no evidence has yet been presented to show that the triose phosphate thus formed may be further degraded.

For this purpose, uniformly labeled C^{14}-glucose

plus hexokinase was incorporated in reaction mixtures with *V. alcalescens* extracts as described in Table 2, and the products were determined. Glucose analysis indicated that 93% (37 μmoles) of the added glucose disappeared. The residual glucose was found in peak 1 from Dowex formate columns (Fig. 1). The C^{14} content of peak 1 was 9.3% of the total C^{14} activity originally added. Glucose oxidase analyses of the peak 1 fractions confirmed that almost all (98.2%) of the C^{14} activity in this peak could be accounted for as glucose. A small amount of fructose was also detected chemically in peak 1 and was calculated to be 2.7% of the total C^{14} activity. In addition,

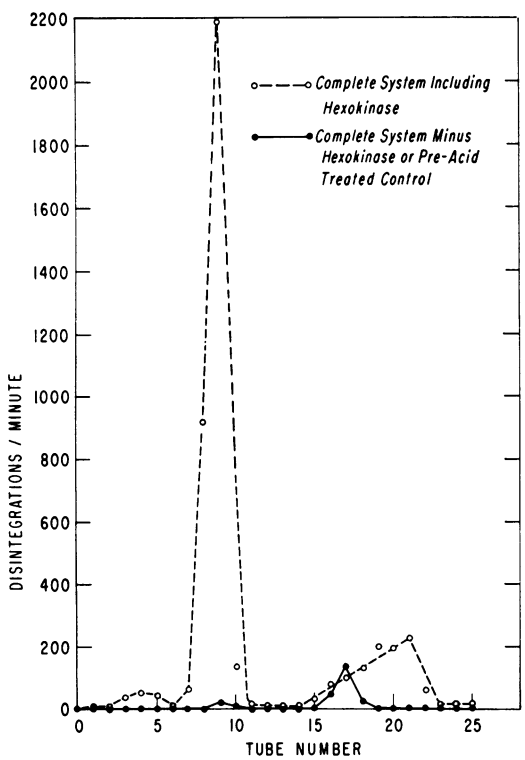

FIG. 2. *C^{14} distribution of eluant fractions from Celite columns. Veillonella alcalescens VH-11 was employed with C^{14} glucose as substrate (see Table 2). Samples (1.5 ml) from reaction mixtures were placed on the columns. Disintegrations per minute represent radioactivity detected in 1 ml from each 10-ml eluate fraction. This also applies for Fig. 3. For solvent elution sequence of Celite columns see Materials and Methods. A trace of an unidentified radioimpurity was present in peak 3 from acid-pretreated controls. Glucose reaction mixtures but without hexokinase gave an elution pattern identical with acid-pretreated controls. Elution pattern from CH_3-C^{14}-OCOOH reaction mixture was identical with that from glucose + hexokinase.*

TABLE 3. *C^{14} balance from HDP**

Compound	Disintegrations/ min × 10^{-4}	Per cent
Initial HDP	379.8	100.0
Residual HDP	169.4	44.6
Products		
Fructose	49.4	13.0
Sugar monophosphates	143.3	37.7
Lactate	8.5	2.2
Acetate	7.4	1.9
Propionate	1.2	0.3
Pyruvate	0.4	0.1
CO_2	3.2	0.8
Total residual HDP + products	382.8	100.6

* All ingredients and experimental conditions were the same as stated in Table 2 except that ATP, glucose, and hexokinase were omitted, and ADP (40 μmoles) and 40 μmoles of HDP with a C^{14} activity of 382.8 × 10^4 dpm were included. The C^{14} activities were derived as in Table 2.

known glucose and fructose appeared exclusively in peak 1 from Dowex formate columns. Sugar monophosphates (G6P and F6P) accounted for 87% and HDP comprised 4.8% of the original C^{14} activity. The presence of sugar monophosphates and HDP in peaks 3 and 4, respectively, was established by the following procedures: column chromatography of known compounds on Dowex-1-formate, phosphate analyses of each eluant fraction in which the phosphate values paralleled the radioactivity, and thin-layer chromatography where complete C^{14} recoveries were obtained from the appropriate spots from thin-layer cellulose plates (see Materials and Methods). Peak 2 (Fig. 1) was not homogeneous and contained the carboxylic acids listed in Table 2. The pyruvate-C^{14} activity was estimated from the chemical analyses of fractions from peak 2 (Fig. 1). Lactate, acetate, and propionate were best resolved from Celite columns in exact correspondence with known compounds. A typical profile obtained from such Celite columns is illustrated in Fig. 2, along with the results from the control reaction mixtures denoted in the figure. Peak 1 consisted of propionic acid and peak 2 of acetic acid. Peak 3 contained lactic acid, although the presence of a small amount of succinic acid is not precluded.

A C^{14} balance from an experiment in which uniformly labeled HDP served as substrate for cell-free extracts of *V. alcalescens* VH-11 is given in Table 3. The C^{14} activities of the compounds listed in Table 3 were derived in the same way as

previously described for the glucose experiments. A comparison of Fig. 1, 2, and 3, and Tables 2 and 3, reveals that the products from the glucose and HDP experiments were qualitatively similar.

However, quantitative differences among the various end products were found. For example, lactate accounted for 15.6 and 41.5% of the carbon in the terminal end products from glucose-C^{14} and HDP-C^{14}, respectively. To determine whether the operation of the latter stages of a glycolytic system could be enhanced, the following experiments were performed. Lactic dehydrogenase (LDH) was included in reaction mixtures to trap the pyruvate formed as lactate and to provide a reduced NAD ($NADH_2$) regenerating system. The reaction mixtures and results are shown in Table 4. In experiment 1, approximately twice as much lactate was formed in the presence of LDH as in its absence, regardless of the substrate used. In experiment 2, a stimulation of lactate production, dependent on the presence of LDH, was again noted. In addition, the substitution of pyruvate and $NADH_2$ for the fructose esters and NAD resulted in the stimulation of lactate production quantitively comparable to the amount of $NADH_2$ added, i.e., 7.5 μmoles. When HDP was the substrate and $NADH_2$ presumably was being generated at the glyceraldehyde-3-phosphate dehydrogenase level, the lactate yield was about 2.5 times that found when pyruvate was the substrate. In the two experiments, comparable protein levels of the two different cell extract preparations were used. Also, when LDH was added with HDP, the formation of lactate was proportional to the time of incubation. In contrast, when F6P was the substrate, the lactate accumulation was found to be highly variable. The last observation was also true in the experiments, previously described, where triose phosphates rather than lactate were assayed.

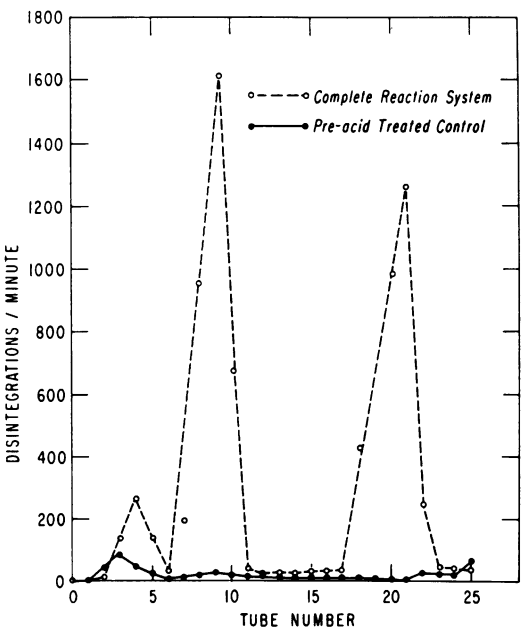

FIG. 3. *C^{14} elution pattern from Celite columns after HDP degradation by Veillonella alcalescens VH-11. A trace of a radioimpurity was present in peak 1 from acid-pretreated controls.*

TABLE 4. *Lactic acid production by Veillonella alcalescens VH-11**

Substrate	Expt 1 (2 hr)		Exp 2 (4 hr)	
	With LDH	Without LDH	With LDH	Without LDH
	μmoles	*μmoles*	*μmoles*	*μmoles*
F6P.....	5.8	3.0	0.7	0
HDP.....	11.7	6.6	20.8	5.5
Pyruvate...	—	—	7.9	0.6

* The reaction mixtures were as follows: substrates, 40 μmoles; phosphate buffer (*p*H 7.0), 100 μmoles; ATP (*p*H 7.0), 20 μmoles; $MgCl_2 \cdot 6H_2O$ (*p*H 7.0), 5 μmole; NAD (with F6P and HDP), 7.5 μmoles; $NADH_2$ (with pyruvate), 7.5 μmoles; lactic dehydrogenase (where indicated), 95,000 units; cell extract protein, 38 mg; total volume, 4.01 ml. Incubation was at 37 C in Thunberg tubes repeatedly evacuated and gassed with N_2. The reactions were stopped by adding 0.1 ml of 6 N H_2SO_4.

DISCUSSION

Although the usual aldolase assay system utilizes HDP as substrate (Taylor, 1955; Christian, 1955), Ling, Byrne, and Lardy (1955) described a coupled enzyme assay involving two reactions.

$$\text{F6P} + \text{ATP} \xrightarrow{\text{phosphofructokinase}} \text{HDP} + \text{ADP} \quad (1)$$

$$\text{HDP} \xrightarrow{\text{aldolase}} \text{dihydroxyacetone phosphate} + \text{glyceraldehyde-3-phosphate} \quad (2)$$

Phosphofructokinase activity was followed by adding purified muscle aldolase and measuring the glyceraldehyde-3-phosphate formed in reaction 2. The same principle was applied in the present work, even though a different analytical technique was used in detecting triose phosphates. However, the aldolase activity was not extrinsic but rather originated from the *Veillonella* extract protein, itself.

A high level of 320 KM units of hexokinase was

purposefully used to convert the 20 to 40 μmoles of glucose to G6P rapidly. The crystalline preparations, although the best available, were contaminated with a series of enzymes so that, in addition to G6P, a small amount of F6P and triose phosphate were also formed. This has already been shown in Table 1 for the glucose plus hexokinase reaction mixture without *Veillonella* extract.

Based on the triose phosphate assays, the following enzymatic activities were demonstrated: (i) a phosphohexose isomerase, (ii) a phosphofructokinase, and (iii) an aldolase. Thus, with the exception of a hexokinase, the activities of a classical glycolytic system at the hexose level are demonstrable. The phosphofructokinase activity was found to be quite variable and dependent on the particular extract used.

To answer the question as to the presence of enzymatic activities capable of catalyzing reactions ascribable to the three-carbon portion of a glycolytic system, the experiments with C^{14}-labeled substrates (and no hydrazine trapping of carbonyl functional groups) should be considered. When HDP-C^{14} was the substrate, 9.8% of the substrate carbon utilized could be accounted for as pyruvate or products derived from pyruvate (i.e., acetate, CO_2, lactate, and propionate). The carbon products from pyruvate with cell extracts, under conditions similar to those used here, were found by McCormick, Ordal, and Whitely (1962) to be acetate and CO_2. Independent unreported experiments in this laboratory have confirmed this, and, thus, the major carboxylic acid products obtained from glucose-C^{14}, HDP-C^{14}, and pyruvate were qualitatively identical. The substitution of glucose-C^{14} for HDP-C^{14} in the reaction mixtures resulted in no detectable utilization of the substrate or accumulation of radioactive end products unless crystalline yeast hexokinase was added. With glucose-C^{14} plus hexokinase, the results were qualitatively identical to those described for HDP-C^{14} as the substrate. However, the overall yield of C^{14} in the aforementioned terminal reaction products was lowered to 2.6% of the glucose-C^{14} utilized. Direct chemical assay of lactate in the experiments with LDH showed that a significant accumulation of lactate occurred with a hexose substrate, such as HDP. In this case, $NADH_2$ may be regenerated because of the enhanced production of lactate in competition with the other pyruvate-degrading activities known to be present in these organisms (McCormick et al., 1962). Thus, cell-free extracts of *V. alcalescens* VH-11, when supplemented with hexokinase and certain cofactors, have been demonstrated to possess all the enzymatic activities required for the conversion of glucose to pyruvate and beyond. The absence of hexokinase offers a reasonable explanation as to why these organisms do not utilize glucose as an energy source.

Since hexokinase appears to be absent and the activities required for the remainder of a glycolytic system are present, the hexose moieties, polymerized in their lipopolysaccharides (Mergenhagen, 1964 and *personal communications*), could not result from direct phosphorylation and subsequent interconversions, but rather they could be synthesized from added carboxylic acids through the functional reversal of the glycolytic system. The data in Table 3 provide presumptive evidence for at least one of the activities required to achieve functional reversal of a classical glycolytic system (Krebs and Woodford, 1965); i.e., there was an accumulation of hexose monophosphates and free fructose accounting for 50.7% of the initial HDP added. Thus, a fructose diphosphatase activity is present. A system for forming phosphoenolpyruvate from nonphosphorylated precursors has yet to be found. At any rate, the G6P generated inside the cells by such a reversed glycolytic system would bypass the requirement for a kinase.

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LITERATURE CITED

BANDURSKI, R. S., AND B. AXELROD. 1951. The chromatographic identification of some biologically important phosphate esters. J. Biol. Chem. **193**:405–410.

BARKER, S. B., AND W. H. SUMMERSON. 1941. The colorimetric determination of lactic acid in biological fluids. J. Biol. Chem. **138**:535–545.

BERGMANN, F. H., J. C. TOWNE, AND R. H. BURRIS. 1958. Assimilation of carbon dioxide by hydrogen bacteria. J. Biol. Chem. **230**:13–24.

BUSH, H., R. B. HULBERT, AND V. R. POTTER. 1952. Anion exchange chromatography of acids of the citric acid cycle. J. Biol. Chem. **196**:717–727.

CHRISTIAN, W. 1955. Aldolase from yeast, p. 315–320. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.

DOUGLAS, H. C. 1950. On the occurrence of the lactate fermenting anaerobe, *Micrococcus lactilyticus*, in human saliva. J. Dental Res. **29**:304–306.

DREYWOOD, R. 1946. Qualitative test for carbohydrate material. Ind. Eng. Chem. Anal. Ed. **18**:499.

FOUBERT, E. L., JR., AND H. C. DOUGLAS. 1948. Studies on the anaerobic micrococci. II. The

fermentation of lactate by Micrococcus lactilyticus. J. Bacteriol. **56**:35–36.

FRIEDEMANN, T. E., AND G. E. HAUGEN. 1943. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. **147**:415–442.

GORDON, C. F., AND A. L. WOLFE. 1960. Liquid scintillation counting of aqueous samples. Anal. Chem. **32**:574.

GORNALL, A. G., C. J. BARDAWILL, AND M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.

GOTTSCHALK, A. 1947. Further observations on the non-fermentability of D-fructopyranose by yeast. Biochem. J. **41**:478–480.

HALL, I. C., AND B. HOWITT. 1925. Bacterial factors in pyorrhea-alveolaris. IV. *Micrococcus gazogenes*, a minute gram-negative, non-sporulating anaerobe prevalent in human saliva. J. Infect. Diseases **37**:112–125.

JOHNS, A. T. 1951*a*. Isolation of a bacterium, producing propionic acid, from the rumen of sheep. J. Gen. Microbiol. **5**:317–325.

JOHNS, A. T. 1951*b*. The mechanism of propionic acid formation by *Veillonella gazogenes*. J. Gen. Microbiol. **5**:326–336.

KINARD, F. E. 1957. Liquid scintillator for the analysis of tritium in water. Rev. Sci. Instr. **28**:293–294.

KORNBERG, A., AND B. L. HORECKER. 1955. p. 323–324. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.

KREBS, H. A., AND M. WOODFORD. 1965. Fructose 1,6-diphosphatase in striated muscle. Biochem. J. **94**:436–445.

KUNITZ, M., AND M. R. MCDONALD. 1946. Crystalline hexokinase (heterophosphatase). Method of isolation and properties. J. Gen. Physiol. **29**:393–412.

LANGFORD, G. C., JR., J. E. FABER, JR., AND M. J. PELCZAR, JR. 1950. The occurrence of anaerobic gram-negative diplococci in the normal human mouth. J. Bacteriol. **59**:349–356.

LEWKOWICZ, X. 1901. II. Recherches sur la flore microbienne de la bouches des nourrissons. Arch. Med. Exptl. **13**:633–660.

LING, K. H., W. L. BYRNE, AND H. LARDY. 1955. Phosphohexokinase, p. 306–310. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.

LOWRY, O. H. 1957. p. 373. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 4. Academic Press, Inc., New York.

MCCORMICK, N. G., E. J. ORDAL, AND H. R. WHITELY. 1962. Degradation of pyruvate by *Micrococcus lactilyticus*. I. General properties of the formate-exchange reaction. J. Bacteriol. **83**:887–898.

MERGENHAGEN, S. E. 1964. Isolation of chemically and immunologically separable lipopolysaccharides from an oral *Veillonella*. Bacteriol. Proc., p. 43.

PRÉVOT, A. R. 1933. Études de systematique bacterienne. I. Lois générales. II. Cocci anaérobies. Ann. Sci. Nat. Botan. Biol. Vegetale, 10 Sér. **15**:23–258.

ROE, J. H., J. H. EPSTEIN, AND N. P. GOLDSTEIN. 1949. A photometric method for the determination of inulin in plasma and urine. J. Biol. Chem. **178**:839–845.

ROGOSA, M. 1964. The genus *Veillonella*. I. General cultural, ecological, and biochemical considerations. J. Bacteriol. **87**:162–170.

ROGOSA, M., AND F. S. BISHOP. 1964. The genus *Veillonella*. II. Nutritional studies. J. Bacteriol. **87**:574–580.

SIBLEY, J. A., AND A. L. LEHNINGER. 1949. Determination of aldolase in animal tissues. J. Biol. Chem. **177**:859–872.

SWIM, H. E., AND M. F. UTTER. 1957. p. 584–595. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 4. Academic Press, Inc., New York.

TAYLOR, J. F. 1955. Aldolase from muscle, p. 310–315. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.

WADE, H. E., AND D. M. MORGAN. 1953. Detection of phosphate esters on paper chromatograms. Nature **171**:529–530.