CARBOHYDRATE METABOLISM OF *BACILLUS LARVAE*[1]

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In recent years considerable attention has been paid to the elucidation of pathways of carbohydrate breakdown differing from that of the well-known Embden-Meyerhof-Parnas (EMP) scheme. Although it is recognized that the stepwise oxidation of glucose via gluconate with or without preliminary phosphorylation is an important means by which a number of aerobic bacteria obtain their energy (Barron and Friedemann, 1941; Entner and Doudoroff, 1952; Wood and Schwerdt, 1953) no data are available in this respect concerning *Bacillus larvae*, the causative agent of American foulbrood, a disease of the larvae of the honeybee. The present study was undertaken therefore to determine the manner in which this organism utilizes glucose.

Stone and Werkman (1937) obtained small amounts of phosphoglyceric acid from glucose dissimilation by *Bacillus subtilis* and *Bacillus mycoides* in the presence of sodium flouride. Aeration of the culture favored formation of this ester. Dedonder and Noblesse (1953) concluded that the hexosemonophosphate oxidative pathway was of importance to *B. subtilis* and *Bacillus megaterium* on the basis of triphosphopyridine nucleotide reduction by extracts of these two organisms and by the formation of pentoses from hexosephosphate. Gary *et al.* (1954), working with the Marburg strain of *B. subtilis*, showed that the nutritional environment of the cells affected the relative activity of the enzymes of the EMP and the hexosemonophosphate (HMP) pathways.

MATERIALS AND METHODS

The following substrates and coenzymes were used: glucose, glucose-6-phosphate[3] (G-6-P), fructose-6-phosphate[3] (F-6-P), fructose-1,6-diphosphate[3] (F-1,6-diP), sodium pyruvate, 6-phosphogluconate[4] (6-PG), adenosine triphosphate[3] sodium salt (ATP), triphosphopyridine nucleotide[3] (TPN) and diphosphopyridine nucleotide[3] (DPN). The barium salts of the phosphates were dissolved in dilute hydrochloric acid, the barium removed with ammonium sulphate and the solutions adjusted to pH 7.0.

Cultivation of cells. One strain of *B. larvae* isolated from an infected honeycomb was used in these studies. The organism was maintained on brain-heart-liver agar (Difco) (Katznelson, 1957). Growth from a 24 hr slant was washed off and transferred to a 300 ml Erlenmeyer flask containing 100 ml of medium composed of the following (in per cent): peptone (Difco), 1.0; yeast extract (Difco), 1.0; glucose, 1.0; KH_2PO_4, 0.05; K_2HPO_4, 0.05; $MgCl_2 \cdot 6H_2O$, 0.01; $MgSO_4$, 0.01; $FeSO_4$, 0.0001; and $MnSO_4$, 0.0001 (all at pH 7.0). The flasks were shaken on a rotary shaker for approx 16 hr at 37 C and the contents transferred to 1,200 ml of medium in 3 L Fernbach flasks. Following incubation on the shaker for 16 hr the cells were harvested and washed twice in tap water.

Preparation of cell extracts. Cell extracts were first prepared by grinding the cells with twice their weight of alumina powder and the gradual addition of 2 parts of cold M/20 phosphate buffer, pH 7.0. During this operation the mortar and contents were maintained at 0 to 5 C. The alumina and cell debris were removed by centrifugation. In later studies sonic extracts were substituted for the alumina ground extracts owing to the failure of the latter to oxidize glucose (although they oxidized a number of other compounds). For the preparation of sonic extracts the cells were suspended in M/20 phosphate buffer pH 7.0, M/20 glycl-glycine pH 7.0 or suitable concentrations of $NaHCO_3$ and treated in a Raytheon 10 KC sonic oscillator for 2 min. After centrifugation of this material for 15 min at 5,500 × G there remained an opalescent

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TABLE 1

Oxygen uptake, carbon dioxide production, and acetate formation by cell free preparations of Bacillus larvae

(In phosphate buffer)

Substrate	O_2	CO_2	Acetate
5 μmoles	*μmoles*	*μmoles*	*μmoles*
Glucose	10.72	10.36	
G-6-P	10.06	10.70	10.65
F-6-P	11.41		
F-1,6-diP	9.42		9.95
6-PG	16.0	19.0	6.45
Pyruvate	2.2	5.5	4.75

supernatant of high protein content which was used immediately or stored at −20 C.

For those experiments involving intact cells, suspensions were prepared in the appropriate buffer to give a reading of 400 in the Klett-Summerson photoelectric colorimeter with a 660 mμ filter.

Chemical methods. Prior to analysis of the vessel contents the residual protein was removed with sulfuric or trichloracetic acids. Hexokinase was determined by measuring the carbon dioxide evolved from bicarbonate buffer in the presence of ATP (Colowick and Kalckar, 1943) and aldolase by the procedure of Sibley and Lehninger (1949). The method of Friedemann and Haugen (1943) or Krebs and Johnson (1937) was used for the determination of pyruvic acid and that of Barker and Summerson (1941) for lactic acid. Inorganic phosphorus was measured with the Fiske-Subbarow reagent (Umbreit *et al.*, 1949). The orcinol reaction was used to indicate the presence of pentose. TPN and DPN reductions were followed by observing the change in optical density at 340 mμ in a Beckman DU spectrophotometer. The volatile acids were separated from the reaction mixture by steam distillation and the distillate treated according to the method of Carroll and Hungate (1954). The acid was identified on the basis of its Duclaux constant.

Manometric techniques. Oxygen consumption and carbon dioxide production were measured by conventional Warburg manometry; all results are recorded with endogenous values subtracted.

RESULTS

In an effort to determine the pathway of glucose utilization by *B. larvae*, a variety of substrates were compared with regard to gas exchange and acetate formation. Intact cells of *B. larvae* harvested from a medium containing glucose, oxidized glucose to completion (5.9 μmoles of oxygen per μmole of glucose). Cell free preparations from either alumina ground cells or sonically treated cells were incapable of complete oxidation of either glucose or its phosphorylated derivatives (table 1). With glucose, G-6-P, F-6-P, or F-1,6-diP 2 μmoles of oxygen were consumed per μmole of substrate and 2 μmoles of carbon dioxide produced. On the other hand with 6-PG approx 3.5 μmoles of oxygen were consumed and 4.0 μmoles of carbon dioxide produced. Fifty ml of the cell free preparation in M/20 phosphate buffer was incubated aerobically with 0.5 mmoles of sodium pyruvate at 37 C for approx 2.5 hr. Acidification of the reaction mixture, steam distillation and subsequent identification of the volatile acid showed the formation of 0.475 mEq of acid with a Duclaux constant of 0.72 (acetic acid 0.7). From 0.2 mmoles of glucose-6-phosphate 0.46 mEq of acid with a Duclaux constant of 0.69 were obtained. One-half mmole of fructose-1,6-diphosphate produced 0.99mEq of acetic acid, and the oxidation of 0.125 mmoles of 6-phosphogluconate yielded 0.160 mEq of acid. The results suggest that under the conditions of these experiments glucose or G-6-P and 6-PG may be oxidized by different pathways as illustrated by the following equations:

$$C_6H_{12}O_6 + 2O_2 = 2CH_3COOH + 2CO_2 + 2H_2O$$

$$C_6H_{12}O_7 + 3\tfrac{1}{2}O_2 = CH_3COOH + 4CO_2 + 4H_2O$$

The initial step common to both pathways, i. e., the phosphorylation of glucose, was demonstrated with sonic extracts by measuring CO_2 production (figure 1) in bicarbonate buffer containing ATP, and by estimating the decrease in 7 min hydrolyzable phosphate, which was found to be approx 30 μmoles. Para-chloromercuribenzoate (PCMB) strongly inhibited this phosphorylation (figure 1).

Evidence of the glycolytic pathway. Since the inhibition of glucose breakdown by flouride or by iodoacetate has been taken as indicative of the operation of the EMP pathway, glucose utilization was studied with and without iodoacetate or sodium flouride (figure 2). The inability of the enzyme preparations to oxidize glucose in the presence of the inhibitors suggested that the EMP pathway was operative.

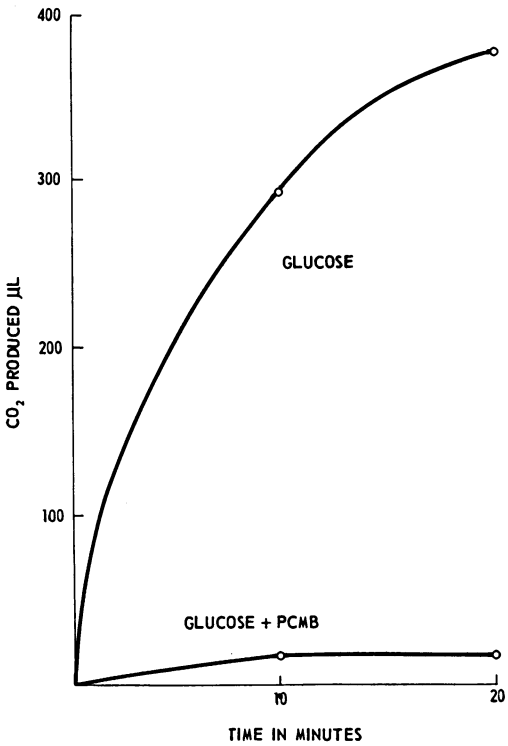

Figure 1. Phosphorylation of glucose with and without parachloromercuribenzoate (PCMB). Vessel contents: 2 ml sonic extract in 0.02 M $NaHCO_3$ and 0.01 M $MgCl_2$, 25 µmoles ATP, 40 µmoles glucose, 200 µmoles sodium fluoride, 2.0 µmoles PCMB. Total volume 2.8 ml. 5% CO_2:95% N_2. 37 C.

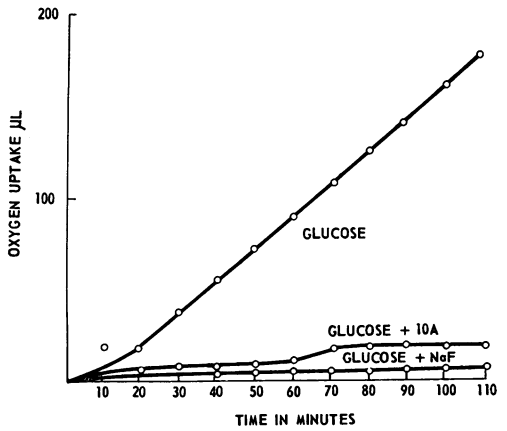

Figure 2. Effect of iodoacetate and sodium fluoride on the utilization of glucose. Vessel contents: 1.5 ml sonic extract in M/20 phosphate, 50 µmoles iodoacetate (IOA), 50 µmoles sodium fluoride (NaF), 5.0 µmoles glucose, 0.2 ml. 10% KOH in center well. Total volume 2.3 ml. 37 C.

The production of lactic acid from glucose provided further evidence for the glycolytic pathway. Under anaerobic conditions in bicarbonate buffer intact cells produced 10.55 µmoles of CO_2 (figure 3) and 9.0 µmoles of lactic acid

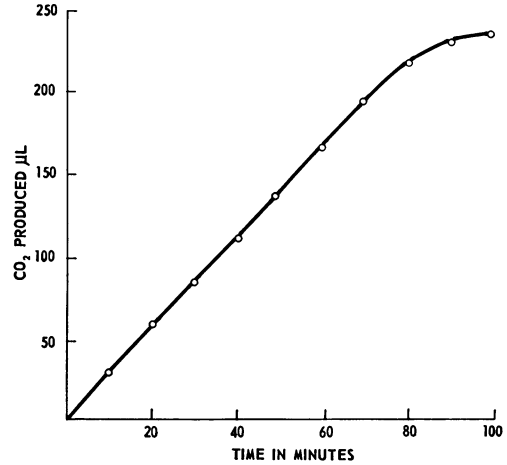

Figure 3. Anaerobic production of CO_2 from glucose by intact cells of *B. larvae*. Vessel contents: 2.0 ml cell suspension in 0.0066 M $NaHCO_3$, 5 µmoles glucose. Total volume 2.1 ml. 5% CO_2: 95%N_2. 37 C.

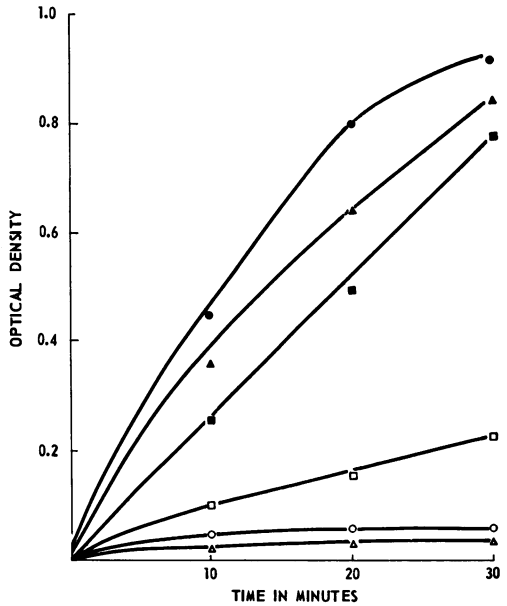

Figure 4. Production of triose phosphate as measured by chromogen formation (Sibley and Lehninger, 1949). Vessel contents: 2.0 ml 0.1 M tris buffer pH 8.6; 0.5 ml 0.56 M hydrazine pH 8.6; 1.0 ml enzyme, 1.0 ml water, 25 µmoles substrate, 25 µmoles ATP, 37 C. ○ glucose, ● glucose + ATP, □ F-6-P, ■ F-6-P + ATP, △ G-6-P, ▲ G-6-P + ATP.

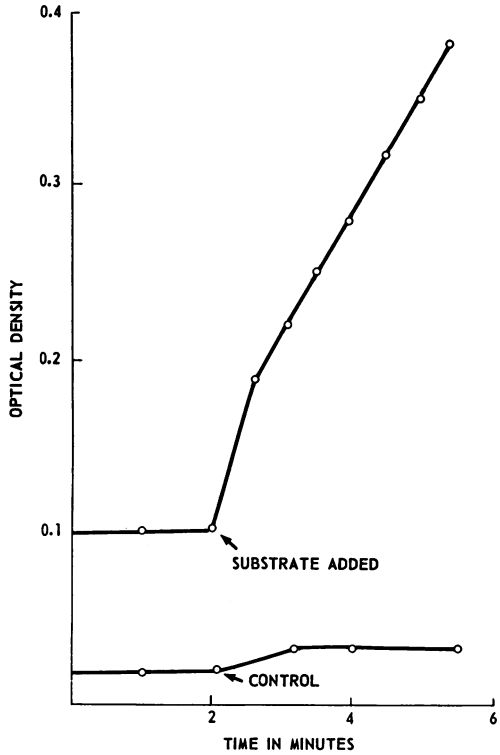

Figure 5. Reduction of DPN by products of fructose diphosphate breakdown. Increase in optical density at 340 mμ. Cuvette contained 0.112 M veronal buffer, 1.0 ml (pH 7.3); 0.173 M arsenate, 0.3 ml; 0.04 M cysteine, 0.3 ml; DPN, 2 mg; eluate, 0.3 ml; substrate, 0.2 ml. Total volume 3.1 ml.

from 5 μmoles of glucose. Under aerobic conditions pyruvic acid accumulated in the presence of arsenite. In one experiment with intact cells in 0.05 M sodium arsenite the ratio of glucose utilized to pyruvate formed was 1:1.5.

The aldolase reaction as determined by the method of Sibley and Lehninger (1949) with glucose, G-6-P, F-6-P or F-1, 6-diP as substrates further suggested that the breakdown of glucose followed the glycolytic pathway. The addition of ATP accelerated the transformation of glucose, G-6-P, and F-6-P to trioses (figure 4).

A DPN-reducing enzyme, possibly glyceraldehyde-3-phosphate dehydrogenase, was demonstrable (figure 5) in eluted extracts of vacuum dried cells when treated by the method of Gary *et al.* (1954). Eight-tenths of a gram of dried material was mixed with 5 ml of cold 0.02 M $NaHCO_3$; 10 mg DPN; 1.0 ml of 0.04 M cysteine and 10 mg versene. The mixture was incubated in an ice bath for 3 hr and maintained at pH 7.0

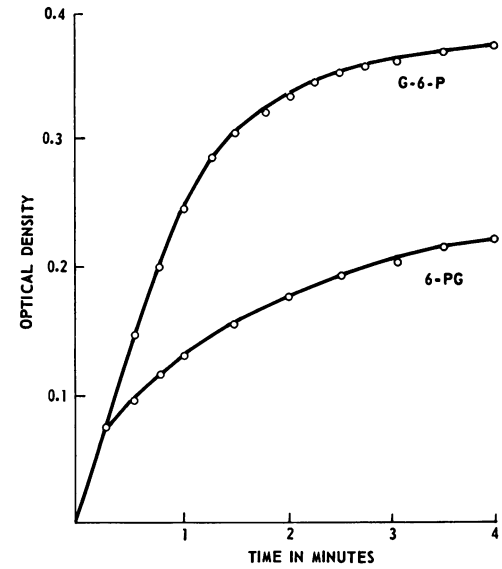

Figure 6. Reduction of TPN by G-6-P and 6-PG dehydrogenases. Increase in optical density at 340 mμ. Cuvette contained 0.25 M glycyl-glycine buffer, 0.4 ml (pH 7.4); $MgCl_2$, 3.0 mg; sonic extract in glycyl-glycine, 0.1 ml; TPN, 0.4 mg; substrate, 5.0 μmoles. Total volume 3.0 ml.

by the periodic addition of NaOH. Since glyceraldehyde-3-phosphate was not available, substrate was prepared by incubating 0.5 ml sonic extract with 0.2 ml M/20 F-1,6-diP for 5 min; the mixture was centrifuged and the supernatant used. A similar preparation without hexose diphosphate served as a control.

Evidence for the hexose monophosphate pathway. The ability of cell free preparations of *B. larvae* to reduce TPN with G-6-P and 6-PG as substrates is shown in figure 6. Table 2 shows gas exchange and pentose formation with G-6-P, 6-PG, in M/20 glycyl-glycine buffer. Analysis of the vessel contents revealed that an orcinol-positive compound which had a maximum absorption at 670 mμ was formed from the G-6-P and the 6-PG.

TABLE 2

Oxygen uptake, carbon dioxide production, and pentose formation by cell free preparations of Bacillus larvae

(In glycyl-glycine buffer)

Substrate	O_2	CO_2	Pentose
5 μmoles	*μmoles*	*μmoles*	*μmoles*
G-6-P.....	4.5	4.2	4.0
6-PG.....	2.0		3.0

DISCUSSION

The metabolic pattern of *B. larvae* is similar to that of many other bacteria in that mechanisms exist for both the glycolytic attack on, and the direct phosphorylative oxidation of, glucose. Further studies on the latter pathway are necessary particularly with regard to the identity of the pentose produced from both glucose-6-phosphate and 6-phosphogluconate, and its metabolism. Studies on the nitrogen metabolism of this organism are also contemplated, as it is considered desirable to obtain a well-rounded picture of the physiology of this organism prior to a consideration of its relationship to its host—the bee larva.

The intermediary metabolism of the honeybee larva at different stages of growth has not been investigated as yet. However, in view of the observation (Katznelson and Jamieson, 1950) that these larvae are susceptible to *Bacillus larvae* only during the first few days after hatching from the egg, it would be interesting to determine if this change in susceptibility is in any way related to, or coincident with, a fundamental change in metabolism. Studies of this nature might, therefore, lead to a better understanding of this host-parasite relationship.

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SUMMARY

The existence of two pathways of carbohydrate metabolism in *Bacillus larvae* has been demonstrated. Through the use of sonic extracts with or without various inhibitors it is concluded that glucose is degraded quantitatively to acetate via the Embden-Meyerhof pathway. Direct or indirect evidence is presented for the participation of hexokinase, phosphohexose isomerase, aldolase, triosphosphate isomerase, diphosphopyridine-nucleotide (DPN)-linked dehydrogenase(s) and enolase. Enzymes involved in the initial oxidative steps of the hexose monophosphate pathway were readily demonstrated.

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