THE MECHANISM OF OBLIGATE AEROBIC GLYCOLYSIS IN BACTERIUM TULARENSE AND OTHER MICROORGANISMS

by

Robert L. Hill
A.B., University of Kansas, 1949
A.M., University of Kansas, 1951

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INTRODUCTION

1. Scope of Problem

During the course of studies on the metabolism of Bacterium tularense a series of experiments demonstrated that glucose was almost completely oxidized to carbon dioxide and water in the presence of air. Pyruvate and acetate were shown to be intermediates of the glucose oxidation. However, in the absence of air, no glucose utilization was found. While this phenomena is not unique with B. tularense when compared to other aerobic microorganisms, the question could be asked, just what is the metabolic pathway of glucose.

Previous workers in the field of bacterial metabolism have demonstrated that glucose might be metabolized by either of two main pathways: the Embden-Meyerhof-Parnas scheme of glycolysis (along with slight modifications which are characteristic to the fermentation scheme of the particular organism) and the hexose monophosphate shunt. The former pathway can function either in the absence or presence of oxygen while the latter pathway requires oxygen and is generally associated with aerobic microorganisms. Since the initial studies on the glucose metabolism of B. tularense were not completely compatible with either the glycolytic scheme or the monophosphate shunt, further experiments were undertaken in hope of describing the pathways of glucose in this bacteria.

As a starting point in the following investigation the decision was made to attempt to demonstrate key enzymes which are characteristic

to either the glycolytic scheme or the monophosphate shunt. Starting with the shunt mechanism, survey experiments demonstrated the lack of glucose-6-phosphate dehydrogenase in enzyme extracts of B. tularense. Since this is one of the most important enzymes of the shunt mechanism, in as much as it represents the first departure of the shunt from glycolysis, it was felt that glucose was not degraded through gluconate. Turning then to the glycolytic scheme, the decision was made to assay B. tularense enzyme extracts for lactic dehydrogenase. The results of such assays, indicated the absence of DPN-dependent lactic dehydrogenase although data were obtained which indicated the presence of a DPN-independent lactic dehydrogenase. This series of experiments provided an important contribution to what is now believed to be the major pathway of glucose in B. tularense.

It is well known that anaerobic glycolysis exists only by virtue of the presence of a DPN-linked oxidation reduction between 3-phosphoglyceraldehyde and pyruvic acid which in turn leads to the production of 1,3-diphosphoglyceric and lactic acids. If the DPN reduction step involving 3-phosphoglyceraldehyde and DPN were present in B. tularense, and nothing comparable to the pyruvate-lactate DPN-linked system were present, then anaerobic glycolysis could not occur and glucose degradation via the glycolytic scheme could only proceed in the presence of air or another comparable eleectron acceptor. To prove this arguement it would be necessary to demonstrate the presence of 3-phosphoglyceraldehyde DPN-dependent oxidation. This reaction was studied and found to exist. Hence the assumption that B. tularense cannot anaerobically utilize glucose via glycolysis due to the lakk of a system which allows reoxidation of reduced DPN, proved correct. In the presence of air no

problem would arise as to the reoxidation of reduced DPN since it is believed that electron pathways from DPN to oxygen exist in this organism. As additional proof of this hypothesis, it was reasoned that B. tularense enzyme extracts should glycolyze if a DPN-reoxidation system is supplied to enzyme extracts. Such experiments were performed using two different reoxidizing systems. The first system attempted was the addition of mammalian DPN-dependent lactic dehydrogenase to enzyme extracts of B. tularense. The second method involved the additon of oxalacetate to enzyme extracts. This would allow glycolysis by virtue of the fact that oxalacetate in its reduction to malic acid by malic dehydrogenase involves the reoxidation of reduced DPN. Both of the methods described allowed rapid glycolysis in enzyme extracts.

Since this type of glucose metabolism has not been previously described, it seemed feasible to attempt to demonstrate a similar scheme in other bacteria. Further experiments have shown that several aerobic microorganisms exhibit a similar pattern of glucose metabolism as that found in B. tularense. Of the bacteria studied, enzyme extracts of Sarcina lutea, Alcaligenes faecalis, Pseudomanas auriginosa, and Bacterium anitratum did not glycolyze either in the absence or presence of DPN-dependent lactic dehydrogenase. On the other hand enzyme extracts of Agrobacterium tumefaciens, Brucella abortus, Mycobacterium phlei, Neisseria perflava, and Rhizobium meliloti glycolyzed only in the presence of DPN-dependent lactic dehydrogenase.

As previously mentioned B. tularense was shown to possess a DPN-independent lactic dehydrogenase. Since only a few cases of this

have been previously reported it was felt that a study of this particular enzyme would be interesting. The distribution of the enzymes seems to follow exactly in line with the absence of a DPN-dependent lactic dehydrogenase. All of the species which glycolysed only in the presence of DPN-dependent lactic dehydrogenase were found to have a DPN-independent lactic dehydrogenase. Partial purification of the enzyme has been achieved using B. tularense enzyme extracts as starting material. While the enzyme in B. tularense is believed to be closely associated with cytochrome b in the bacterial cell, the enzyme is not a cytochrome as is the case in certain yeast. Attempts to demonstrate a stimulation of dehydrogenase activity by the addition of flavin coenzymes were not successful and no flavin can be cleaved from the enzyme by ordinary procedures.

2. Historical

a. Glycolysis.

The latter part of the nineteenth century and the first third of the present century saw the development of a series of reactions which together constitute a detailed description of the metabolism of glucose. While it is not to be inferred that everything is known concerning the possible pathways of glucose, it is accepted that more detailed metabolic information is known for glucose than any other common metabolite. The accepted series of reactions that constitute one of the major pathways of glucose is presented in Figure 1. This series of events, known as glycolysis, is found in almost universal distribution with respect to various members of the animal kingdom. Quite comprehensive reviews of glycolysis as it exists in cells and tissues

have been written (1,2,3). Since this dissertation is primarily concerned with bacterial glycolysis, some mention will be made as to the previous work demonstrating the distribution of this phenomena in various bacterial species.

Before proceeding some clarity should be established with respect to the term glycolysis. It is to be emphasized that this term describes the series of events in metabolism, from glucose (or glycogen) to pyruvate, as is shown in Figure 1. Any further degradation of pyruvic acid is characteristic to the animal cell studied, and is not to be considered part of glycolysis. Thus yeast and mammalian muscle accumulate lactate during glycolysis while certain bacteria are known to accumulate such compounds as ethanol, butyric acid, propionic acid, succinic acid, and acetic acid, all of which are the direct result of pyruvic acid. In contrast to glycolysis, fermentation of glucose involves part of the glycolytic mechanism to pyruvate and further, the production of some end product of pyruvate.

Establishment of the glycolytic scheme can be approached in three ways: (a) the isolation of intermediates, (b) study of the utilization of the glycolytic intermediates, and (c) the study of individual reactions of glycolysis. The latter involves the demonstration of the particular enzymes shown in Figure 1. The most suprising aspect of bacterial glycolysis is the lack of unequivocal evidence for the existance of the glycolytic scheme. Only in the case of Escherishia coli have most of the enzymes and reactions been well characterized. In spite of this it seems accepted by many workers

in the field, that glycolysis as found in yeast and muscle is the primary glucose degradation pathway for many bacteria, especially those which carry on an anaerobic glucose utilization.

Tables I, II, and III present a summary of the information available concerning the presence of glycolysis in various bacteria. It will be noted that very little information has accumulated with respect to any one species other than E. coli.

In any consideration of glycolysis, emphasis should be placed on the DPN-$DPNH_2$ coupled oxidation-reduction reaction. It is established that anaerobic glucose utilization via glycolysis can proceed only by virtue of the presence of this reaction. Inspection of Figure 1 shows that DPN is reduced during the oxidation of 3-phosphoglyceraldehyde. Since it is also known that DPN is limiting in biological systems, then some means of reoxidation of reduced DPN must occur before any appreciable amount of glyceraldehyde oxidation can take place. Many different compounds act as hydrogen acceptors for reduced DPN. In the case of yeast, muscle and some bacteria, pyruvate acts as the acceptor along with the DPN-linked lactic dehydrogenase and the end result of the hydrogen transfer is oxidized DPN and lactate. Other bacteria can produce different hydrogen acceptors. Indeed it seems that the difference in the accumulated products of fermentation between various bacteria, exists due to the ability of bacteria to synthesize a wide range of hydrogen acceptors from pyruvic acid (2).

b. Hexose Monophospate Shunt

Until the last ten or fifteen years the classical Embden-Meyerhof scheme of glycolysis was felt to represent the major path of glucose in bacteria as well as other animal tissues. It is now accepted that glucose can undergo direct oxidative attack whereby the first carbon of the glucose is oxidatively decarboxylated. This pathway for glucose, known as the hexose monophosphate shunt or the glucose-gluconate shunt is presented in Figure II. As is the case for glycolysis, the shunt mechanism seems to be widespread and has been observed in bacteria (4), mammalian tissues (5), molds (6), and lebedev juice (7). It is felt that one of the most important features of the shunt mechanism is the production of ribose which could serve as a precursor to that carbohydrate found in nucleic acids (8).

Warburg and Christian in 1933 (9) reported the production of 6-phosphogluconate from glucose-6-phosphate and demonstrated that TPN was necessary as the coenzyme. Lipmann (10) in 1936 proposed that D-arabinose-5-phosphate was the degradative product of 6-phosphogluconate with 2-keto-6-phosphogluconate as an intermediate which underwent oxidative decarboxylation.

Bacterial production of 2-keto gluconate was demonstrated in 1935 by Bernhauer and Gorlich (11) but only in the last few years has this observation been reinvestigated. Cohen and Raff (12) in 1951 showed that E. coli adapted to gluconate degraded this substrate to a pentose phosphate with 6-phosphogluconate and 2-keto-gluconate as intermediates. Stokes and Campbell in the same year showed that

Pseudomonas aeruginosa oxidized glucose to 2-keto gluconate via gluconate. They were unable to demonstrate the formation of phosphorylated intermediates. This observation has been substantiated by Wood and Schwerdt (13) in the case of Pseudomonas fluorescens. The latter workers however did demonstrate the utilization of many phosphorylated hexose intermediates of glycolysis as well as demonstrate the presence of a number of glycolytic enzymes. The exact relationship between the role of phosphorylated hexoses and gluconate shunt intermediates is not known at this time. It is also of interest to note that Wood and Schwerdt were able to identify the seven carbon carbohydrate, sedoheptulose, during ribose-5-phosphate utilization. Although the exact role of this septulose is not known it has been observed in similar experiments on yeast, liver, and spinach (14). Certainly the complete function and scope of the hexose monophosphate shunt is not at all clear at this time.

In contrast to the work of Stokes, Wood, et al, Entner and Duodoroff (4) have demonstrated that Pseudomonas saccharophilo degrades glucose through gluconate involving phosphorylated intermediates. Furthermore the 6-phosphogluconate appears to be degraded to pyruvic and 3-phosphoglyceric acids.

A rather unique situation is thought to exist on Leuconostoc mesenteroides, the heterolactic fermenting bacteria. Molar equivalents of carbon dioxide, ethanol, and lactic acid are produced during glucose fermentation (16). It appears that ethanol and carbon dioxide conceivably arise from anaerobic breakdown of glucose

through the shunt mechanism which is coupled with some appropriate hydrogen acceptor (17). Thus glycolysis in this case does not account for the observed anaerobic breakdown of carbohydrate. This seems to accentuate the need for positive confirmation of the glycolytic scheme before assigning this phenomena to fermenting bacteria.

c. Lactic Dehydrogenase.

The accentuation of glycolysis as the primary mechanism of glucose degradation in bacteria has led to a more thorough study of DPN-dependent lactic dehydrogenase than the less well characterized DPN-independent lactic dehydrogenase. DPN-dependent lactic dehydrogenase has been recognized since the early part of this century but it was not until 1920 that Thunberg pointed out the donor function of lactic dehydrogenase (18) and Stephenson eight years later obtained the first cell-free preparation from E. coli (19). The DPN-dependency of the enzyme is well established (20). The work of Straub has led to the crystalization of the lactic dehydrogenase apoenzyme (21). Undoubtedly this enzyme exists in many bacteria which are capable of lactic acid fermentation. The belief however, that the lactic dehydrogenases from one bacteria to another are identical, must be accepted with caution in light of the observed isomeres of lactic acid produced in fermentation mixtures. S. Lactis is known to produce the D(-) lactate, and L. meserentoides only the L(+) lactate, while Lactobacillis pentoaceticus produces a racemic mixture (22).

The lactic dehydrogenase which operates without the DPN coenzyme have been reported in yeast and some bacteria. Bach, Dixon and Zerfas reported the purification of an enzyme from yeast which retained the identity of cytochrome b throughout purification procedures (23). Several workers have demonstrated a lactic dehydrogenase which seems to be associated with neither cytochrome b nor DPN. Barron and Hastings in 1933 studied the oxidation of lactic acid by alpha-hydroxyacid oxidase (24), which was isolated from Neisseria gonnorhea. While this enzyme oxidized many alpha-hydroxy acids, the greatest reaction rates were observed with lactate. Edson in 1947 reported the occurrence of DPN-independent lactic dehydrogenase in Mycobacterium phlei and demonstrated that the enzyme was probably flavin linked (25). A similar situation has been shown to exist in the mold, Penecillium chrysogenum (26). This enzyme is characterized by an extremely high Michaelis constant indicating low substrate-enzyme affinity. Also the flavin coenzyme appeared to be freely dissociable from the apoenzyme, which is unlike that found in Mycobacteria phlei.

Figure 1.

Metabolic Pathways in Glycolysis

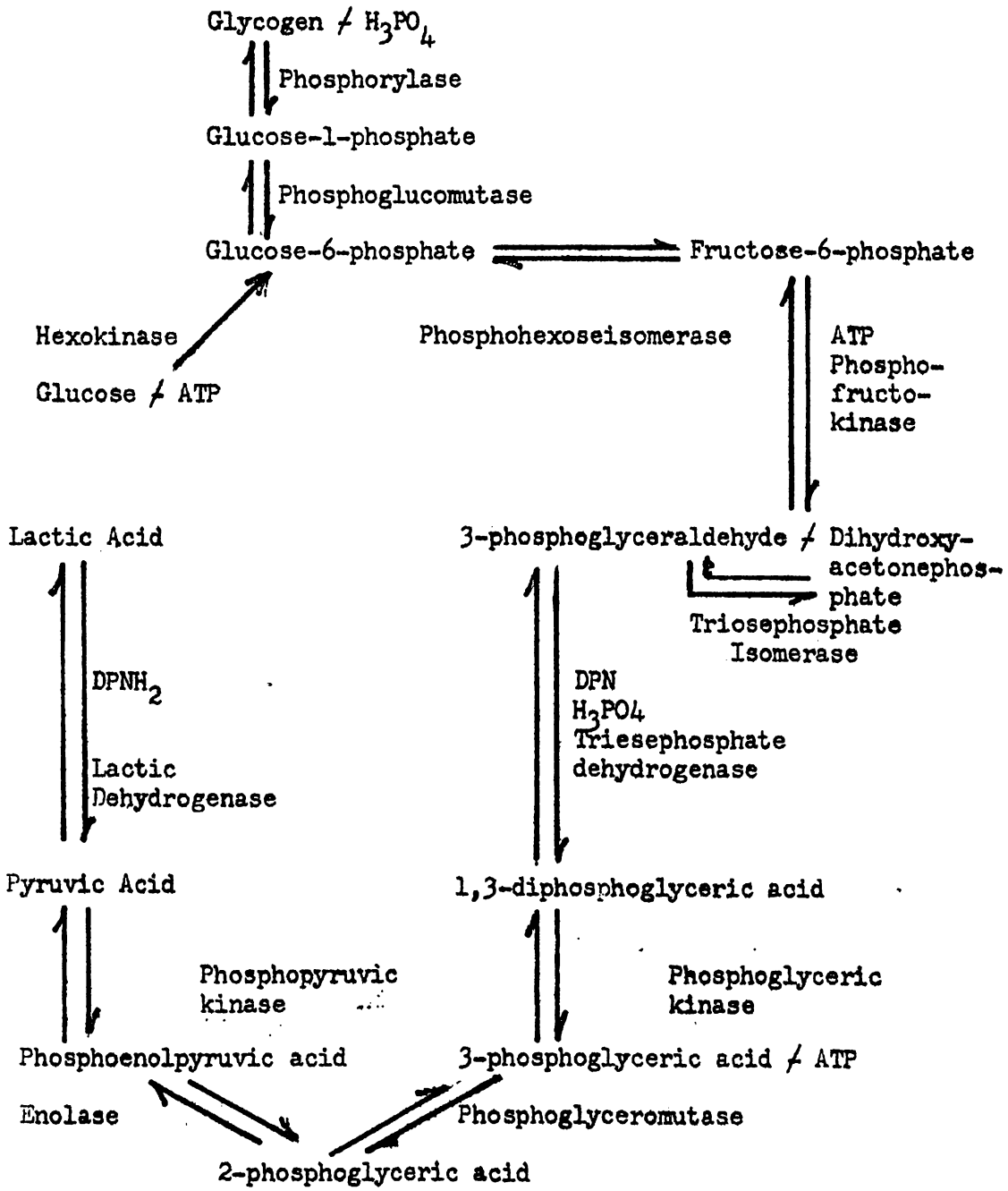


Figure 2.

Metabolic Pathways in the Glucose Monophosphate Shunt.

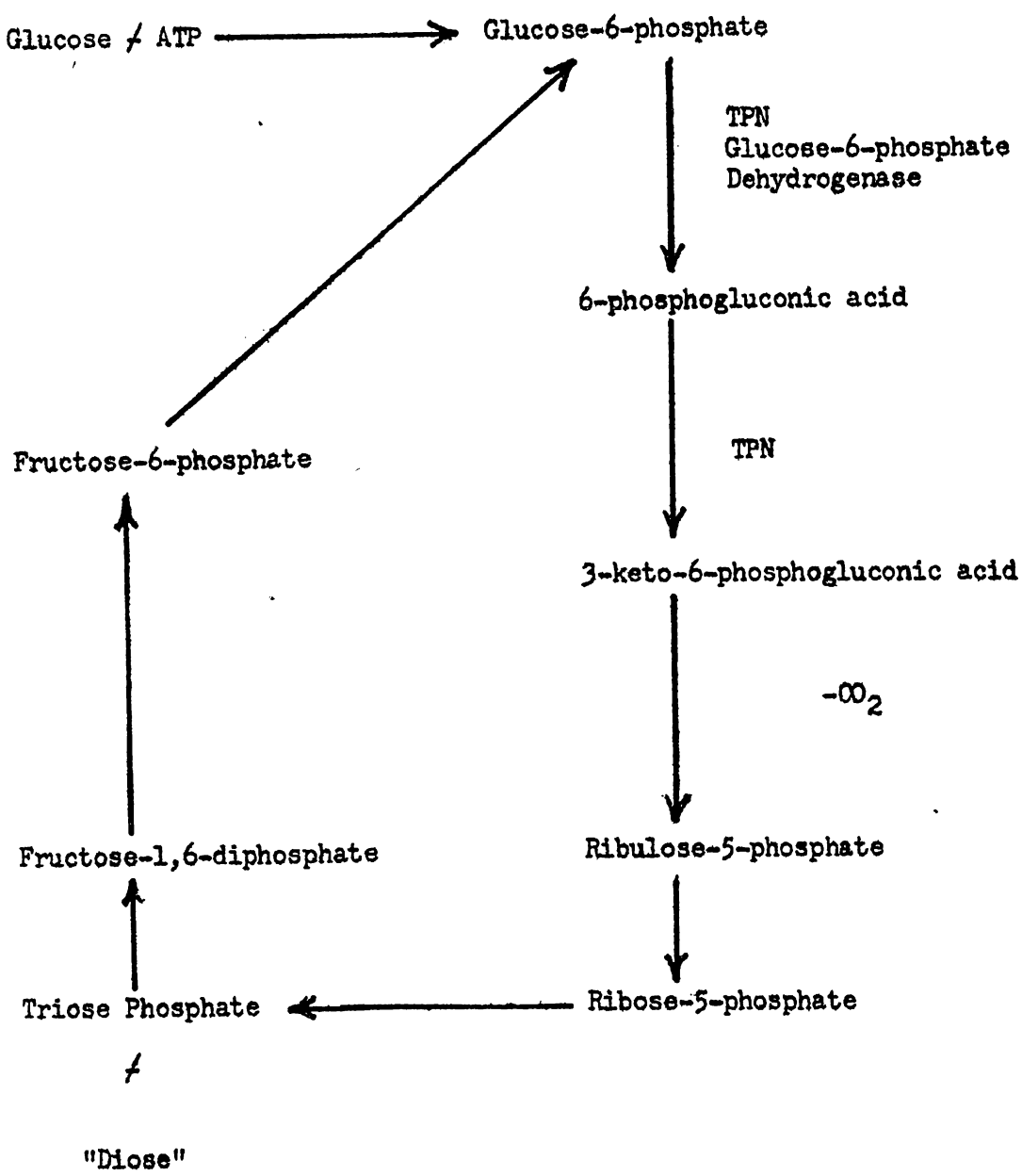


Table I

THE DISTRIBUTION OF GLYCOLYTIC ENZYMES IN BACTERIA

Enzyme	Bacteria	Reference
Hexokinase	E. coli	(27)
	Pseudomonas putrefaciens	(28)
Phosphohexoisomerase	E. coli	(29,30)
	Pseudomonas fluorescens	(13)
Phosphofructokinase		
Aldolase	E. coli	(31)
	Clostridium perfringens	(32)
	Pseudomonas fluorescens	(13)
Triosephosphate Isomerase	E. coli	(31)
	Pseudomonas fluorescens	(13)
Triosephosphate dehydrogenase	Leuconostoc meserentoides	(16)
	E. coli	(33)
	Pseudomonas fluorescens	(13)
Phosphoglyceric kinase	E. coli	(33)
Phosphoglyceromutase	E. coli	(34)
Enolase	Propionibacterium pentosacaum	(35)
Phosphopyruvic kinase		

Table II

THE ISOLATION OF GLYCOLYTIC INTERMEDIATES FROM BACTERIA

Intermediate	Bacteria	Reference
Glucose-6-phosphate	Brucella suis	(36)
	E. coli	(37)
	Streptococcus faecalis	(38)
Fructose-6-phosphate	B. suis	(36)
	E. coli	(30)
Fructose-1,6-diphosphate	B. suis	(36)
3-phosphoglyceraldehyde		
1,3 diphosphoglyceric acid		
3-phosphoglyceric acid	Propionibacterium pentosaceum	(39)
	Aerobacter aerogenes	(39)
	E. coli	(39)
	Serratia marcescens	(39)
	Many others	(39)
2-phosphoglyceric acid		
Phosphoenolpyruvic acid		

Table III

UTILIZATION OF GLYCOLYTIC INTERMEDIATES BY VARIOUS BACTERIA

Intermediate	Bacteria	Reference
Glucose-6-phosphate	Pseudomonas fluorescens	(13)
Fructose-6-phosphate	Pseudomonas fluorescens	(13)
Fructose-1,6-Diphosphate	E. coli	(40)
	Pseudomonas fluorescens	(13)
3-phosphoglyceraldehyde		
1,3-diphosphoglyceric acid		
3-phosphoglyceric acid	Propionibacterium pentosaceum	(41)

METHODS

1. Bacteriological

a. Bacterium tularense.

Except where indicated, strain Sm was used throughout the entirety of the experimental procedures. Two separate experiments dealing with the less pathogenic strains, Jap and 38 are described. All of the cultures of B. tularense were maintained, grown, and handled in precisely the same manner.

Stock cultures were maintained on glucose-cysteine-blood agar as described by Downs, et al (42). Liquid cultures were grown on casein hydrolysate-decamin medium of the following composition: 1% NaCl, 2% Glucose, 2% Acid hydrolyzed casein (vitamin free), 0.3% Decamin[1], 0.2% Cysteine·HCl, 0.2 micrograms thiamin/ml, 0.0225 M potassium phosphate buffer, pH 6.5, 0.0002 M $MgSO_4$, 0.0004 M $Cacl_2$, $2X10^{-7}$ M $FeSO_4$ and $2X10^{-7}$ M $MnSO_4$. The medium was adjusted to pH 6.5 with KOH and subsequently autoclaved at 15 lbs./in.^{2} and 125° C. for fifteen minutes.

Whole cells were obtained by inoculation of fresh medium with a 24 hour culture in a ratio of 5 ml of culture to 100 ml of medium. After growth at 37°C. on a reciprocating shaker, the cells were collected by centrifuging, washing, and resuspending in 0.9% NaCl.

Cell free extracts were prepared as follows: the cells from 400 ml of an 18 hour growth were collected, washed once with 0.9% NaCl and resuspended in 0.1 M potassium phosphate buffer, pH 7.4,

[1]Autolyzed Yeast, Vice Products Co., Chicago, Illinois.

to a final volume of either 20 or 40 ml. The packed cell volume was about 10 ml. The whole cell suspension was then cooled to below 10°C. and disintegrated for thirty minutes in a Raytheon Sonic disintegrator at a plate voltage of 100 volts and 9000 cycles. The temperature during the disintegration never exceeded 10°C. The resulting suspension was centrifuged for thirty minutes at 3000 X g to remove the small amount of sedimentable material. The final supernatents before dialysis contained from 1.9 to 2.3 mgmN/ml in the case of resuspension of the cells to a volume of 40 ml. After dialysis the nitrogen content dropped to 1.4 to 1.8 mgm N/ml. In the case of resuspension to a final volume of 20 ml, approximately twice the nitrogen content was observed. Before use in the enzymatic assays the cell-free extracts were dialysed at 10°C. for 18 hours against two changes of 1 liter of distilled water.

b. Escherichia coli.

Strain #4157 of the American Type Culture Collection was used. The cells were obtained by 24 hour growth in Difco-Nutrient Broth at 37°C. using the same shaking procedures as described for B. tularense. Enzyme sonic extracts were prepared in the same manner as described for B. tularense except that the plate voltage was increased to 160 volts. Although considerable sedimentable material was found at the end of thirty minutes disintegration, the supernatants were found to have high glycolytic activity. Before use, the enzyme extracts were dialysed as described above.

c. Pseudomonas aeruginosa

The stock strain of the Department of Bacteriology of the University of Kansas was used. Good growth was obtained in Difco-Nutrient Broth. Growth procedures and sonic extract preparation were identical to that described for E. coli.

d. Alcaligenes faecalis.

The stock strain of the Department of Bacteriology of the University of Kansas was used throughout. Growth and sonic extract preparations were the same as described for E. coli.

e. Sarcina lutea.

The stock strain of the Department of Bacteriology of the University of Kansas was used throughout. Growth and sonic extract preparation were identical to that described for E. coli.

f. Bacterium anitratum

Strain #B5W3 was obtained from the Michigan State Department of Health through the courtesy of Dr. William Ferguson. Growth was obtained with glucose enriched nutrient broth and the sonic extracts were prepared as described for B. tularense. As in the case of the three former bacteria, only 60-70% disintegration was obtained, though a high protein concentration was present in the final supernantants.

g. Mycobacterium phlei.

Strain #10142 of the National Type Culture Collection was used. Good growth was obtained in the medium which will be described for B. abortus, using incubation procedures as described for B. tularense. Sonic extracts were prepared from 1500 ml of an 18 hour

growth using 100 plate volts and a 45 minute disintegration period. Approximately 75% disintegration was found and the final dialysed supernatants had a nitrogen content of 0.604 mgm N/ml.

h. Brucella abortus.

Strain #19 was obtained through the courtesy of Dr. Cora Downs of the Department of Bacteriology of the University of Kansas. Good growth occurred in the medium described by McCollough, et al.(43). Sonic extracts were prepared from 1500 ml of medium incubated as described for B. tularense. Sonic extracts were prepared as previously described using a 45 minute disintegration period. While incomplete disintegration was found the final dialysed supernatants were found to have 1.95 mgm N/ml. This is high compared to values for other bacteria used.

i. Agrobacterium tumefaciens

Strain #A6 of the Department of Bacteriology of the Iowa State College was obtained through the courtesy of Dr. David Paretsky, of the Department of Bacteriology of the University of Kansas. Good growth was obtained in glucose enriched nutrient broth following the incubation procedures as described for B. tularense. Sonic preparations were prepared from 1000 ml of cells resuspended in 40 ml of 0.1M potassium phosphate buffer, pH 7.4. Incomplete disintegration was found though the final dialysed supernatants had a nitrogen content of 1.37 mgm N/ml. All other disintegration procedures were identical to those described for B. tularense.

J. Pastuerella pestis.

Strain #A-1122 (avirulent) was obtained from Dr. Sanford Elberg of the University of California, Berkely, California. Good growth was found using glucose enriched heart infusion broth of the following composition: 50% Infusion from Beef Heart (Difco), 1% Bacto-Tryptose, 0.5% NaCl and 2% Glucose. The glucose was autoclaved and added separately. The cells obtained after incubation for 18 hours at 37°C. with shaking, were collected and sonic extracts were prepared exactly as described for B. tularense except that a 45 minute disintegration period was used. Approximately 80% cell destruction was obtained, and the final dialysed supernatants had a nitrogen content of 1.68 mgm N/ml.

Neisseria perflava

Strain #12 was obtained through the courtesy of Dr. Michael Pelczar of the Department of Bacteriology of the University of Maryland. Good growth was found using the following medium: 1.5% Proteose-peptone #3 (Difco), 0.2% glucose, 1.0% Starch, 0.5% NaCl, 0.3% di-sodium hydrogen phosphate, and 1.0% Gelatin. The medium was adjusted to pH 7.3, filtered, and autoclaved for 15 minutes at 125°C.,15 lbs./ in^2. Growth from 1500 ml of medium, incubated as described for B. tularense, was used for preparation of sonic extracts. Disintegration was the same as that described for B. tularense except that a 45 minute disintegration period was used. The final dialysed supernatants had a nitrogen content of 1.16 mgm N/ml.

1. Rhizobium meliloti

Strain #9930 of the American Type Culture Collection was used. Good growth was obtained using the medium described by Allison and Hoover (44). The cells from 1500 ml of growth at 37°C. were used for preparation of the sonic extracts. While 45 minute disintegration periods were used, the remainder of the sonic disintegration procedures were the same as described for B. tularense. The final supernatants had a nitrogen content of 1.87 mgm/ml.

2. Chemical

Gas exchanges were measured in a Warburg respirometer according to standard procedures (45). Glycolysis assays were performed by the method of LaPage and Schneider (46). Lactic dehydrogenase assays were performed by one of three methods: a) the bicarbonate-ferricyanide method of Quastel and Wheatley (47), b) the ultraviolet absorption of reduced DPN at 340 mu., and c) the reduction of 2,6 dichlorophenol indophenol as reported by Haas (48). Glucose-6-phosphate dehydrogenase was assayed by the method of Haas (48) as well as by the method of Alivisatos and Denstedt (49). Transamination activity was measured using the assay procedures of O'Kane and Gunsalus (57). Carbon dioxide fixation was measured by both the methods of Kaltenbach and Kalnitsky (58) and Ochoa (59).

Glucose determinations were performed by the method of Nelson (50). Pyruvate was determined according to the procedure of Lu (51). Acetate and other volatile acids were determined according to the chromatographic procedures of Kennedy and Barker (52). Steam distillation of volatile acids was performed according to Friedemann (56).

3. Materials

Lactic dehydrogenase was prepared according to Biochemical Preparations (53). The enzyme was not crystallyzed but was purified to the extent that it was free of glycolytic enzymes. DPN (90% purity), TPN (10% purity) and ATP (sodium salt of 95% purity) were commercial preparations. Fructose-1,6-diphosphate was purified by preparation of the strychnine salt (54). Oxalacetic acid was prepared from the sodium salt of the diethyl ester by the procedure of Krampitz and Werkmen (55). All other substrates were commercial preparations of C.P. quality, unless stated otherwise in the text of the experiment.

EXPERIMENTAL

1. Carbohydrate Metabolism of Bacterium tularense.

a. Aerobic and Anaerobic Dissimilation of Glucose.

Three methods of attack were used to help determine the gross metabolism of glucose in this organism: a) Incubation of whole cell suspensions in a glucose-phosphate environment and the subsequent determination of glucose disappearance and acid formation both anaerobically and aerobically, b) Measurement of acid production from glucose as determined in a carbon-dioxide-bicarbonate buffer system, and c) the assay for glycolysis in cell free extracts of Bacterium tularense.

Incubation of whole cells with glucose-phosphate medium was performed in the following manner. The cells from 20ml of a four hour growth of B. tularense in casein-decamin medium were washed once and resuspended in ten ml of medium of the following composition: 0.01 M potassium phosphate buffer, pH 6.8, 0.15 M NaCl, and glucose as indicated in Table IV. The cell suspensions were gassed with nitrogen for fifteen minutes or left open to the atmosphere as indicated. Sterile conditions were used throughout. The media were sterilized by filtration through sintered glass ultra-fine bacteriological filters. The tubes were then incubated with shaking at 37°C. for 18 hours. Glucose concentrations were measured at zero and 18 hours. As shown in Table IV no glucose was utilized in the absence of air while 80-90% utilization occurred in the presence of air.

Experiments with whole cells in glucose-bicarbonate medium under an atmosphere of 5% CO_2-95%N_2 were attempted in the hope of showing acid production under anaerobic conditions. Bicarbonate concentrations which allowed an enviromental pH of either 6.8 or 7.4 were used in conjunction with 0.01 M potassium phosphate buffer at the desired pH. Both four and eighteen hour growths of whole cells were used. No acid production could be found as indicated by the lack of carbon dioxide evolution. No control experiments were performed with another organism which was known to be a good acid producer.

Cell-free enzyme extracts were prepared by sonic disintegration as described under Methods. Such extracts were assayed for glycolysis using the following experimental conditions. To Warburg flasks designed for gassing, the following solutions were added: 0.15 ml, 0.5 M $KHCO_3$, 0.30 ml 0.4M nicotinamide, 0.15 ml, 0.15 M sodium pyruvate, 0.10 ml 0.01 M ATP, 0.10 ml DPN (6mgm/ml), 0.20 ml 0.1M $MgCl_2$, 0.10 ml 0.3M glucose, 0.50 ml fructose-1,6-diphosphate (6mgmP/ml), 0.15 ml 0.2M KF, and 0.25 ml 0.1M potassium phosphate buffer pH 7.4. 1.0 ml of sonic extract was placed in the side arm and 0.2 ml of water was placed in the center well. The other solutions were in the main flask comppartment. The flasks were then gassed for fifteen minutes with 95% N_2-5% CO_2, incubated for five minutes for temperature equilibration and the sonic extract tipped from the side arm. The gas exchange during the first five minutes was ignored and zero readings were taken at five minutes after tipping of the sonic extract. Carbon dioxide production measurements were taken for at least forty minutes.

TABLE IV

Glucose Utilization by Whole Cells of Bacterium tularense

Tube #	Gas Phase	Glucose Concentration		Glucose Utilization
		Initial	After Incubation	
		mM/ml	mM/ml	mM/ml
1	Air	0.0104	0.0003	0.0101
2	Air	0.0115	0.0018	0.0097
3	Nitrogen	0.0122	0.0122	0.0
4	Nitrogen	0.0122	0.0122	0.0

The tubes contained ten ml of cells suspended in medium of the following composition: 0.01 M potassium phosphate buffer, pH 6.8, 0.011 M Glucose and 0.15M NaCl.

TABLE V

Glucose Utilization and Acid Production by Bacterium tularense

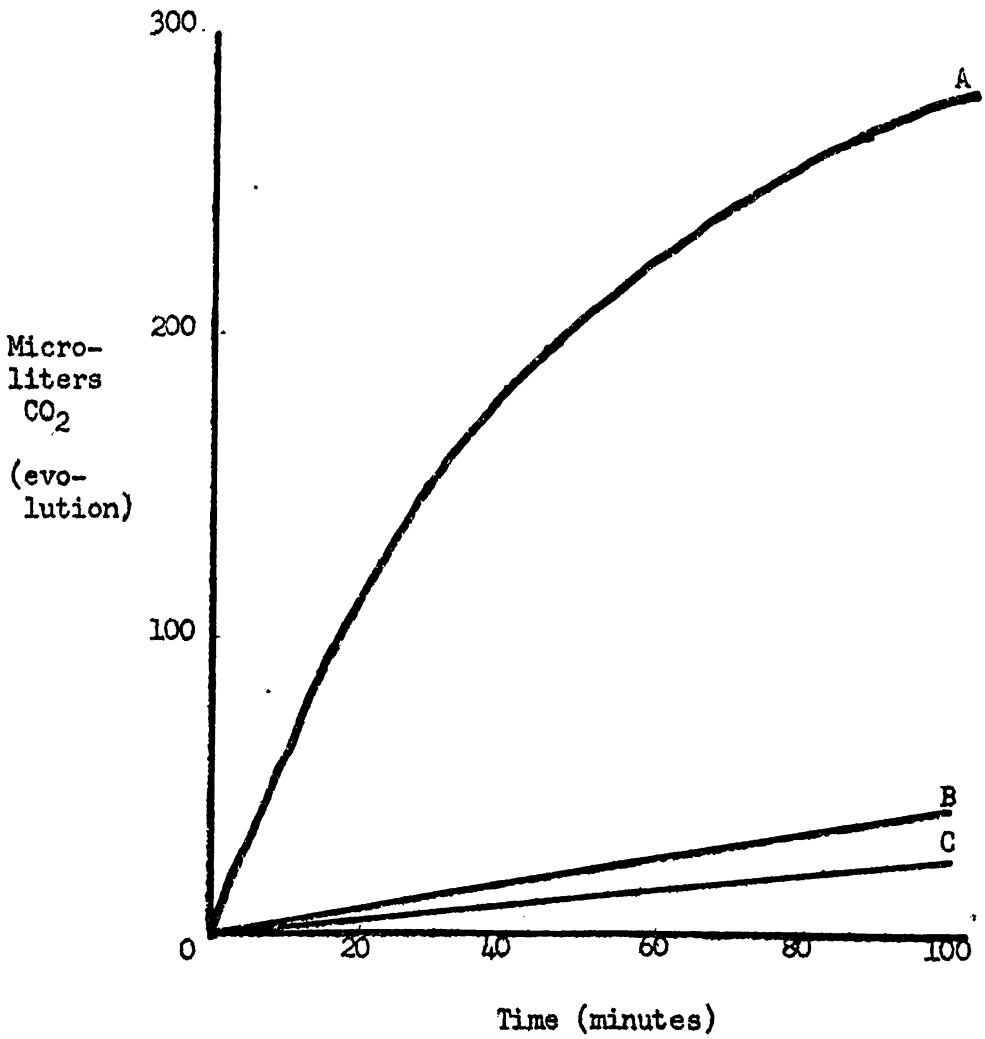
Experiment	Glucose Concentration		Glucose Utilized	Volatile Acid Produced	Hours Incubated
	Initial	After Incubation			
	mM/ml	mM/ml	mM/ml	mM/ml	
2	0.0063	0.0028	0.0025	0.001	24
3	0.0081	0.0012	0.0072	0.0017	12

The tubes contained ten ml of cell suspended in 0.02 M potassium phosphate buffer pH 6.5, 0.13 M sodium chloride and glucose as indicated in the Table.

FIGURE 3

Glycolytic Activity of Bacterium tularense and Escherichia coli

Sonic Extracts



Explanation of the Assay Procedure Found in the Text.

Curve A- E. coli
Curve B- B. tularense
Curve C- B. tularense and E. coli, endogenous

E. coli sonic extracts were compared to those of B. tularense. As seen in Figure 3 no glycolytic activity was present in B. tularense sonic extracts while E. coli source extracts glycolysed at a rapid rate.

b. Intermediates of Glucose Oxidation.

After the preliminary experiments just described, studies were initiated to determine the route of aerobic glucose dissimilation. These experiments were performed in the following manner: The cells from 20 ml of an 18-24 hour growth of B. tularense from liquid casein-decamin medium were suspended in 10 ml of a solution of 0.1% glucose, 0.02 M potassium phosphate buffer, pH 6.5 and 0.13 M NaCl. The cells were then allowed to incubate with shaking for 15-20 hours. Sterile conditions were used throughout the experiment. At the end of the incubation period glucose determinations were made and volatile acids were collected by steam distillation after acidification of the incubated solution to pH 2.0. The volatile acids were titrated with 0.01 N sodium hydroxide using Brom Thymol Blue indicator. Non-volatile acids possibly left in the residue after steam distillation were extracted with ether for 72 hours in a continuous ether extraction apparatus. Titration of the ether extract after removal of the ether showed that no non-volatile, ether soluble acids were produced. The volatile acids, after initial titration were redistilled and exactly neutralized with ammonium hydroxide. The resluting ammonium salts were then concentrated in vacuo to 1-2 ml and chromatographed by ascending paper chromatography. The method used consists essentially of the application of 15-20 micrograms of volatile acid in the form of

its ammonium salt to paper that has been previously treated with 1% exalic acid and allowed to dry in air. The acid salt is then submitted to ascending movement on the paper using 5% NH_4OH in 95% ethanol as solvent. After 8-10 hours the solvent has moved 15-20 inches and the paper is dried at 80°C., for 3 to 5 monutes. The paper is then sprayed with Brom Phenol Blue indicator. The acid salts appear as blue spots on a yellow background. With this method acetic acid was found to be the only volatile acid present. Control acid chromatograms were also run using the ammonium salts of formic, acetic, propionic, and butyric acids. The volatile acid fraction also gave a positive lanthanum nitrate spot test for acetic acid as described by Feigl. Table V summarizes the results of these experiments.

To determine if pyruvate is involved as one of the intermediates in glucose utilization, the following experiment was performed. The cells from 100 ml of an 18-20 hour growth of B. tularense in casein-decamin medium, were collected and resuspended in 50 ml of the glucose-phosphate-saline solution described above. The cells from another 100 ml of medium were resuspended in 50 ml of the same solution, to which was added sodium arsenite to a concentration of 0.001 M. The cells were then incubated with shaking for 21 hours. Glucose, pyruvate, and volatile acids were determined. The results are presented in Table VI. Pyruvate is seen to accumulate in the presence of arsenite.

TABLE VI

Glucose Utilization and Pyruvate Production by Bacterium tularense.

Tube #	Glucose Concentration		Glucose	Pyruvate Concentration		Arsenite
	Initial	Final	Utilized	Initial	Final	Concentration
	mM/ml	mM/ml	mM/ml	mM/ml	mM/ml	M
1	0.0072	0.0038	0.0034	0.0	0.0	0.0
2	0.0083	0.0070	0.0013	0.0	0.009	0.001

The tubes contained 50 ml of cells suspended in 0.02 M potassium phosphate buffer pH 6.5, 0.13 M sodium chloride, and glucose and arsenite as indicated in the Table.

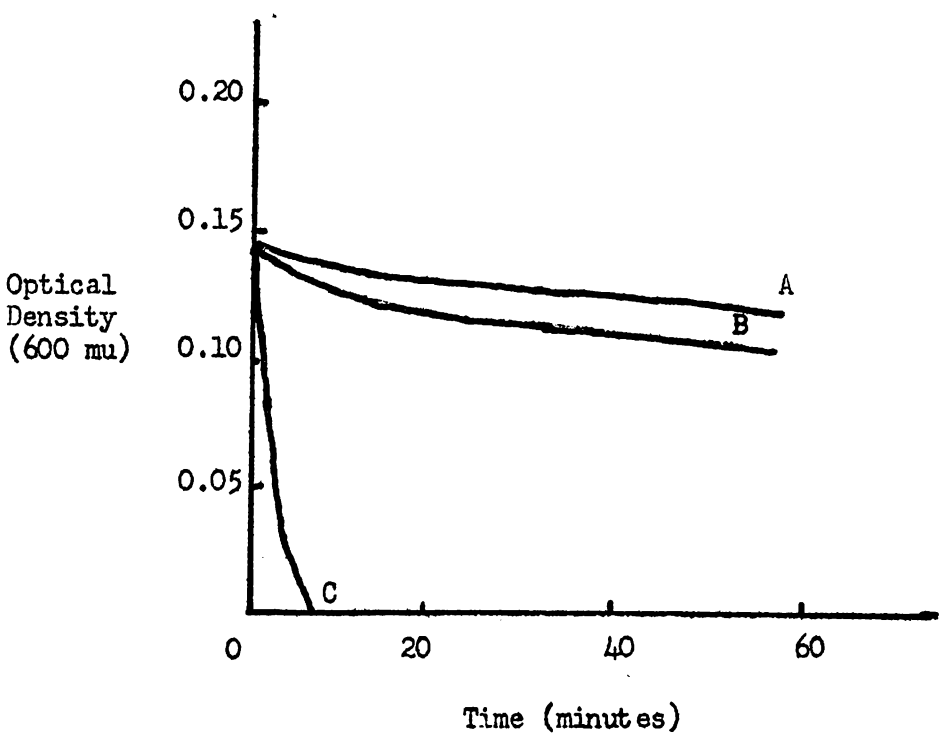
c. Assay for Specific Enzymes in B. tularense Sonic Extracts

1) Glucose-6-phosphate dehydrogenase

Experiments were now directed along the line of attempting to demonstrate why glycolysis was not present in B. tularense. With this in mind assays for various enzymes were made. It was reasoned that if glycolysis were not present but the hexose monophosphate shunt was operative, then sonic extracts should contain glucose-6-phosphate dehydrogenase. This enzyme represents the first departure of glucose-6-phosphate from the glycolytic scheme. Dye reduction experiments, designed after the 2,6 dichlorophenolindophenol method of Haas were performed. The following assay mixtures were used: 1.0 ml glucose-6-phosphate, 1.75 mgm/ml dissolved in 0.015 M potassium phosphate buffer pH 8.3; 1.0 ml TPN, 0.06 mgm/ml; 2.0 ml 2,6-dichlorophenolindophenol 0.02 mgm/ml dissolved in 0.015 M potassium phosphate buffer pH 8.3; 2.0 ml potassium phosphate buffer 0.015 M, pH 8.3; and 1.0 ml sonic extract (dialysed) which was diluted 1:10 with pH 8.3, 0.015 M phosphate buffer. The above solutions were mixed in spectrophotometer tubes designed for use in conjunction with the Coleman, Jr., Spectrophotometer. Sonic extract was added at zero time and readings were made for at least twenty minutes at 600 mu. As a control experiment, assay was also made for glutamic acid dehydrogenase by adding 1.0 ml glutamic acid, 2.0 mgm/ml, to the above assay mixture in the place of glucose-6-phosphate. The results as seen in Figure 4 indicate high glutamic acid dehydrogenase activity but no glucose-6-phosphate dehydrogenase activity.

Figure 4

Glucose-6-Phosphate Dehydrogenase and Glutamic Acid Dehydrogenase Activities in Bacterium tularense Sonic Extracts as Measured by 2,6 Dichlorophenol Indolphenol Reduction.



Curve A-Glucose-6-Phosphate Dehydrogenase System, Complete, Minus TPN, Minus Glucose-6-phosphate
Curve B-Glutamic Acid Dehydrogenase System Minus Glutamic Acid
Curve C-Glutamic Acid Dehydrogenase System Complete

Assay Procedure Described in Text.

Corroborative experiments for the absence of glucose-6-phosphate dehydrogenase were performed by assay of sonic extracts in the bicarbonate-ferricyanide manometric assay system. The following assay system, patterned after that of Alivisatos and Denstedt (see Methods), was used. To Warburg flasks designed for gassing, the following solutions were added: 0.5 ml 0.15 M potassium Bicarbonate, 0.5 ml 0.0114 M potassium ferricyanide, 0.3 ml 0.4 M nicotinamide, 0.2 ml TPN 0.06 mgm/ml, 0.5 ml water, and 0.5 ml sonic extract. The sonic extract was present in the side arm and was tipped into the other reagents in the center well after fifteen minutes of gassing with 95% N_2-5% CO_2 and a five minute temperature equilibration period. The first five minute readings were ignored. Carbon dioxide production was measured for 180 minutes at the end of which time no evolution was found. This substantiated the above dye reduction experiments which also demonstrated the lack of glucose-6-phosphate dehydrogenase in the sonic extracts of B. tularense.

2) Lactic dehydrogenase.

Since one of the most important enzymes of the hexose phosphate shunt mechanism was found to be absent, it was thought advisable to attempt to demonstrate key enzymes present in the anaerobic glycolytic scheme. The first enzyme chosen was lactic dehydrogenase. Assay for this enzyme was made in three different ways: a) dye reduction with 2.6 dichlorophenolindophenol, b) measurement of DPN reduction at 340 mu, and c) the bicarbonate ferricyanide assay system as described above for glucose-6-phosphate dehydrogenase assays.

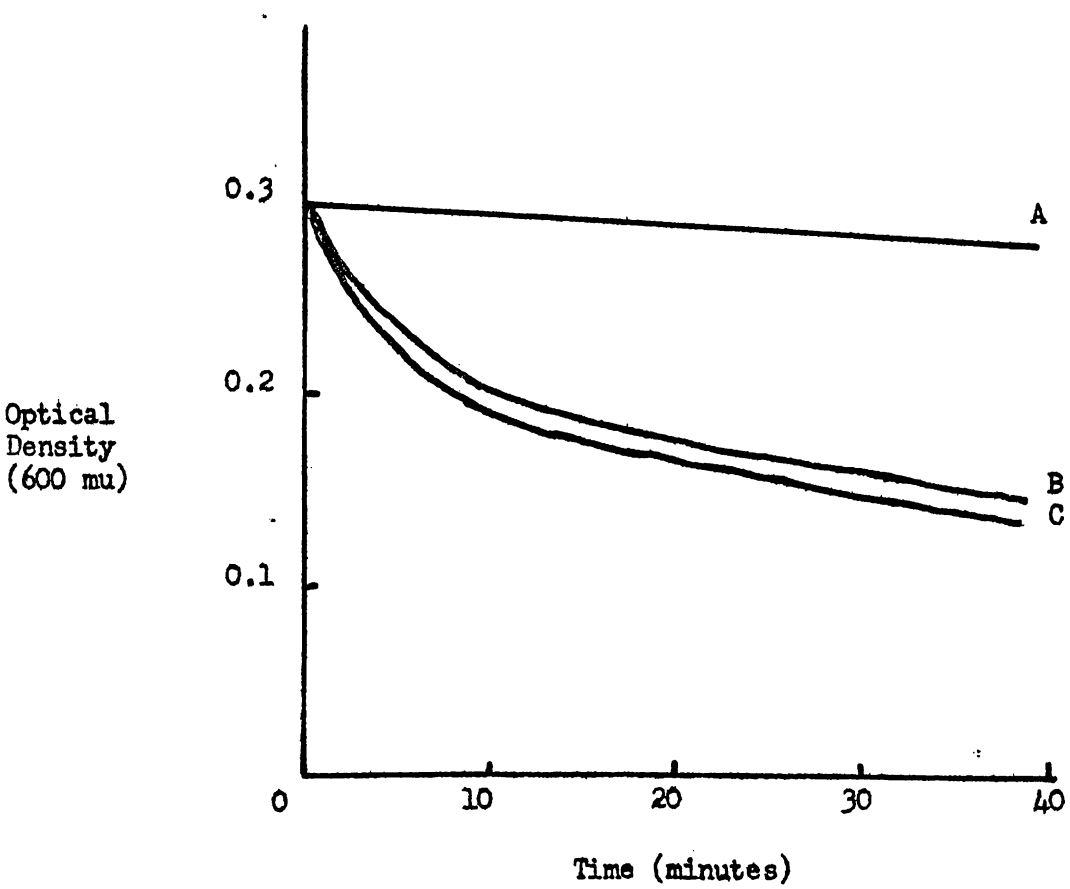
Dye reduction assay systems had the same composition as described above except that changes in pH and concentrations of components were made. The following solutions were added to Coleman, Jr., Spectrophotometer tubes: 5.0 ml 2,6-dichlorophenolindophenol 1 mgm %, 0.5 ml 0.1M sodium DL-lactate, pH 7.4, 0.5 ml DPN 0.003 M, 1.0 ml potassium phosphate buffer 0.1 M, pH 7.4 and 0.3 ml sonic enzyme extract. The extract was added at zero time and readings were made at 600 mu. Lactate and/or DPN were omitted as indicated in Figure 5. It is seen that while lactic dehydrogenase was present, no DPN-dependency was demonstrable.

Absorption studies at 340 mu were made using the following constituents added to standard quartz Beckman Model DU cuvettes: 1.0 ml 0.1 M sodium DL-lactate pH 7.4, 0.1 ml DPN (6 mgm/ml), 1.2 ml 0.1 M potassium phosphate buffer pH 7.4 and 0.7 ml sonic enzyme extract. Lactate was omitted in the second cuvette and a third cuvette containing all of the above constituents except DPN was used as the blank. The selector switch under these conditions was set at 0.1 and the slit width at 1.8 mm. Lactate was added at zero time. Over a fifty minute incubation period no DPN reduction was observed as indicated by lack of absorption at this wavelength. Under similar conditions DPN-dependency can be shown for 3-phosphoglyceraldehyde dehydrogenase.

Bicarbonate-ferricyanide manometric experiments were performed under the following conditions: to Warburg flasks designed

Figure 5.

Lactic Acid Dehydrogenase Activity of Bacterium tularense Sonic Extracts as Measured by 2,6-Dichlorophenol Indophenol Reduction.



A-no Lactic Acid or DPN
B-Lactate + DPN
C-Lactate, no DPN

Assay procedures described in text

for gassing were added the following; 0.50 ml 0.15 M $KHCO_3$, 0.50 ml 0.0114 M potassium ferricyanide, 0.30 ml 0.4 M nicotinamide, 0.50 ml 0.78 M sodium DL-lactate, pH 7.4, 0.30 ml DPN (0.003 M), 0.70 ml water and 0.50 ml of sonic enzyme extract. The sonic extract was tipped from the side arm after the flasks had been gassed for fifteen minutes with 5 % CO_2-95 % N_2 and a further five minute period for temperature equilibration had elapsed. The first five minute gas exchanges were ignored. Lactate and/or DPN were omitted where indicated in Table VII. The results indicated that while lactic dehydrogenase was present no DPN requirement was necessary.

3) Aldolase-3-phosphoglyceraldehyde Dehydrogenase System

The observation that DPN-dependent lactic dehydrogenase is absent in the sonic extracts, led to the following line of reasoning in an attempt to explain the lack of anaerobic glycolysis in B. tularense. Since no DPN-dependent lactic dehydrogenase is present in B. tularense, then this organism would be incapable of carrying out the DPN coupled oxidation-reduction that normally occurs in anaerobic glycolysis. During the oxidation of 3-phosphoglyceraldehyde, DPN reduction takes place. The DPN, under anaerobic conditions, must be reoxidized and this is usually performed by DPN-coupled lactic dehydrogenase. If the DPN-reduction step could take place in B. tularense, and no DPN reoxidation system were present, then glycolysis could only exist in B. tularense as an aerobic pathway. This assumes that some oxidation pathway between reduced DPN and oxygen exists.

Table VII

Lactic Dehydrogenase Activity of Sonic Extracts of *Bacterium tularense*.

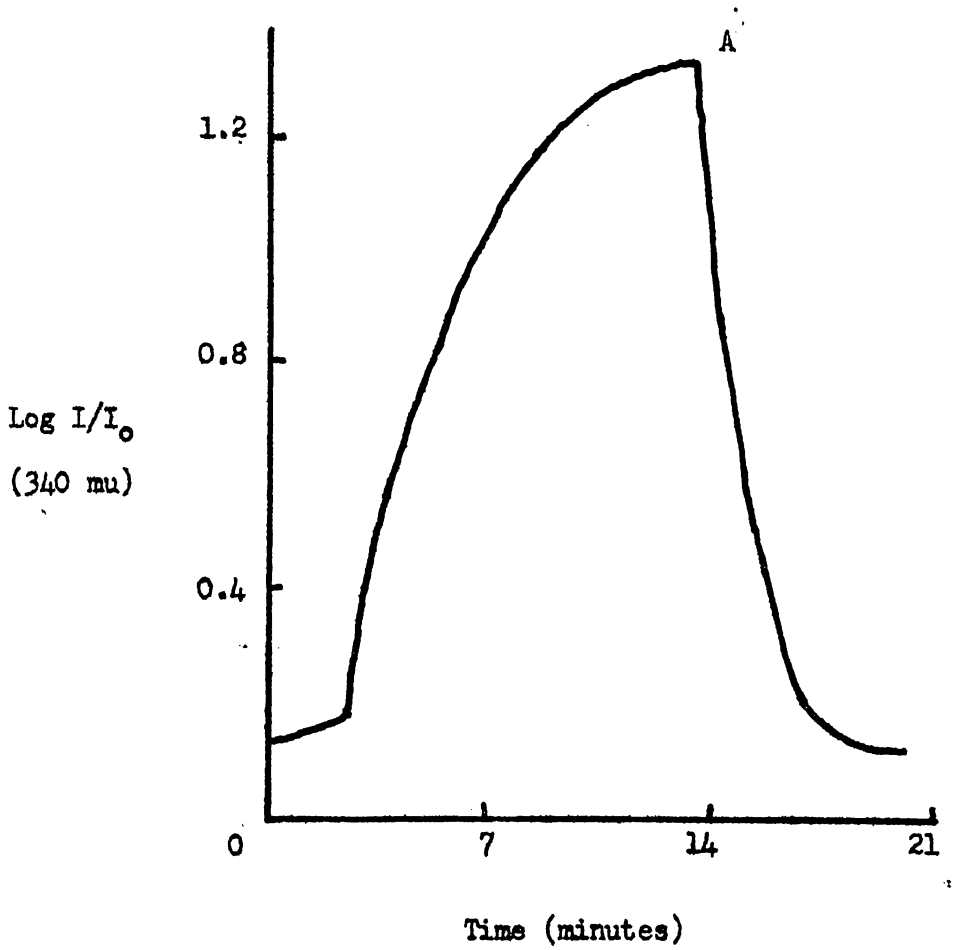
Flask #	Present in Flask		Microliters CO_2 evolved per 120 minutes
	DPN	Lactate	
1	+	+	252
2	-	+	264
3	+	-	55
4	-	-	61

Procedure described in text

In order to prove the above hypothesis for the lack of anaerobic glycolysis in B. tularense, the DPN-linked oxidation must be demonstrated. To do this, advantage was taken of the methods of Warburg and Christian in the design of the experiment. They reported that aldolase assays can be performed by the incubation of 3-phosphoglyceraldehyde dehydrogenase, DPN, fructose-1,6-diphosphate and aldolase preparation, and subsequent examination of the reaction mixture at 340 mu. Reduced DPN production would indicate the extent of aldolase activity on the fructose-1,6-diphosphate since one of the products of aldolase action is 3-phosphoglyceraldehyde. This latter compound, in the presence of DPN and the proper dehydrogenase, would produce phosphoglyceric acid and reduced DPN, causing an absorption at 340 mu. The following reaction mixture, observed at 340 mu as described under the lactic dehydrogenase experiments, was used: 1.0 ml fructose-1,6-diphosphate, 6 mgm/ml; 0.1 ml DPN, 6 mgm/ml; 0.9 ml 0.1 M potassium phosphate buffer, pH 7.4; and 1.0 ml of sonic extract. The absorption curve obtained is shown in Figure 6. That the absorption seen is due to reduced DPN, was substantiated by the rapid decrease in absorption after the addition of oxaloacetate to the reaction mixture at point A. Malic dehydrogenase is known to be present in the sonic extracts, and would allow conversion of the added oxaloacetate to malate with the simultaneous oxidation of reduced DPN.

Figure 6.

Reduction of DPN by Sonic Extracts in the Presence of Fructose-1,6-Diphosphate



25 micromoles of oxaloacetate added at point A

Procedure Explained in Text

d. The Effects of Oxaloacetate and DPN-dependent Lactic Dehydrogenase on the Glycolytic Activity of Sonic Extracts.

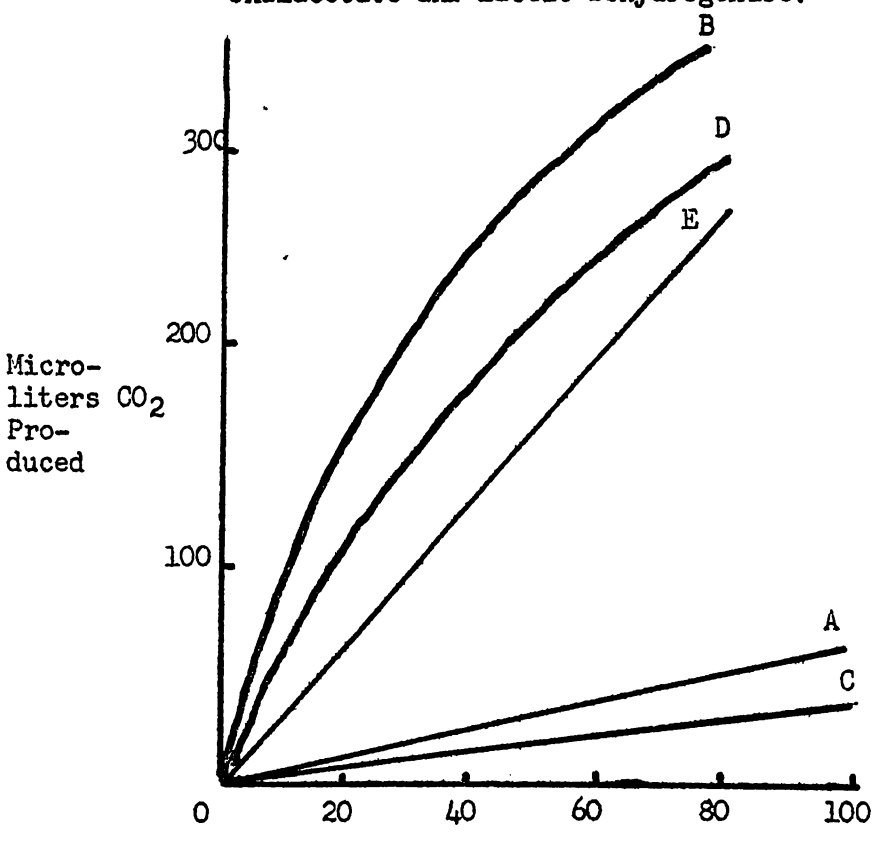
Under the assumption that the glycolytic system of B. tularense is complete except for a mechanism which is capable of reoxidation of reduced DPN, it should be possible to induce glycolysis by the addition of either (1) DPN-linked (mammalian) lactic dehydrogenase or (2) oxaloacetate. The results of experiments attempting to produce glycolysis in sonic extracts are seen in Figure 7. The conditions for these experiments were the same as described for the assay of glycolysis in sonic extracts under Aerobic and Anaerobic Dissimilation of Glucose. Lactic dehydrogenase prepared from fresh beef heart was suspended in 0.1 M potassium phosphate buffer pH 7.4, and added to the assay system at the expense of this buffer. Oxaloacetate was added to the assay system in a similar manner. Since the figure shows glycolysis present after either the addition of the lactic dehydrogenase or the oxaloacetate, it can be inferred that the glycolytic mechanism of B. tularense is complete except for a method of reoxidation of reduced DPN.

e. Transamination in Sonic Enzyme Extracts.

Since reoxidation of reduced DPN was shown to take place in the presence of oxaloacetate, experiments were undertaken to determine if a source of oxaloacetate supplied from other substrates would have the same reoxidation action. Two logical sources of

Figure 7.

Glycolytic Activity of Bacterium tularense and Escherichia coli Sonic Extracts in the Presence and Absence of Added Oxalacetate and Lactic Dehydrogenase.



Curve A-B. tularense Sonic Extract
Curve B-E. coli Sonic Extract, E. coli Sonic Extract + Oxalacetate, E. coli Sonic Extract + Lactic Dehydrogenase
Curve C-B. tularense and E. coli Sonic Extracts Endogenous
Curve D-B. tularense Sonic Extract + 50 Micromoles oxalacetate
Curve E-B. tularense Sonic Extract + 0.25 ml Lactic dehydrogenase (10 mgm/ml)

oxalacetate can be obtained in metabolizing systems under anaerobic conditions; 1) transamination of aspartic acid with alpha-keto glutaric acid or pyruvic acid, and 2) carbon dioxide fixation with pyruvic acid. In either case, if whole cells of B. tularense were capable of carrying out one of these processes when supplied the proper substrate materials, then anaerobic utilization of glucose should occur.

Initial experiments were carried out to determine the transamination activity of sonic extracts. Two types of transamination were assayed for; 1) pyruvic-aspartic acid transamination and 2) aspartic acid-alpha-keto glutaric acid transamination. On incubation for fifteen minutes at 37°C. of aspartic acid, pyruvic acid and sonic extract in Warburg flasks under a nitrogen atmosphere, high transamination activity was demonstrated as indicated by the aniline citrate method of analysis for oxalacetic acid. Appropriate control experiments showed no oxalacetate production from either of the substrates alone. The similar incubation of alpha-keto glutaric acid and aspartic acid also showed a high activity in sonic extracts as determined by the aniline citrate method of analysis for oxalacetate. The results of these assays are presented in Table VIII.

f. The Effect of Aspartic Acid or Alpha-Keto Glutaric Acid and Aspartic Acid on the Glycolytic Activity of Sonic Extracts.

TABLE VIII

Transamination in Sonic Extracts of Bacterium tularense

Flask #	Substrate Present			ul CO_2 evolved	Corrected ul CO_2
	Pyruvate	a-ketoglutarate	aspartate		
1	+	-	+	691	314
2	+	-	-	330	---
3	-	-	+	355	---
4	-	+	+	680	303
5[1]	+	-	+	377	---

The main chamber of Warburg flasks designed for gassing contained 1.0 ml Sonic Extract, 0.5 ml potassium phosphate buffer pH 7.4, 0.2 ml water. One side arm contained 0.25 ml 0.4 M pyruvic acid and 0.25 ml 0.8 M aspartic acid. The second side arm contained 0.5 ml 50% aniline cirtate. The flasks were gassed with 100% nitrogen for ten minutes, equilibrated at 37°C. for five minutes and the aspartic-pyruvic mixture tipped to start the reaction. After fifteen minutes incubation, the aniline citrate was added and carbon dioxide production measured until no further evolution occurred.

[1]No Sonic Extract Present

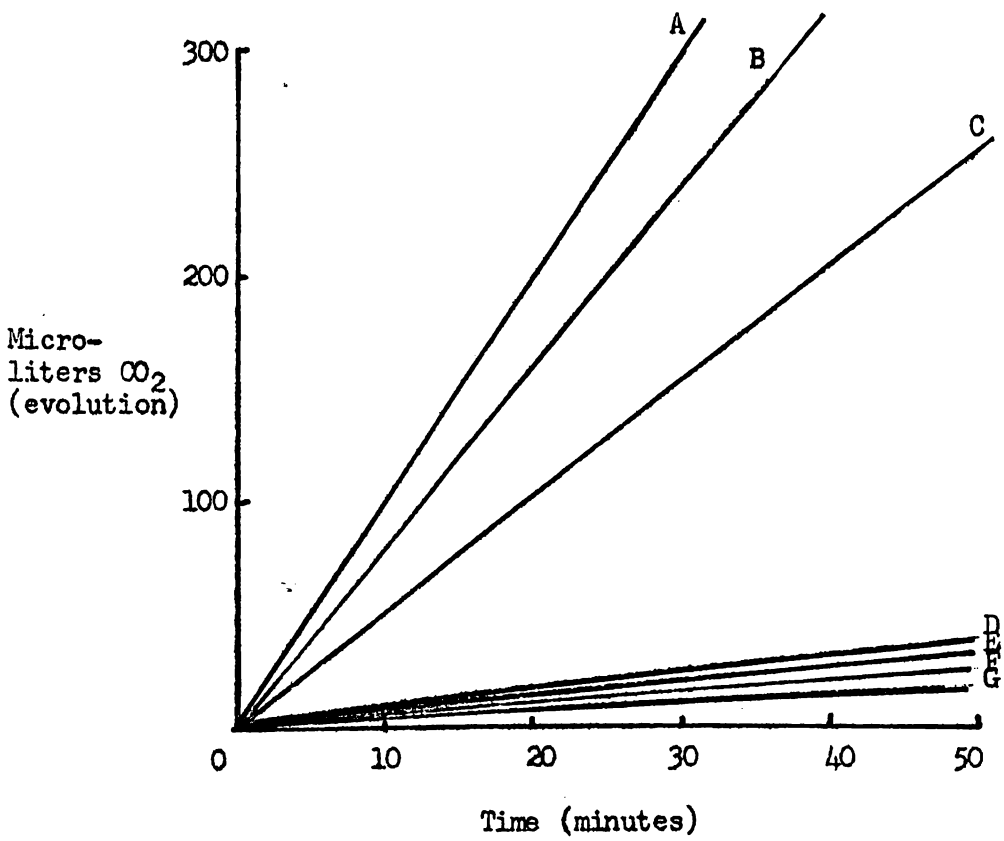
Attempts were now made to induce glycolysis in sonic extracts by the addition of transaminase system substrates which would allow oxalacetate production. Using the glycolysis assay system as described above in the presence of aspartic acid and either pyruvic acid or alpha-keto glutaric acid, considerable glycolysis was demonstrated. Slight modifications in the glycolysis assay were increasing the pyruvic acid added from 0.15 ml of 0.15 M to 0.25 ml of 0.4 M, and the addition of 0.3 ml of pyridoxal phosphate, 20 micrograms ml. The final volume in the flasks was 3.5 ml as compared to 3.0 ml in the normal assay system. The results of the experiment are shown in Figure 8. It is seen that good transaminase activity is present in the sonic extracts.

g. Attempts to Couple Glycolysis with Transamination in Whole Cells of Bacterium tularense.

Since the above experiments indicated a high transamination activity in sonic extracts it was thought feasible to attempt to couple transamination with glycolysis in whole cells. This would allow the production of axalacetate which could react anaerobically with malic dehydrogenase and allow the reoxidation of reduced DPN. The following experiments were designed in hope of demonstrating the described coupling. 200 ml of a 24 hour growth of cells from casein-decomin medium was washed and resuspended in 40 ml of 0.15 M NaCl. Five ml of the concentrated cells were added to solutions containing glucose, potassium phosphate buffer pH 7.4,

Figure 8

Transamination Coupled with Glycolysis in Sonic Extracts of Bacterium tularense.



Curve A-Aspartic Acid + Pyruvic Acid
Curve B-Aspartic Acid + Pyruvic Acid + Alpha-ketoglutaric Acid
Curve C-Oxalacetic Acid (25 micromoles)
Curve D-Alpha-ketoglutaric Acid + Pyruvic Acid
Curve E-Alpha-ketoglutaric Acid
Curve F-Aspartic Acid
Curve G-No substrates

Assay Procedures Explained in Text

aspartic acid and either pyruvic or alpha-keto glutaric acids. The final volume of the resulting substrate-cell suspensions was 10 ml. The final concentrations of the substrates were, 0.0056 M glucose, 0.05 M potassium phosphate buffer, pH 7.4, 0.03 M aspartic acid and either 0.02 M pyruvic or alpha-keto glutaric acids. The cells were incubated with shaking at 37°C. for fifteen hours under an atmosphere of nitrogen. Glucose determinations were made before incubation and after the fifteen hour incubation period. Sterile conditions were used throughout the experiment. No glucose disappearance was found which indicated the lack of coupling between the transamination and glycolytic systems. These results were obtained on two seperate days using different cell preparations.

h. Carbon Dioxide Fixation with Pyruvic Acid in Sonic Extracts

As mentioned above in section g, oxaloacetate could be produced in sonic extracts by fixation of carbon dioxide with pyruvic acid. This type of reaction is known to follow two pathways in bacteria. The first type of fixation involves the production of oxaloacetate directly from the two primary substrates. The second type of fixation involves the direct production of malic acid from the two substrates in the presence of reduced DPN. In any case both systems would involve the oxidation of reduced DPN. The former reaction wouldallow the production of oxaloacetate which could then be reduced to malic acid with the concommitant oxidation of reduced DPN while the latter reaction involves the direct productian of malate from pyruvic acid, reduced DPN and carbon dioxide.

The first type of fixation involving the direct production of oxaloacetate was assayed for in the following manner. To Warburg flasks designed for gassing were added; 0.5 ml 0.95 M $KHCO_3$, 0.5 ml 0.4 M sodium pyruvate, 0.5 ml 0.45 M potassium phosphate buffer pH 8.1, 0.3 ml 0.58 M $MgSO_4$, 0.5 ml of sonic extract, and 0.2 ml of water. The flasks were gassed for fifteen minutes with 5 % CO_2- 95 % N_2 gas mixture, and incubated for forty-five minutes at 37°C. with shaking. At the end of this time 0.7 ml of 50 % aniline citrate was added from the side arm of the vessels. Carbon dioxide evolution as produced by the decarboxylation of the oxaloacetate was then measured in the Warburg respirometers. In neither dialysed nor undialysed freshly prepared sonic extracts was any carbon dioxide evolution found. This is indicative of the lack of carbon dioxide fixation by this pathway in sonic extracts.

The second type of carbon dioxide fixation, supposedly catalysed by the "malic" enzyme of Ochoa, was assayed for in the following manner. Carbon dioxide production was measured in Warburg flasks containing the following components; 1.0 ml potassium phosphate buffer 0.24 M, pH 6.0; 0.1 ml 0.1 M $MnSO_4$; 1.0 ml 0.18 M sodium malate; 0.8 ml of sonic extract and either 0.1 ml of DPN or TPN (6 mgm/ml). Carbon dioxide evolution was measured under aerobic conditions. In no case was any carbon dioxide production shown.

Thus the possibility of coupling glycolysis with carbon dioxide fixation is not very likely in light of the absence of any type of fixation in sonic extracts. It is possible that the enzyme systems

involved are very labile and/or are destroyed during the sonic disintegration. This was arbitrarily ruled out on the basis of the high activity of normally labile enzymes demonstrated to be present in the extracts.

2. Carbohydrate Metabolism of Aerobic Bacteria Similar to Bacterium tularense

Since the type of obligate aerobic glycolysis seen in B. tularense has not been previously described, it seemed advisable to investigate other bacterial species in order to determine the distribution of this phenomenon. At first the only absolute requirements in choosing bacteria for study were that they be aerobic and utilize glucose. After failure to demonstrate the phenomenon under study in a few microorganisms possessing only these two characteristics, further choice was based on the followin criteria, all of which are properties of B. tularense; 1) predominantly aerobic and capable of glucose utilization; 2) parasitically intracellular with respect to its host or directly related to species possessing this property; and 3) possession of a DPN-independent lactic dehydrogenase.

The measurement of the obligate aerobic glycolytic properties of all of the species studied was made by determining the glycolytic rate of sonic extracts both in the absence and presence of mammalian lactic dehydrogenase.

The first species of bacteria selected for study were Sarcina

lutea, Alcaligenes faecalis, Pseudomonas aeruginosa and Bacterium anitratum, all possessing the common property of being predominantly aerobic and utilizing glucose. The sonic extracts of each of these bacteria were assayed seperately and compared to a positive control of B. tularense in the presence of lactic dehydrogenase. As seen in Figure 9 none of these bacteria responded to lactic dehydrogenase.

In the light of the failure to demonstrate obligate aerobic glycolysis in any of the above species,additional bacteria were selected on the basis of the criteria listed above. Agrobacterium tumefaciens the causitive agent of the cancer-like growth of plants, crown gall, was selected since it is known to possess an intracellular phase of parasitism. Brucella abortus, the pathogen causing undulent fever, was selected on the same basis. Rhizobium meliloti was also selected for its intracellular nature and while it is not pathogenic like the two former species, it is symbiotic with its normal plant hosts. Mycobacterium phlei and Neisseria perflava were selected on the basis that both Mycobacteria and Neisseria are reported to contain active lactic oxidase systems. It is interesting to note that both of the latter species are directly related to bacteria which are definite intracellular parasites, Mycobacterium leprae and Neisseria gonorrhea. Pastuerella pestis was also chosen on the basis of its intracellular phase of infection and its close morphological similarity to B. tularense. Two less virulent strains of B. tularense Jap and 38, were also studied

in order to determine if obligate aerobic glycolysis is present in more than one strain of B. tularense.

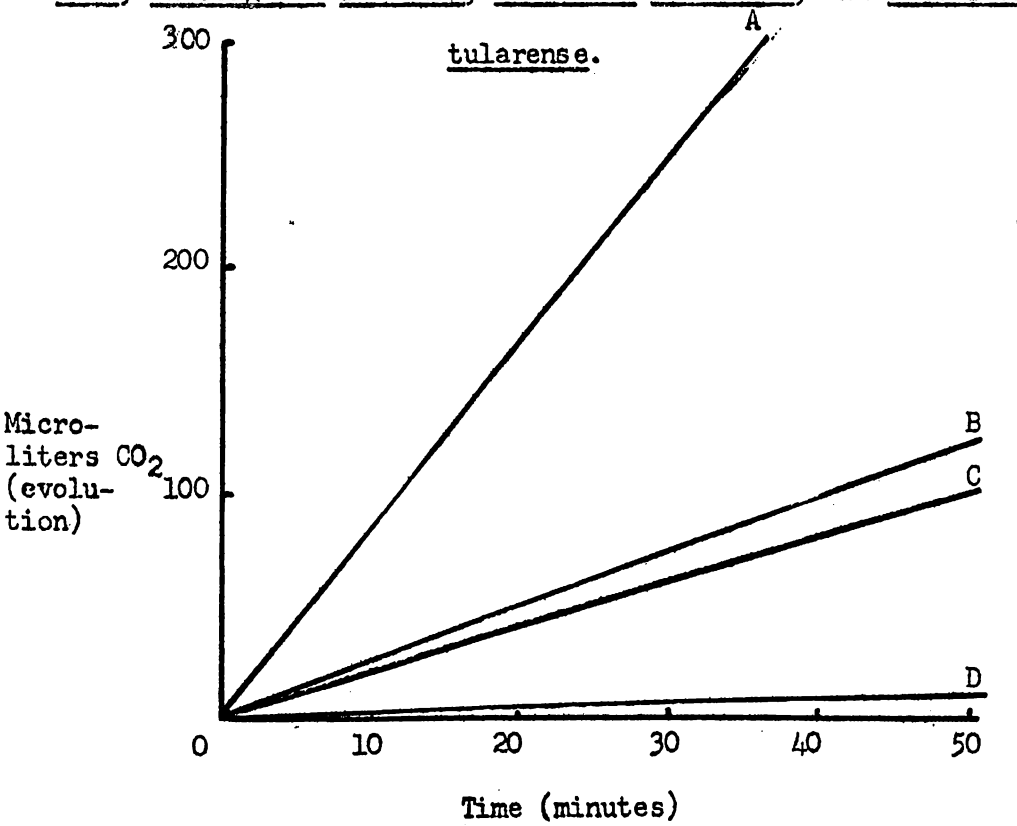
The results of the glycolysis assays are shown in Figures 10 and 11. The rate of carbon dioxide evolution from the assay system on the basis of mgm nitrogen/ hour is seen in Table IX. Except for one species, it is seen that all of the bacteria selected, either on the basis of intracellular parasitism or DPN-independent lactic dehydrogenase were found to glycolyze only in the presence of added lactic dehydrogenase. The lone exception is P. pestis, which glycolyzed equally as well in the absence of the mammalian dehydrogenase as in its presence.

3. Lactic Dehydrogenase Activity of Bacteria Showing Glycolysis in the Presence of DPN-Dependent Lactic Dehydrogenase

Since the bacteria just described possess the obligate aerobic glycolysis seen in B. tularense, it should be of interest to study the DPN-dependency of the lactic dehydrogenase possibly present in these organisms. Lactic dehydrogenase assays were performed by the bicarbonate-ferricyanide manometric method previously described. As shown in Table X, no DPN-dependency could be found for the dehydrogenase present in any of the bacterial extracts.

Figure 9

The Effect of Lactic Dehydrogenase (mammalian) on Glycolytic Activity of Sonic Extracts of Sarcina lutea, Pseudomonas aureginosa, Alcaligenes faecalis, Bacterium anitratum, and Bacterium tularense.

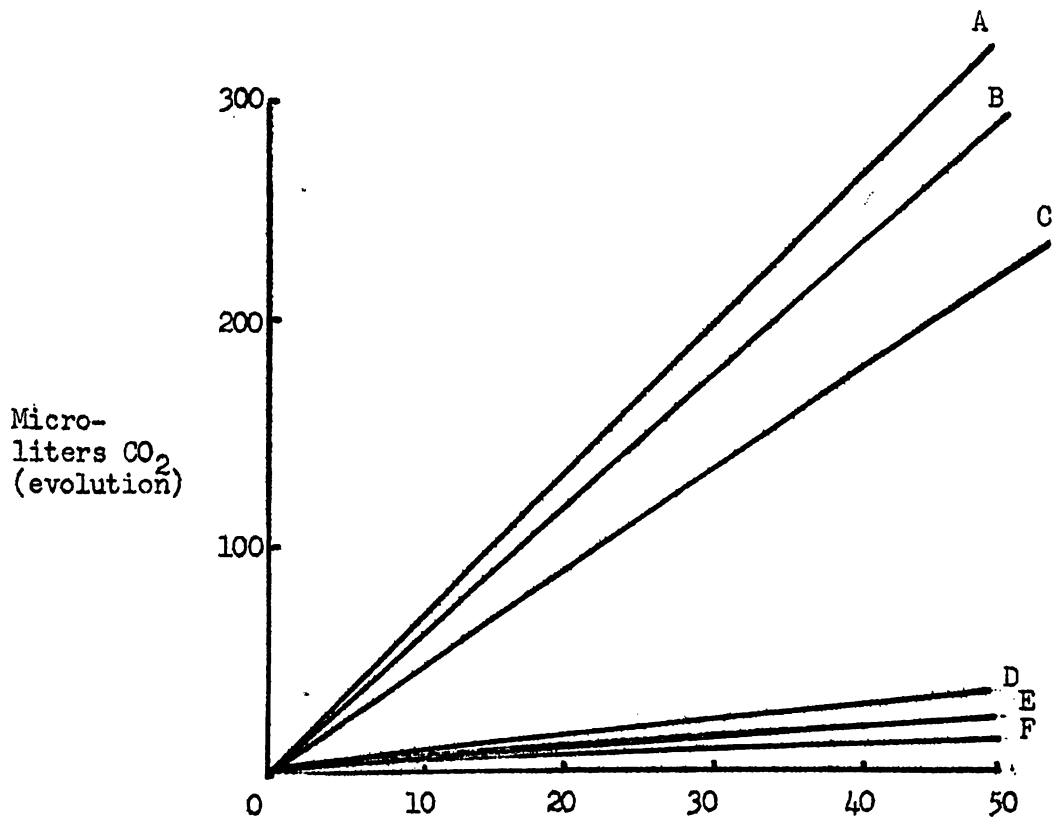


Curve A-B. tularense + Lactic Dehydrogenase
Curve B-B. anitratum
Curve C-B. anitratum + Lactic Dehydrogenase
Curve D-P. aureginosa, S. lutea, and A. faecalis in the absence and presence of Lactic Dehydrogenase

Assay Procedures Explained in Text

Figure 10

The Effect of Lactic Dehydrogenase (mammalian) on Glycolytic Activity of Sonic Extracts of Brucella abortus, Mycobacterium phlei and Agrobacterium tumefaciens.



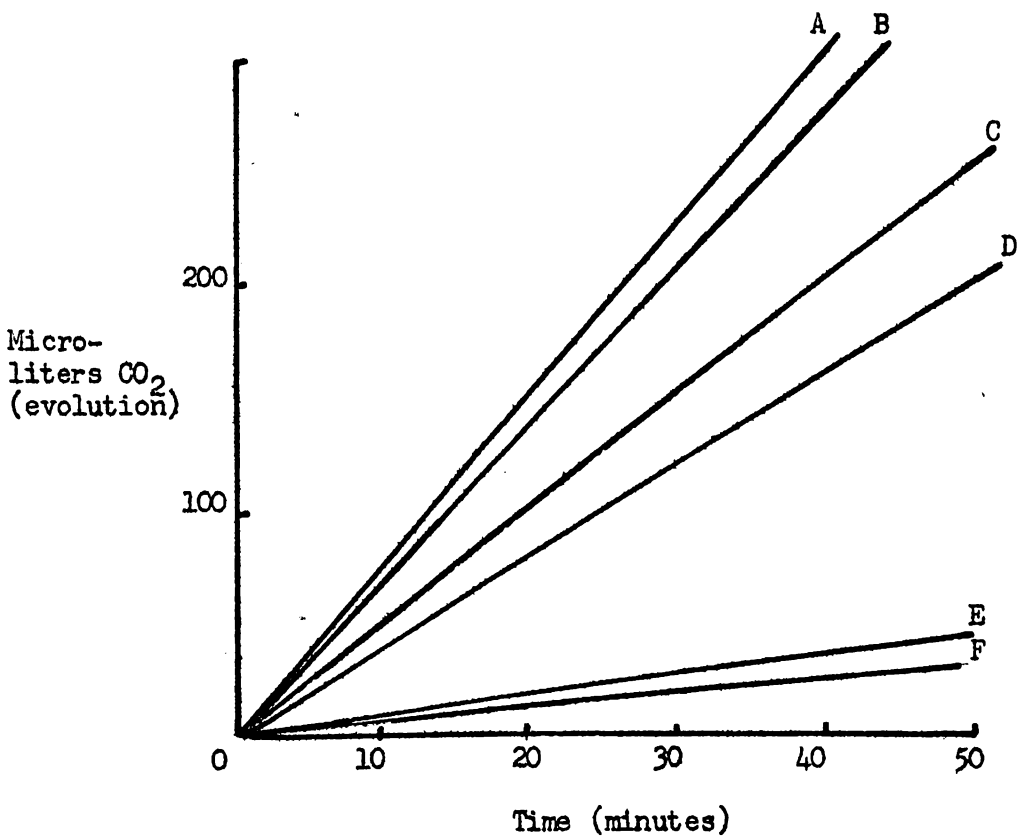
Curve A-A. tumefaciens + Lactic Dehydrogenase
Curve B-B. abortus + Lactic Dehydrogenase
Curve C-M. phlei + Lactic Dehydrogenase
Curve D-B. abortus
Curve E-A. tumefaciens
Curve F-M. phlei

1 ml of Sonic Preparation Assayed in Each Case.

Procedure as Described in Text.

Figure 11

The Effect of Lactic Dehydrogenase (mammalian) on the Glycolytic Activity of Sonic Extracts of Pastuerella pestis, Neisseria perflava and Rhizobium meliloti.



Curve A-P. pestis + Lactic Dehydrogenase
Curve B-P. pestis
Curve C-R. meliloti + Lactic Dehydrogenase
Curve D-N. perflava + Lactic Dehydrogenase
Curve E-N. perflava
Curve F-R. meliloti

1 ml of Sonic Extract Assayed in Each Case.

Procedure Described in Text.

TABLE IX

Glycolysis Assays of Various Bacterial Species

Organism	Strain	No Additions Micro-liters CO_2/mgm N/hr.	+ Lactic Dehydrogenase Microliters CO_2/mgm N/hr.
P. aeruginosa	KU	0.0	0.0
A. faecalis	KU	0.0	0.0
S. lutea	KU	0.0	0.0
E. coli	#4157	108.0	108.0
B. tularense	Sm	12.9	52.8
B. tularense	Jap	15.6	42.0
B. tularense	38	10.2	40.3
B. abortus	A19	9.0	72.8
A. tumefaciens	A6	17.0	184.5
M. ohlei	#10142	0.0	98.5
R. meliloti	#9903	17.6	64.4
N. perflava	12	21.0	55.0

TABLE X

Bicarbonate Ferricyanide Assays for Lactic Dehydrogenase in Various Bacterial Species

Organism	Strain	DPN + Lactate Microliters CO_2/mgm N/hr.	Lactate Microliters CO_2/mgm N/hr.
B. tularense	Sm	60.3	80.2
A. tumefaciens	A6	234.	220.
B. abortus	A19	103.	92.
M. phlei	#10142	60.3	101.
N. perflava	12	540.	550.
R. meliloti	#9930	95.	85.

4. Lactic Dehydrogenase of Bacterium tularense

Since a DPN-independent lactic dehydrogenase has been reported in only one species of yeast, one species of mold and two species of bacteria, it was thought of interest to determine some of the properties of this enzyme which is found in B. tularense.

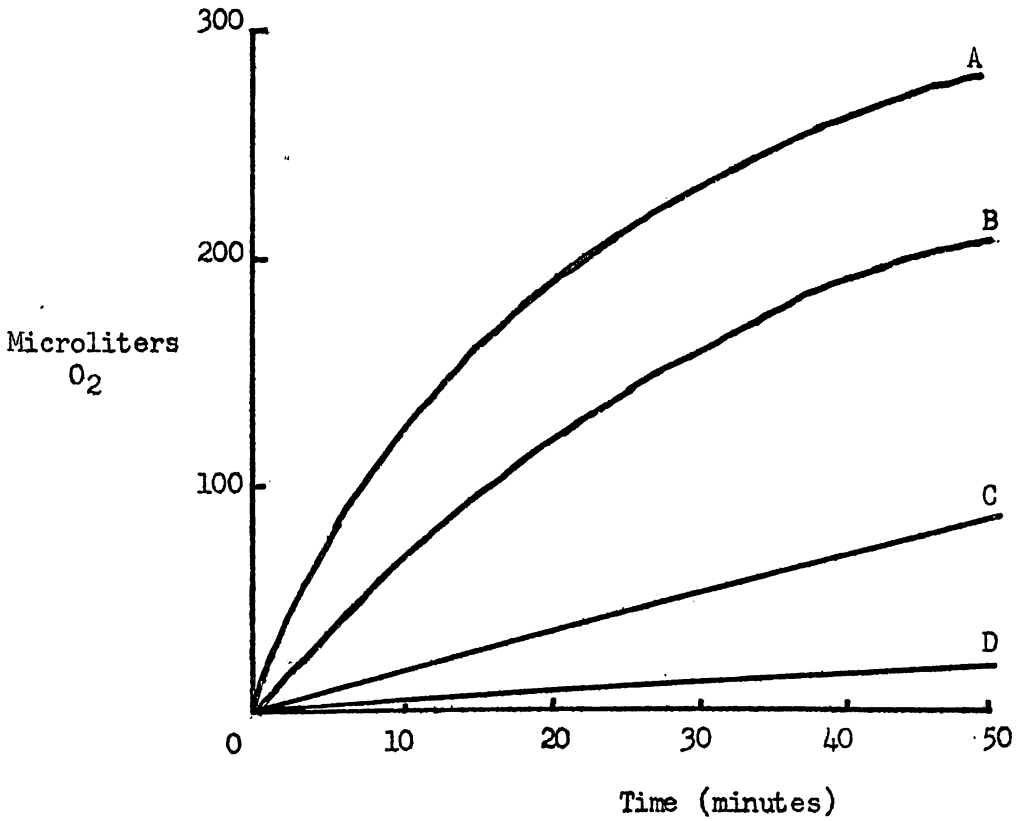
a. Lactic Dehydrogenase as Part of an Oxidase System

Since the original assay systems which were used for the identification of lactic dehydrogenase would not detect how this enzyme functions with electron transferring enzymes, survey studies were made to determine the oxidative activity of whole cells and sonic extracts, using DL-lactate as the substrate. Whole bacterial suspensions were found to have no ability to oxidize lactate in the presence of air at pH values of 6.0, 7.0 and 8.0. Sonic extracts that were freshly prepared, either dialysed or undialysed, showed no oxygen uptake in the presence of lactate at pH 7.4.

In the light of these findings, attempts to show lactate oxidation were made using extracts which were fortified with coenzyme A, DPN, cocarboxylase and magnesium sulfate. These cofactors were used since they were shown to stimulate pyruvate oxidation in whole sonic extracts. If the equilibrium of the lactate-pyruvate reaction were far in favor of the lactate, the lactate oxidation could be increased by oxidizing any pyruvate which was formed from the lactate. As shown in Figure 12, these additions had definite results although the oxygen uptake rates were very slow compared to substrates of the Kreb Cycle.

FIGURE 12

Lactic Oxidase Activity of Bacterium tularense Sonic Extracts



Curve A-15 Micromoles Succinic Acid
Curve B-15 Micromoles Pyruvic Acid
Curve C-500 Micromoles Lactic Acid and Cofactors
Curve D-500 Micromoles Lactic Acid and Endogenous Respiration

Data for curves A,B,and D obtained from flasks which contained in addition to substrate, 1.5 ml 0.1M potassium phosphate buffer pH 7.4 and 1.0 ml of sonic extract. 0.2 ml 10% KOH in center well. Total volume equals 3.2 ml. Gas phase Air. Temperature at 37°C. Data for curve D from flasks containing above reagents plus 0.3 ml coenzyme A concentrate from liver 2.5 mgm/ml, 0.2 ml cocarboxylase 0.05 mgm/ml, 0.1 ml DPN 6 mgm/ml and 0.2 ml $mgSO_4$ 0.1M.

The enzymes and cofactors which are involved in the lactic oxidase system have been briefly studied and some statement can be made with respect to the properties of the system. Early experiments with whole dialysed extracts demonstrated that cytochrome b was the only cytochrome present in B. tularense (60). This identification was achieved with both a highly sensitive spectrograph and the more simple Zeiss Hand Spectroscope. It was noted that the addition of lactate to sonic extracts caused the reduction of cytochrome b. This could be interpreted to mean that cytochrome b is one of the electron acceptors in the lactic oxidase system. Further proof of this concept would be the separation of the lactic dehydrogenase from the cytochrome b and then recombination of the two components to form the complete functional oxidase system. Attempts to separate the cytochrome b and the dehydrogenase were successful according to procedures which will be described for the purification of the dehydrogenase. However, recombination of the active dehydrogenase and the cytochrome b did not restore the oxidase activity as determined by manometric oxygen uptake measurements. Even though this is negative information, cytochrome b is probably a part of the oxidase system since the purified dehydrogenase was unable to act on lactate in the presence of oxygen.

b. Purification of Lactic Dehydrogenase from Whole Sonic Extracts

Many procedures were used in an attempt to purify the dehydrogenase. It was apparent from many observations that the dehydrogenase was associated with the "particulate material" present in the whole sonic extracts. This material was consistantly precipitated

from the whole extract when submitted to either ammonium sulfate or alcohol protein fractionation procedures, and consequently neither of these methods afforded good purification. The problem seemed to be one of solubilization of the dehydrogenase away from the "particulate material". (It might be mentioned that experiments by Fellman and Mills (60) showed that the particulate material contained all of the cytochrome b and succinoxidase activity and resembled to some extent the particulate material obtained by fractionation of mammalian cells to obtain such components as nuclei, mitochondria, etc. The precise designation of this particulate material from B. tularense is impossible although it is easily separated from the small amount of soluble protein by centrifugation at 101,300 X g for one hour.)

The first attempts at purification were made using procedures which were designed to solubilize the "particulate material". Whole sonic extracts were treated with desoxycholic acid (sodium salt) at concentrations ranging from 0.048 to 1.6 %. Considerable clearing of the extract was visible and after centrifugation at 3,000 X g for 1 hourthe resulting supernatents possessed some dehydrogenase activity. At 1.6 % desoxycholic acid, only 33 % of the original activity was found while at 0.048 % desoxycholic acid, about 60 % of the activity remained. The clearing that was seen was assumed to be due to the solubilization of the particulate material. Since it was felt that the activity that was seen in the desoxycholate treated extracts was due to particulate material that was not solubilized, this method of attack was discarded.

Some attempt at purification was made using protamine sulfate precipitation procedures. It has been reported that protamine can precipitate nucleic acid-like material from sonically prepared bacterial extracts, thus leaving soluble enzymes in solution (61). If the lactic dehydrogenase were a soluble enzyme this technique could be used. On treating whole sonic extracts with protamine sulfate at levels of 2 mgm per ml of extract, a heavy precipitate was formed which contained all of the dehydrogenase activity. This supports the contention that the dehydrogenase is associated with the particulate material and is not a soluble enzyme. It is reported that protamine can be extracted away from the precipitate by dilute sulfuric acid solutions but this was not attempted in light of the rather harsh acid treatment which would be involved.

The procedure of purification that proved most successful was freeze-thawing of sonic extracts at pH 6.25. Such a procedure was devised from the observation that mitochondrial preparations can be solubilized by freeze-thawing techniques and that sonic extracts which were routinely used over a period of weeks and which had been frozen and thawed, tended to precipitate a certain amount of protein material. On centrifugation of the insoluble material all of the cytochrome was found in the precipitate and the majority of the dehydrogenase activity was found in the yellowish supernatent solution. The supernatent contained no cytochrome material. Another important observation was that crude sonic extracts tended to precipitate out of solution at pH values lower than 7.4 and tended to

redissolve at higher pH values (about 8). On the basis of this information the following procedure for purification was devised and was subsequently shown the most successful method yet attempted. Crude sonic extracts were dialysed as described under Methods and adjusted to pH 6.25 with a few ml of dilute hydrochloric acid. At this point a heavy cloudiness appeared in the sonic extract. The extract was then stored at -10° C. for 1 week, thawed and made 0.1 M with respect to dipotassium hydrogen phosphate, by the addition of the solid salt directly to the cold extract. (The pH at this point was 7.8-8.0.) The extract was then centrifuged at 20,000 X g for two hours. A large precipiate was obtained (Fraction I) which contained all of the cytochrome material originally present in the crude sonic extract, and was completely devoid of any dehydrogenase activity. The supernatent (Supernatent I) was a clear yellowish solution which contained all of the dehydrogenase activity seen in the original whole extract. After storing Supernatent I for a few days at -10° C., more insoluble material was obtained. This yellowish insoluble material (Fraction II) was found to contain no dehydrogenase activity. The remaining supernatent (Supernatent II) contained all of the dehydrogenase activity. Quantitative bicarbonate-ferricyanide assays for lactic dehydrogenase were performed on each fraction and supernatent obtained. The results of these assays as seen in Table XI indicate that some twenty-fold purification can be obtained by these procedures. Possibly ammonium sulfate or alcohol procedures could be applied to Supernatent II achieving some further degree of purification.

Table IX

Activity of Purified Lactic Dehydrogenase Preparations from Sonic Extracts.

Material	Microliters CO_2/mgm N/hr.
Whole Sonic Extract	40.2
Fraction I	0.0
Supernantent I	476.0
Fraction II	0.0
Supernantent II	785.0

c. Michaelis Constant Determinations for the Purified Enzyme Using D L-Lactate as the Substrate.

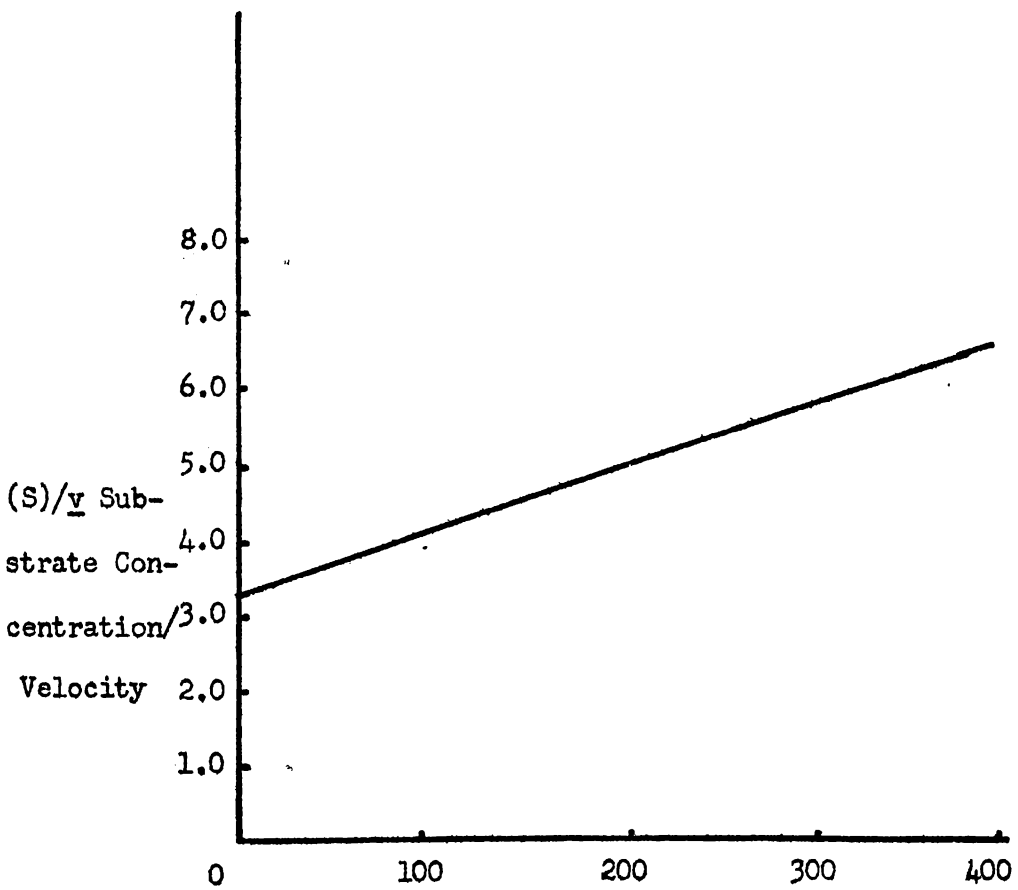
Since the rates of dehydrogenase activity on DL-lactate are known to be low, it was thought of interest to determine the enzyme-substrate affinity by measurement of the Michaelis constant. Supernatent II was used as the enzyme source in conjunction with the bicarbonate-ferricyanide manometric assay previously described. DL-lactate was used at levels ranging from 50 to 500 micromoles per flask. When the results of the assays were plotted according to the procedure of Lineweaver and Burke (62), the curve shown in Figure 13 is obtained. Maximum velocity values calculated from the graph gave a value of 55.3 micromoles/ml/hour and a Michaelis constant of 1.82×10^{-1} M. While these are high values, they substantiate the suspected low enzyme substrate affinity for the dehydrogenase and DL-lactate.

d. Substrate Specificity of the Dehydrogenase

Due to the observed high Michaelis constant, it seemed advisable to determine the substrate specificity for the dehydrogenase and other hydroxy acids. While only a limited number of hydroxy acids were available, those listed in Table XII were tested. Malic and glycolic acids were shown to be slowly utilized by the lactic oxidase system of N. gonorrhea. Alanine was tested as a measure of the possible amino acid oxidase activity of the purified enzyme. Green, et. al., (63) reported that purified L-amino acid

FIGURE 13

Michaelis Constant Determination With DL-Lactate



Bicarbonate-Ferricyanide Assay as described in Text.

TABLE XII

Substrate Specificity of Purified Lactic Dehydrogenase Preparation.

Substrate	Microliters CO_2/mgm N/hr.
DL-Lactic Acid	785.0
L-Malic Acid	0.0
Glycolic Acid	0.0
Beta-Hydroxy Butyric Acid	2762.0
Alanine	0.0

Initial concentrations of all substrates was 500 micromoles/flask except beta-hydroxy butyrate which was 50 micromoles/flask. Calculations based on rates for first ten minutes of reaction.

oxidase possesses in some cases a higher affinity for lactate than for certain amino acids. Beta-hydroxy butyrate was tested since it is an important intermediate in fatty acid oxidation. It is seen that only DL-lactate and beta-hydroxy butyrate were found to have any substrate value and suprisingly the beta-hydroxy butyrate was the best substrate tested.

e. Studies on the Prosthetic Group of the Dehydrogenase

Since all of the dehydrogenase enzymes reported in the literature are linked with some prosthetic group such as DPN, a flavin or a cytochrome, it was reasoned that one of these cofactors is probably associated with the lactic dehydrogenase found in B. tularense. DPN and a cytochrome function can be ruled out on the basis of previous experiments which demonstrated the full dehydrogenase activity in the absence of both cytochrome b and DPN. Procedures for the cleavage of a flavin from a flavoenzyme are reported by Horecker (64). Using his procedures no flavin could be detected and the precipitate obtained during the process retained all of the dehydrogenase activity seen in the starting material. Dialysis against acidified water over a period of two days tended to destroy all of the dehydrogenase activity of the starting material. The addition of flavin adenine dinucleotide, flavin monophosphate and riboflavin did not reactivate the dialysed material. Thus while no flavin can be detected, this cofactor should not be ruled out until all flavins are shown to be absent in the purified enzyme. This should be possible on the basis of very sensitive flavin determinations which are available (65).

DISCUSSION

Data have been presented which indicate the absence of a system in B. tularense which is capable of reoxidation of reduced DPN normally produced during anaerobic glucose dissimilation. In mammalian systems, reoxidation of reduced DPN is a function of lactic dehydrogenase while in bacterial fermentations reduced DPN oxidation is a function of many well defined dehydrogenases, among which are lactic, alcohol and alpha-glycerophosphate dehydrogenase. It is unlikely that any of these latter dehydrogenases is functional in B. tularense due to the complete lack of anaerobic glucose utilization in both whole cells and sonic extracts. Apparently the only normal reoxidizing system which is functional in B. tularense, is the electron transfer from reduced DPN through the cytochrome carriers to oxygen. This excludes anaerobic conditions and thus offers an explanation for the original observation that B. tularense can easily utilize glucose in the presence of air but cannot utilize glucose in its absence.

It is also seen that the addition of three separate substances to B. tularense sonic extracts will allow glycolysis to take place. DPN-linked mammalian lactic dehydrogenase allowed glycolysis since it could utilize pyruvate as an electron acceptor from reduced DPN. Oxaloacetate when either added directly to sonic extracts or when provided to the extracts by transamination of aspartic acid with pyruvic acid, also allowed glycolysis. The oxaloacetate acts as an electron acceptor from reduced DPN by virtue of the presence of DPN-linked malic dehydrogenase in the sonic extracts. Thus all of the methods of

producing a glycolyzing system in B. tularense sonic extracts provide the extract a means of reoxidation of reduced DPN.

The phenomenon of obligate aerobic glycolysis does not appear to be unique with B. tularense but is also characteristic of other microorganisms. Bacteria which have been shown to possess this phenomenon have the following properties all of which are characteristic of B. tularense: 1) predominantly aerobic, utilizing glucose, 2) pathogenic (or directly related to pathogenic species) and intracellular during their parasitic phase of infection, and/or 3) possessing a DPN-independent lactic dehydrogenase. The first requirement covers a whole multitude of species, and bacteria chosen only on this basis do not necessarily possess an obligate aerobic glycolysis. This is shown in the case of the four species studied in this work, viz., P. aeruginosa, A. faecalis, S. lutea, and B. anitratum. None of these organisms possesses either requirement two or three even though they are predominantly aerobic in their growth requirements and utilize glucose. However all of the bacteria chosen which possessed properties two and three along with property one, were shown to have the obligate aerobic glycolysis seen in B. tularense. The single exception was the case of an avirulent strain of P. pestis which glycolysed in the absence of added lactic dehydrogenase equally as well as in its presence. Bacteria shown to be similar to B. tularense in their utilization of glucose were A. tumefaciens, B. abortus, N. perflava, M. phlei, and R. meliloti. The first two speices are intracellular pathogens (66,67) and the latter

organism, while not a pathogen exists in an intracellular symbiotic relationship with its host (68). N. perflava and M. phlei are not intracellular species but are known to possess active DPN-independent lactic dehydrogenase systems (25,26). These latter two species are also directly related to definite intracellular species, viz., M. leprae and N. gonorrhea.

The relationship between virulence or pathogenicity and the presence of obligate aerobic glycolysis can be briefly mentioned. It is seen that three strains of B. tularense all possess the phenomena of obligate aerobic glycolysis and roughly to the same extent. One of these species is some 10,000 times less virulent than Sm and the other completely avirulent to most mammalian hosts. Completely avirulent N. perflava used in this work possessed the effect in discussion as did the non-pathogenic M. phlei. On the basis of this one might conclude that obligate aerobic glycolysis can be carried throughout a whole genus irrespective of virulence or pathogenicity. On the other hand a completely avirulent strain of P. pestis did not have obligate aerobic glycolysis which forces one to ask whether the fully virulent P. pestis strains are similar to the avirulent strain. Unfortunately this problem cannot be reconciled due to the high pathogenicity of virulent P. pestis cultures.

Another interesting relationship which might allow some speculation is that of obligate aerobic glycolysis and intracellular parasitism. It is well known that intracellular rickettsiae and viruses use the enzymatic machinery of their host cells to carry out their energy

utilizing reproductive processes. Whether a similar situation exists in intracellular bacteria is certainly not known but is an interesting problem to consider. For example, this work shows that B. tularense and several other pathogens have a DPN-independent lactic dehydrogenase, thus dissallowing the possibility of anaerobic glycolysis. In times of oxygen lack within a host cell it might be feasible to suppose that the lactic dehydrogenase of the host cell (DPN-dependent) might be utilized by the bacteria to carry out an anaerobic glucose utilization. Attempts to demonstrate glycolysis by whole cells of B. tularense in the presence of mammalian lactic dehydrogenase were unsuccessful. Thus while no experimental evidence for a virus-like parasitism exists it is a possibility which should not be disregarded without some thought.

Perhaps one of the most frustrating problems concerning the distribution of the phenomenon of obligate aerobic glycolysis was the lack of time, and suitable technique to study this effect in many intracellular pathogens. Mention can be made of many bacterial species which are definite intracellular pathogens and which might possibly possess obligate aerobic glycolysis. M. leprae, the causative agent of leprosy is a definite intracellular pathogen and is certainly worthy of study as is its very close relative, M. tuberculosum, the non-intracellular causative agent of tuberculosis. Table XIV presents bacteria which have not been studied but which might possibly possess the property of obligate aerobic glycolysis as found

TABLE XIV

Bacteria Which Might Possibly Possess Obligate Aerobic Glycolysis As Found in Bacterium Tularense.

Organism	Glucose Utilization	Aerobic Nature	Intracellular Nature	Type of Lactic Dehydrogenase
Mycobacterium tuberculosum	(69)	(82)	(—)	(25)
Mycobacterium leprae	(69)	(82)	(83)	(—)
Neisseria gonorrhea.	(71)	(86)	(77)	(26)
Neisseria intracellularis	(78)	(78)	(75)	(--)
Bartonella bacilliformis	(82)	(82)	(79)	(--)
Listeria monocytogenes	(80)	(82)	(—)	(—)
Donovania granulomatis	(81)	(82)	(81)	(--)
Actinobaccilus lignieresi	(82)	(82)	(--)	(--)
Malleomyces mallei	(85)	(82)	(--)	(--)
Noguchia granulosis	(84)	(82)	(--)	(--)
Gaffkya tetragena	(86)	(86)	(--)	(--)

in B. tularense. The Table also presents known properties of the bacteria which show the similarity between these organisms and the bacteria which have already been shown to possess the obligate aerobic glycolytic phenomenon. Whether other intracellular pathogens such as the viruses and rickettsiae have a similar glycolytic impairment as found in B. tularense cannot be answered until techniques become available for the cultivation of large numbers of these organisms.

The concept of obligate aerobic glycolysis reported to be present in a number of bacterial species is not entirely consistent with previous information reported by other workers. B. tularense, the key bacteria in this study, has been reported to "ferment" various carbohydrates including glucose (68). This information was based on the color change of agar growth medium containing acid-base indicators. It has been pointed out that this type of study does not indicate anaerobic carbohydrate utilization since no strict means of excluding air is taken. Furthermore it does not take into account possible acid-base reactions which might result from reactions other than those of the carbohydrate present in the growth agar. Certainly information such as obtained from acid-base indicators in growth agar should not be accepted unequivically as evidence for a particular biochemical pathway, however useful such experiments are in differentiating various bacterial species.

The present work is not in conflict with those studies which have been made on various species of Mycobacteria. Edson (69) points out that the acid fast bacteria possess the chief enzymes of the Embden-

Meyerhof scheme. He also has demonstrated that these bacteria do not possess glycolysis though he does not account for this biochemical lack on the basis of the absence of a DPN-linked enzyme capable of reoxidation of reduced DPN normally produced during glycolysis (69). It is interesting to note that Hanks and Gray (70) have attempted to classify the Mycobacteria on the basis of their metabolism. They point out that in a scale extending from the saprophytic species up through the tubercle bacillus to M. leprae, there is a tendency for the organisms to lose the ability to grow on artificial nutrient media as well as becoming obligatory intracellular parasites. In the strictest biochemical sense however, no exact metabolic differences have been shown by these workers. It would seem that the utilization of sonic extracts in the study of the metabolism of the various species of Mycobacteria might bring to light more subtle biochemical differences.

While no metabolic studies have been performed on the glycolysis of N. perflava, Barron and Miller (71) have shown that N. gonorrhea utilizes glucose aerobically, pyruvate, lactate, and acetate appearing as intermediates in the oxidation. While lactate is associated with the aerobic breakdown of glucose it could possibly arise from the alpha-hydroxy acid oxidase that is found in very high concentrations in this bacteria. Thus studies which have been performed on the glycolysis of Neisseria, are not inconsistent with the data obtained in the present study.

The metabolic nature of Rhizobia is also not too clear but what work has been performed indicates that anaerobic glucose utilization

does take place resulting in the production of butyrate, lactate, acetate and carbon dioxide (72). Wilson has confirmed this finding using root nodule suspensions (73). While R. trifolii was used in the studies which showed a glucose fermentation and R. meliloti was used in the present study, it is highly probable that they are very similar metabolically. Further work with R. meliloti might clarify the discrepancy.

B. abortus has been shown to convert 80% of the glucose in an aerated glucose-tryptose medium to carbon dioxide with appreciable quantities of acetic acid also formed (74). Only traces of formic acid, lactic acid and ethanol were found. Roessler, et al, (36) have reported that B. suis will glycolyze anaerobically. These workers have reported the isolation and identification of fructose diphosphate, fructose 6-phosphate, glucose-1-phosphate, and glucose-6-phosphate from sonic extracts of B. suis which were incubated in a mineral mixture containing glucose and ATP. While about 15% of the added glucose was utilized in 4 hours, no indication is made whether any glycolytic intermediate below fructose diphosphate was found. If these three-carbon intermediates were not found then the data reported in this thesis would not be inconsistant with their findings. It is possible that B. suis does differ from the B. abortus studied in the present work.

In the case of A. tumefaciens no strict biochemical studies on its glucose utilization have been made. It is known that sugars are

utilized with the major product found to be carbon dioxide.(76). This appears to be similar to the observations made with B. tularense, B. abortus and N. gonorrhea.

One of the most striking features of obligate aerobic glycolysis is the observation that all bacteria possessing the phenomenon also possess a DPN-independent lactic dehydrogenase. The exact function of this dehydrogenase as it exists in these bacteria is not known, although it certainly does not possess the unique $DPNH_2$ reoxidizing property as seen in lactic acid fermenting organisms. While this enzyme has been indentified for the first time in B. tularense, N. perflava, R. meliloti, B. abortus, and A. tumefaciens, time allowed only a brief study on the dehydrogenase in a single species, B. tularense.

The lactic dehydrogenase in B. tularense seems to be associated with cytochrome b and possibly other factors to form a lactic oxidase system. However the oxidase system is not nearly as active as the succinic oxidase system also known to be present in B. tularense (60). The first attempts to isolate the dehydrogenase from sonic extracts led to the observation that it was closely associated with insoluble particulate material which was much like that found in mammalian cells. Thus solubilization of the dehydrogenase away from the particulate material was achieved in much the same manner that is used for the solubilization of particulate enzymes of such tissues as mammalian liver, kidney, and heart.

Some of the properties of the lactic dehydrogenase have been studied. It appears to be associated with none of the coenzymes previously reported for other DPN-independent lactic dehydrogenases. It is neither cytochrome linked as found in yeast (23) nor has it yet been shown to be associated with a flavin as found in gonoccoci (26), M. phlei (25) and P. chrysegenum (24). No amino acid oxidase activity is seen in purified preparations and the only hydroxy acid metabolized besides lactate is beta-hydroxy butyrate. Its Maechalis constant is very high showing an extremely low enzyme-substrate affinity. This is very similar to the observed substrate-enzyme affinity seen in P. chrysogenum.

SUMMARY

1) An explanation is presented for the observed inability of B. tularense to utilize glucose anaerobically.

2) The explanation is based on the observation that reduced DPN normally produced during glucose dissimilation cannot be reoxidized by the enzymes of B. tularense except in the presence of air.

3) This obligate aerobic glycolysis is found in various bacteria which are very similar to B. tularense in their biochemical and growth requirements. Those bacteria showing the phenomenon of obligate aerobic glycolysis are B. abortus, A. tumefaciens, M. phlei, R. meliloti, N. perflava and two different strains of B. tularense.

4) Since no anaerobic glucose utilization was found in B. tularense it is assumed that the enzymes which are normally responsible for reduced DPN oxidation during glucose fermentation are absent. However it has been shown that all of the bacteria which possess the obligate aerobic glycolysis also possess a DPN-independent lactic dehydrogenase.

5) Some of the properties of the DPN-independent lactic dehydrogenase found in B. tularense have been studied. It is definitely not cytochrome linked as seen in yeast and as yet is not shown to be associated with a flavin as is the lactic dehydrogenase of other microorganisms.

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