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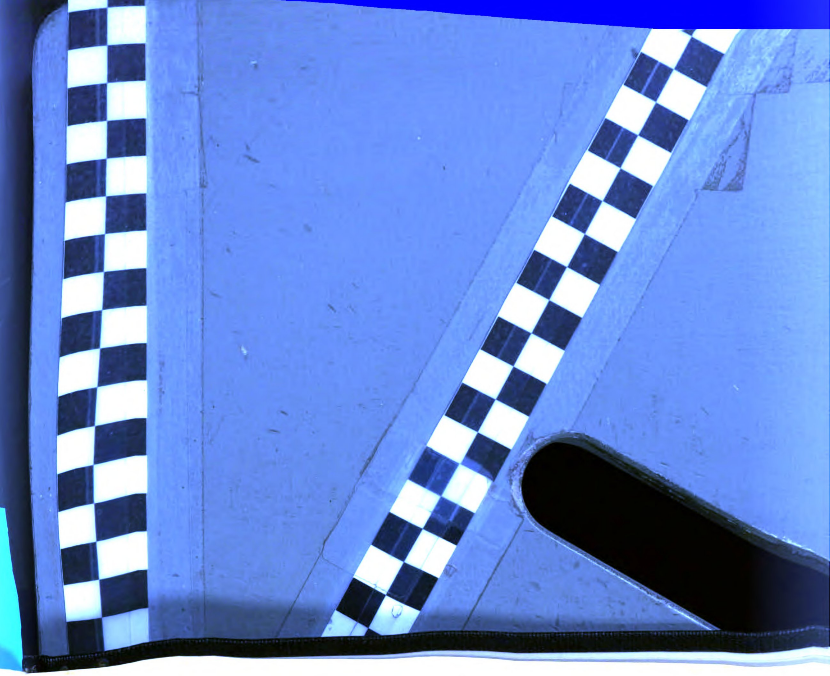

A STUDY OF THE TERMINAL RESPIRATION OF *THIOBACILLUS THIOPARUS*

By

Robert Chauncey Cooper

AN ABSTRACT

A study has been made on the terminal respiration of Thiobacillus thioparus. Whole cell suspensions showed some oxygen uptake in the presence of tricarboxylic acid cycle intermediates. This uptake appeared to be directly proportional to the endogenous rate. Unsupplimented cell free extracts demonstrated no activity against these organic intermediates

Evidence has been presented for the presence of various enzymes of the tricarboxylic acid cycle. No direct evidence could be obtained for the presence of cis-aconitase or fumarase. Tests for the condensing enzyme, citrogenase, were inconclusive. Citrate was shown to be active in the metabolism of T. thioparus but the actual mechanism involved in its formation was not clear. The enzyme, citratase, appeared to be absent.

From the results obtained it appears that T. thioparus has a terminal respiration similar to that found in many heterotrophic

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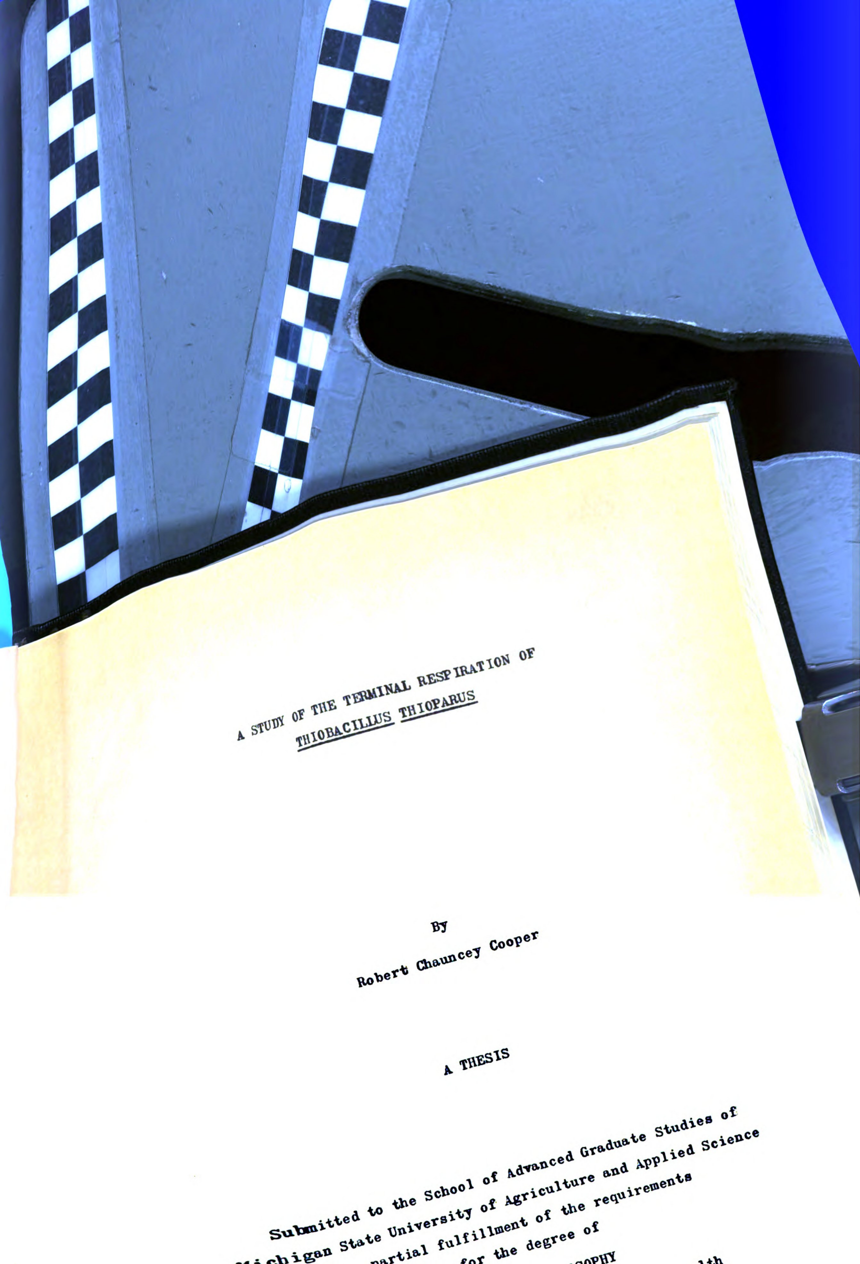

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THIOBACILLUS THIOPARUS

By

Robert Chauncey Cooper

A THESIS

Submitted to the School of Advanced Graduate Studies of
ichigan State University of Agriculture and Applied Science
partial fulfillment of the requirements
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Results

The concept of chemoautotrophy was formulated by Winogradsky (1887,1890) on the basis of his studies of the filamentous sulfur bacteria and the nitrifying bacteria. The fact that living cells can multiply and sustain themselves on completely inorganic materials gives rise to many questions concerning such an organism's metabolism and its relationship to heterotrophic organisms.

Chemoautotrophs are generally defined as those organisms which use only inorganic sources of carbon for their life processes. This definition may be too limiting; would it be incorrect to state that an organism is not an autotroph simply because it needs a minute trace of a vitamin or similar growth factor? Woods and Lascelles (1954) feel that too strict a definition gives rise to many new classification problems. For example, certain strains of *Hydrogenomonas* fit into this category but by all other criteria are living autotrophically. These workers feel that the definition should be changed to include those organisms whose main source of carbon is derived from inorganic sources. Umbreit (1947) defines autotrophs as those organisms whose sole source of energy is derived from the oxidation of inorganic materials which are irreplaceable and in which carbon dioxide is the sole source of carbon. In defining these organisms Werkman (1951) feels it is better to sense the differentiation than to define it. Stanier *et al.* (1957)

emolithotroph rather than chemoautotroph in order to

nd still allow separation of organ-

izable substrate

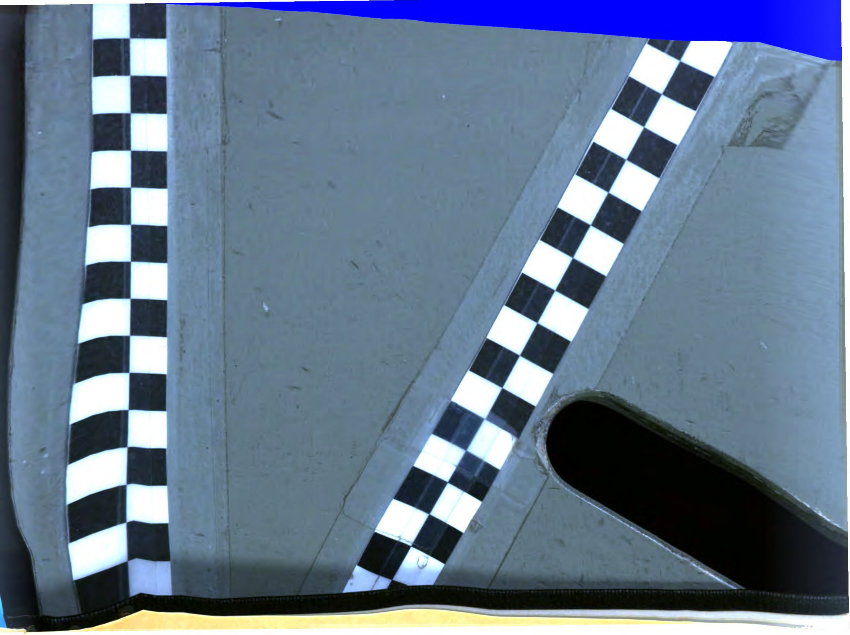
Regardless of the term used, all the definitions imply a high degree of synthetic ability by these organisms since the bulk if not all the organic material of the cell originates from inorganic sources.

How do the organisms that fall into the above classification carry out their metabolic processes? Except for their special ability in assimilating inorganic carbon, it may be incorrect to assume that their basic metabolic patterns are different than those found in heterotrophic organisms. On the assumption of uniformity in nature it would be advantageous to approach the metabolism of autotrophic bacteria by considering it in terms of metabolic patterns already known to exist in other microorganisms. It may be best to seek activities that are common to chemolithotrophs and heterotrophs. Some small similarities do exist such as the existence of certain lithotrophic activities which are common to heterotrophs, as in <u>Escherichia coli</u> for example, in which hydrogen + carbon dioxide → formate or succinate + carbon dioxide → alpha-ketoglutarate (Utter and Wood, 1951). Some heterotrophic bacteria can utilize carbon dioxide as the main hydrogen acceptor for anae-

All these facts indicate that definite similarities in the metabolism of these organisms do exist.

However, at present there are few actual facts concerning the metabolic patterns of autotrophic microorganisms. Knowledge of these patterns would clarify the relationship of these organisms to other bacteria and how they fit into the evolutionary scheme of bacterial development. Information of this kind may also be useful as another step in showing metabolic continuity among various forms of life.

The purpose of this work is to give some evidence that a chemolithotrophic organism, <u>Thiobacillus</u> <u>thioparus</u>, has a terminal oxidation system similar to many heterotrophic microorganisms.



LITERATURE REVIEW

The genus *Thiobacillus* includes nine species (Bergy 1957) of chemolithotrophic bacteria which oxidize sulfur compounds to sulfate. Some members of this group are facultative autotrophs but the majority are obligate autotrophs. The four common species of this genus are *Thiobacillus thioparus*, *T. novellus*, *T. denitrificans*, and *T. thiooxidans*.

Winogradsky (1887, 1888) described certain sulfur bacteria, *Thiothrix sp.*, and this work gave impetus to the study of the sulfur bacteria. Nathanson (1903) described a group of bacteria found in sea water which oxidized hydrogen sulfide or sodium thiosulfate and reduced carbonic acid to form intracellular materials. These organisms grew on a completely inorganic medium and did not accumulate free sulfur intracellularly which differentiates them from those described by Winogradsky. Beijerinck (1904) confirmed Nathansons work using a completely inorganic medium with thiosulfate as the energy source and named the organisms *Thiobacillus*. In this instance he isolated two organisms and described them as *T.*

The occurrence in sewage of a sulfur oxidizing organism which fixed carbon dioxide and turned the medium acid to methyl orange was reported by Lockett (1914). Waksman and Joffe (1921, 1922) described the isolation of an organism which can tolerate conditions of low pH, even lower than pH one. They named this organism *Thiobacillus thiooxidans* and it is probably identical to the one described by Lockett. *Thiobacillus novellus* differs from all the organisms described in that it is a facultative lithotroph. This organism was isolated from soil by Starkey (1934) and will grow on ordinary laboratory media as well as media which contain no organic material. A facultative autotroph which oxidizes sulfur and is also a facultative anaerobe has been reported (Tyulpanova, 1930). This organism seems to bear the same relationship to *T. denitrificans* that *T. novellus* does to *T. thioparus*. It is not listed in *Bergy's Manual*.

All of these organisms are small gram negative rods, usually motile by means of a polar flagellum. The special metabolic activities of these organisms have stimulated some workers to search for morphological characteristics which might differentiate the autotrophic from the heterotrophic bacteria. Umbreit and Anderson (1942) studied *T. thiooxidans* using the electron microscope and reported that cultures of this organism are not a homogenous entity

contain cells in all stages of growth and that there exists a

hich is differentiated from the internal proto-

the cell structure of *T. thio-*

ther bacteria

consisting of a gram negative protoplasm containing one or more large vacuoles and that the cells are actively motile.

These organisms appear to be wide-spread in soil and fresh and and sea water. Bunker (1936) reports T. thioparus to be widely distributed in coastal waters, marine muds, fresh water and soil. These organisms are not found in all soils as reported by Starkey (1935) who found sulfur oxidizing bacteria in only two out of twenty-nine samples tested.

The position of these organisms in bacterial taxonomy has been questionable for some years. Breed and Conn (1938) stated that the general assumption that such bacteria are the most primitive form of life may well be erroneous and moved that these autotrophs be taken out of the Nitrobacteriacae and considered either as an independent family or as a tribe in the family Bacteriacae. Bergy's Manual listed these organisms in the Nitrobacteriacae group until 1957 at which time they were given new status in the order Pseudomonadales, family Thiobacteriacae. This last classification seems most reasonable because their gram reaction, size, shape and flagellation places them in close relation to the pseudomonads.

The thiobacilli are of practical significance in a number of ways. They play a role in the sulfur cycle in which they oxidize reduced sulfur to sulfate which in turn can be reduced by such organisms as Desulfovibrio sp. Gleen and Quastel (1953) feel that the thiobacilli as well as some heterotrophs supply intermediates to soil which may play a beneficial role in the manganese up-take of certain plants. Reports have been made that T. thiooxidans

causes the corrosion of sulfur-containing pipe line seals. Frederick and Starkey (1948) state that this is a minor problem, but have shown that thiobacilli will attack some pipe sealing mixtures. Certain members of the group are thought to cause the corrosion of concrete (Parker and Prisk, 1953) and T. denitrificans has been reported by Robotonova et al. (1950) to break down crude asphalt. The acidic properties of mine drainage is usually ascribed to members of the thiobacilli (Colmer and Hinkle, 1947). Temple and Delchamps (1953) have postulated a sequence of synergistic events which cause acid mine water. The hypothesized synergism exists between T. ferrooxidans, an organism which can oxidize iron or thiosulfate, and T. thiooxidans as follows:

Non-biological: $FeS_2 + H_2O + 3\frac{1}{2}\ O_2 = FeSO_4 + H_2SO_4$

Biological-(T. ferrooxidans): $2FeSO_4 + \frac{1}{2}O_2 + H_2SO_4 = Fe_2(SO_4)_3 + H_2O$

As the $Fe_2(SO_4)_3$ is formed it reacts with finely divided pyrite present as follows:

Non-biological: $Fe_2(SO_4)_3 + FeS_2 = 3FeSO_4 + 2S$

Biological(T. thiooxidans): $S + 1\frac{1}{2}O_2 + H_2O = 2H^+ + SO_4$

oxidation of elemental sulfur.

Beijerinck (1904) proposed the following as the over-all reaction carried out by T. thioparus when thiosulfate is oxidized:

$$(1)\quad Na_2S_2O_3 + \tfrac{1}{2}O_2 = Na_2SO_4 + S^{\circ}$$

This is a rather simple equation and has been proposed in more detail by Starkey (1935) as:

$$(2)\quad 5Na_2S_2O_3 + H_2O + 4O_2 = 5Na_2SO_4 + H_2SO_4 + 4S^{\circ}$$

This equation seems to hold true for all the strictly aerobic species of thiobacillus. Thiobacillus denitrificans under anaerobic conditions utilizes nitrate rather than oxygen in the following manner (Foster, 1951):

$$(3)\quad 6KNO_3 + 5S + 2H_2O = K_2SO_4 + 4KHSO_4 + 3N_2$$

All of these oxidations result in the formation of sulfate with the production of acid. In the case of equations (1) and (2) sulfur is also an end product.

From their experiments with T. thiooxidans Vogler and Umbreit (1941 a,b) concluded that the organism must be in direct physical contact with the sulfur particle before sulfur oxidation can take

(1956) demonstrated that elemental sulfur was oxidized more rapidly in shaken than stationary medium. This indicates that direct contact may not be required as proposed by Umbreit <u>et</u> <u>al</u>. Knaysi (1943) found vacuoles in cells of <u>T. thiooxidans</u> to contain both volutin and sulfur and proposed that these materials might be mistaken for fat globules.

It seems curious that these organisms which use sulfur in their metabolism should in turn produce free sulfur as an end product. Starkey (1935a) demonstrated that 40 percent of the oxidation products of <u>T. thioparus</u> was elemental sulfur and 60 percent sulfate. He concluded that this elemental sulfur arises by a specific biological reaction which is part of the mechanism of thiosulfate oxidation. Work has been done since which indicates that the sulfur is produced by a non-biological mechanism. Tamiya <u>et</u> <u>al</u>. (1941) state that sulfur is produced by a non-biological decomposition of pentathionate to tetrathionate and sulfur. He sums up the oxidation of thiosulfate in the following reactions:

(4) $S_2O_3^{=} + 0.25O_2 + 0.5H_2O = 0.5S_4O_6^{=} + O\bar{H}$

(5) $2S_4O_6^{=} = S_3O_6^{=} + S_5O_6^{=}$

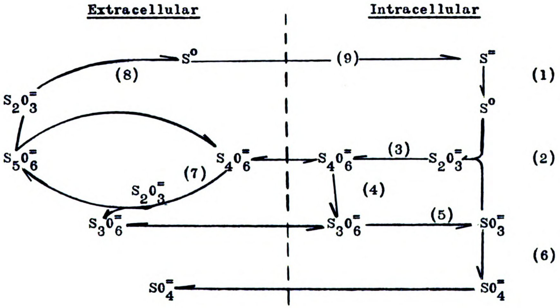
(6) $S_5O_6^{=} = S_4O_6^{=} + S^{o}$

(7) $S_3O_6^{=} + H_2O = S_2O_3^{=} + SO_4^{=} + 2H^{+}$

(8) $S_3O_6^{=} + 2O_2 + 2H_2O = 3SO_4^{=} + 4H^{+}$

Only reactions (4) and (8) are thought to be biological. The

remainder are carried on by spontaneous chemical reactions. Vishniac (1952) and Vishniac and Santer (1957) agree with Tamiya *et al.* that sulfur produced by *T. thioparus* is a result of purely non-biological mechansims, but disagree in that they feel thiosulfate oxidation proceeds through the intermediate formation of tetra, tri and dithionates. Using *T. thioparus* these workers have postulated the following pathway for sulfur oxidation:



In this scheme sulfide enters the cell (9) and is oxidized to sulfur (1). The sulfur undergoes an enzymatic condensation with sulfite (2) to form thiosulfate which is oxidized (3) to form tetrathionate. Thetrathionate is oxidized (4) to trithionate and

to form trithionate and pentathionate. This reaction is catalized by thiosulfate. The pentathionate, in the presence of thiosulfate will produce sulfur and tetrathionate (8). This scheme will explain all the phenomena noted in growing cultures. Reaction (1) has been observed in other organisms; reactions (3) and (4) have been observed in T. thioparus. Reactions (7) and (8) are known inorganic phenomena. Reactions (2),(5),(6) and (9) are conjecture on the part of the authors.

The method by which elemental sulfur gets into the cell is open to much speculation. Starkey (1937,1937a) has reported the ability of T. thioparus and T. thiooxidans to reduce elemental sulfur to hydrogen sulfide. This reaction may point to the presence of active sulfhydryl groups in the sulfur bacteria such as found in glutathione. This may be the means of transport in reaction (9) above and may be schematically represented as follows:

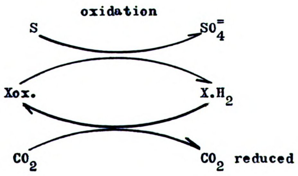
$$\text{Glutathione} \cdot \text{SH} + \text{HS}. \text{Glutathione} + S^{0} =$$
$$\text{Glutathione} \cdot \text{S-S}. \text{Glutathione} + 2H^{+} + S^{=}$$

Starkey stated that this was an unlikely mechanism because, according to his calculations, it was thermo-dynamically impossible.

T. thiooxidans could oxidize sulfur in the absence of CO_2 and subsequently these same cells will fix CO_2 in the absence of sulfur. The amount of CO_2 fixed in the absence of sulfur is limited to about 40μL CO_2/100 gms. of bacterial nitrogen. This work showed that the oxidation of sulfur and the fixation of CO_2 were independent though related entities. This worker also felt that some of the oxygen of the final product, sulfate, arises from carbon dioxide. Vogler and Umbreit (1943) continued this work and indicated that the oxidation of sulfur is coupled with the transfer of inorganic phosphate from the medium to the cells and that CO_2 fixation returns inorganic phosphate to the medium. This was apparent in their results which showed that the oxidation of sulfur in the absence of CO_2 removed inorganic phosphate from the medium and similarly the same cells, freed of sulfur, and in a CO_2 atmosphere, added inorganic phosphate to the medium. It was postulated that the energy of sulfur oxidation was trapped and transported in the form of phosphate esters. Newburgh (1954) repeated this work in regards to the uptake of inorganic phosphate but felt that the simultaneous presence of CO_2, oxygen and sulfur is required since he could not show it to be independent of any of these substances. He also showed that CO_2 fixation can be separated from sulfur oxidation but that no inorganic phosphate liberation could be demonstrated. Baalsrud and Baalsrud (1952) report in their work with various spe-

however, had been observed during the oxidation of thiosulfate. Umbreit (1954) repeated the work he had done in 1943 since many questions had been raised about his conclusions. This later work upheld his original hypothesis.

LePage and Umbreit (1943) isolated a number of organic materials from T. thiooxidans. Among the isolates was a compound very similar to adenosine triphosphate (ATP). Later (1943a) these workers defined this compound as ATP and stated that it contained ribose-3-phosphate rather than the usual ribose-5-phosphate of bacteria. However, Barker and Kronberg (1954) made a very careful analysis of the ATP found in T. thiooxidans and discovered it to be adenosine-5-triphosphate. They could find no evidence for any other type of ATP. Vishniac and Ochoa (1952) suggest that the oxidation of sulfur may be coupled to electron carriers which in turn may reduce CO_2 within the cell. Oxidation of these electron carriers could yield high energy phosphate compounds and this esterification could account for the disappearance of phosphate from the medium. A diagramatic representation of such a scheme follows:



The "X" in the above scheme represents the electron carrier. The

reoxidation of which may yield the energy for CO_2 fixation. This electron carrier could possibly be an iron containing system since Vogler et al. (1942) have shown that azide and similar compounds inhibit sulfur oxidation. More recently Marqulies et al. (1958) have shown that T. thioparus consumes less oxygen when oxidizing thiosulfate in the presence of buffers other than phosphate. Thiosulfate and tetrathionate disappear and chromatography shows the accumulation of sulfur containing compounds not found when phosphate is present. This seems to indicate that phosphate is involved in the oxidation of some intermediate which is produced during the oxidation of thiosulfate to sulfate.

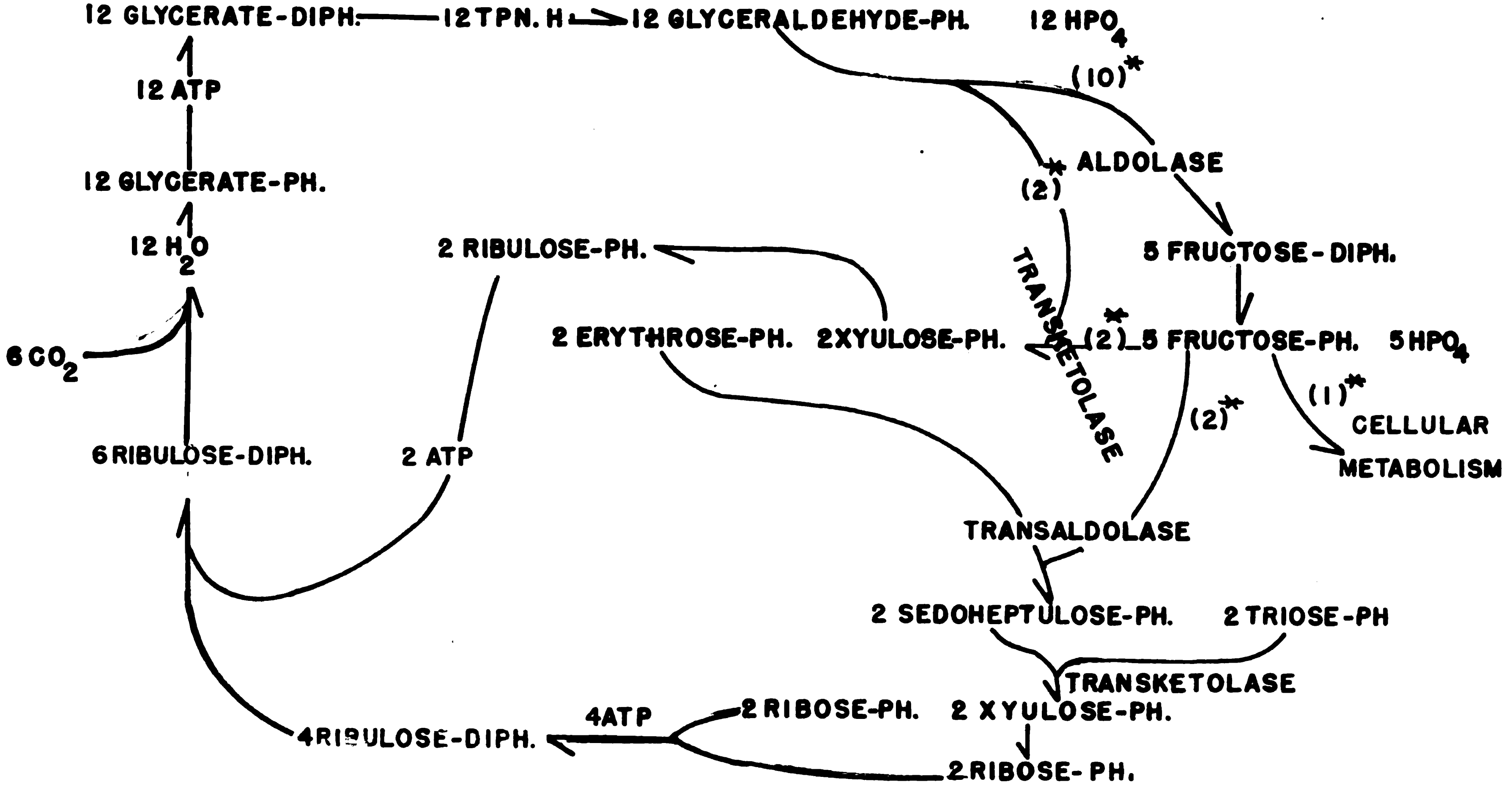
The organism's system of sulfur oxidation and energy exchange appears to be extremely inefficient. As stated previously, T. thioparus produces 40 percent sulfur and 60 percent sulfate. The production of the former appears to be a great energy loss. In continuing this work Starkey (1935) calculated that the machine efficiency of this organism was quite low since the cell utilizes only 4.7 percent of the energy liberated in the oxidation of sulfur. The photosynthetic process, calculated in the same manner, is 80 percent efficient, a considerable difference. Starkey also

organisms to heterotrophs by the isolation of known metabolic intermediates. Starkey (1925) demonstrated that glucose disappears from the sulfur medium during active growth but is not removed in the absence of sulfur. He also found that ammonia is the only source of nitrogen. Urea, peptone and amino acids could act as neither a nitrogen nor carbon source. Vogler (1942) showed that T. thiooxidans has an endogenous oxygen uptake in the absence of sulfur. He stated that this indicated the utilization of organic materials which must have been previously synthesized by the chemosynthetic process. Such growth factors as nicotinic acid, pantothenic acid, biotin, riboflavin, thiamin and pyridoxine were isolated from cell extracts by O'Kane (1942). None of these compounds alone or in combination stimulated growth when added to the growth medium. Because of the endogenous respiration in T. thiooxidans, LePage (1943)proposed that there must be a storage substance which sustains life. He determined this to be a polysaccharide containing glucose,mannose and some other reduced material. In the same experiments in which LePage and Umbreit (1943) isolated ATP they also isolated, from T. thiooxidans: fructose 1-6 diphosphate, phosphoglyceric acid, fructose-6-phosphate, glucose-6-phosphate, glucose-1-phosphate and presumptive evidence for the presence of coenzyme I. From this work they concluded that the internal metabolism of

acids were tested. They either inhibited respiration or had little or no effect. Rittenberg *et al.*(1950) succeeded in obtaining a mutant of *T. thiooxidans* by ultraviolet irradiation which required yeast extract for growth. This again points to a heterotroph-like metabolism. Skarznski *et al.*(1956) isolated a new cytochrome for *T. thioparus* which they called cytochrome S and which appeared to be different from any known cytochrome. Aubert *et al.*(1958,1958a) and Milhaud *et al.*(1958) demonstrated a cytochrome C in *T. denitrificans*. This is of interest since this organism is a facultative anaerobe and the cytochrome transports electrons to nitrate or oxygen depending upon the method of growth. All of this work keeps adding to the general fund of knowledge but it has not been well organized into any definitive scheme.

Until recently the mechanism of carbon dioxide fixation in chemoautotrophic bacteria had remained obscure. However, the work of Calvin on photosynthesis has opened the door to this problem. Calvin and Massini(1952) working with algae and using $C^{14}O_2$ looked for the first compound they could find in which the labeled carbon dioxide was incorporated. This compound was phosphoglyceric acid. Bassham and co-workers (1954) demonstrated that ribulose 1-5 diphosphate was the acceptor compound for CO_2 in green plants. These

FIGURE I. A CYCLIC MECHANISM FOR CARBON DIOXIDE FIXATION AND THE REGENERATON OF THE CARBON DIOXIDE ACCEPTOR. (ADAPTED FROM BASSHAM _ET AL._, 1954)



*THE NUMBER IN PARENTHESIS INDICATES THE NUMBER OF MOLECULES INVOLVED IN THE REACTION.

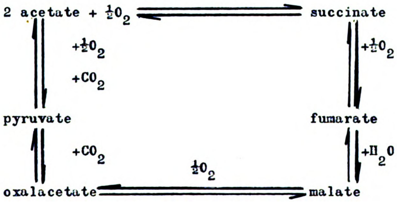
Since it seemed feasible that chemoautotrophs might fix CO_2 in a similar manner, a number of workers turned in this direction. Santer and Vishniac (1955) demonstrated that extracts of _T._ _thioparus_ only fix CO_2 in the presence of ribulose diphosphate. Using Using labeled carbon dioxide they were able to isolate phosphoglyceric acid containing a labeled carbon. Trudinger (1955,1956) found that extracts of _T._ _denitrificans_ fixed $C^{14}O_2$ into the carboxyl group of 3 phosphoglycerate in the presence of either ribose-5-phosphate plus ATP or ribulose 1-5 diphosphate alone. It was found that the cell extracts contained: phosphoglycerokinase, triosephosphate dehydrogenase, aldolase hexosediphosphatase, hexosemonophosphate isomerase and enolase; these extracts would synthesize hexosephosphates from phosphoglycerate, ATP and reduced DPN. The extracts also converted hexosediphosphate into a mixture of pentose, heptulose and tetrose phosphates. Aubert and co-workers (1956) independently obtained similar results to those of Trudinger. These results seemed to leave little doubt in the minds of these workers that the chemolithotrophic sulfur bacteria fixed carbon dioxide in the same manner as green plants. This scheme would fit much of the data which have been presented in the past. The organism requires a good deal of energy, as ATP, to initiate and perpetuate this cycle. This may account for the slow growth of these organisms and point to the fact that the energy derived from chemical oxidation is not as available as that from light energy. Also the considerable amount of inorganic phosphate released upon the conversion of ATP $\longrightarrow$ ADP could account for the increase in

inorganic phosphate seen upon CO_2 fixation with *T. thiooxidans* (Vogler *et al.* 1943 and Umbreit, 1954).

An interesting finding was made by Fuller (1956) when he demonstrated that *Esherichia coli* grown on xylose or arabinose in the presence of 5 percent carbon dioxide and 95 percent air can form two molecules of phosphoglycerate from ribulose 1-5 diphosphate. This finding could point toward an important link between the heterotroph and the autotroph.

Since this work deals with terminal oxidation in *T. thioparus*, a brief review of these mechanisms in bacteria is necessary. As the tricarboxylic acid cycle is common to higher plants and animal cells it was reasonable for the various interested workers to investigate bacteria for this or similar mechanisms of aerobic metabolism. Much of the earlier work raised some question as to whether the terminal cycle in bacteria was the same as the tricarboxylic acid cycle of Krebs or the dicarboxylic acid cycle of Thunberg and Knoop. Slade and Werkman (1943) working with *Aerobacter indologenes* grown in the presence of glucose demonstrated the condensation of C^{13} labeled acetate to form succinic acid. By tracing the heavy carbon they demonstrated that the carbon to carbon linkage created in the condensation involves the carbon atom originally present in the methyl group of acetic acid. They also

These results seemed to indicate the presence of a Thunberg-Knoop system. Weinhouse and Millington (1947) demonstrated that yeast may oxidize acetic acid through the Thunberg-Knoop condensation process starting with the oxidative condensation of two molecules of acetate to succinate. Ajl (1950) presented data which indicated that the tricarboxylic acid cycle does not function in E. coli and A. aerogenes since pyruvate was oxidatively decarboxylated to acetate and the latter underwent a series of cyclic reactions without the participation of cis-aconitate or alpha-ketoglutarate. Barron et al. (1950), working with Corynebacterium creatinovorans, stated that because of a complete lack of citrate oxidation, the failure to obtain citric acid in the presence of acetate and oxalacetate, and the complete inhibition of acetate oxidation by malonate, which could be partially relieved by fumarate, no other obvious pathway could exist except the dicarboxylic acid cycle. This cycle is as follows:



Dagley et al. (1951) observed the same phenomenon using A. aerogenes and stated that the dicarboxylic acid cycle must also exist in

these organisms. Studies were made on the comparative patterns of acetate oxidation by citrate grown and acetate grown A. aerogenes (Ajl, 1951). It was shown that this organism, when grown on citrate, gives evidence of a conventional tricarboxylic acid cycle; when grown on acetate, a dicarboxylic acid cycle seems to be operative. This seems to indicate that the oxidation of acetate may be a function of the substrate upon which the organism is grown and the possibility of both cycles existing in the same cell. To this point all the work cited favors the presence of a dicarboxylic acid cycle in bacterial respiration.

However, Novelli and Lipmann(1950) prepared cell-free extracts of E. coli which synthesized citric acid when reacted with acetate, oxalacetate, ATP and coenzyme A (CoA). This is one of the first demonstrations of a citrate producing system in bacteria which is similar to that of animal cells. Soon after a purified enzyme system was isolated from E. coli which catalized the reduction of isocitric acid to alpha-ketoglutarate and carbon dioxide (Ajl and Kamen, 1951). This system required TPN for electron transport. This marked the first clear-cut observation of the conversion of a tricarboxylic acid to alpha-ketoglutarate in bacteria. This work has also been repeated using E. freundi (Barban and Ajl, 1952). Lara and Stokes (1952) have shown that E. coli strains which cannot use citrate as the sole source of carbon are capable of

compound. The majority of earlier work, in which much evidence accumulated for a dicarboxylic acid cycle in bacteria, was carried out using whole cells alone.

Campbell *et al.* (1953),working with *Pseudomonas aeruginosa*, found a deviation from the conventional tricarboxylic acid cycle. They found that citrate was being broken down into a two carbon and four carbon compound, probably succinate and glyoxylate. They felt that the deviation was initiated with cis-aconitate. Wheat *et al.* (1954), apparently noted the same deviation in *E. coli* although they only described it as the break down of citrate, in the absence of CoA, into two and four carbon compounds. The stoichiometry of this reaction was carried out by Smith and Gunsalus (1955) in which they demonstrated that the deviation originated with isocitrate and not cis-aconitate as follows:

$$\text{Isocitrate} \longrightarrow \text{Glyoxylate} + \text{Succinate}$$

The enzyme catalizing this reaction is called isocitratase. These authors have shown this enzyme to exist in the following organisms: *Pseudomonas aeruginosa*, *P. flourescens*, *P. putrifaciens*, *Azotobacter agile*, *A. vinelandii*, *A. aceti*, *E. coli*, *Serratia marcesens* and *Micrococcus lysodeikticus*.

Recently a malate synthetase reaction has been shown to occur in some bacteria (Wong and Ajl,1957). This reaction forms malate from acetate and glyoxylate as follows:

$$\text{Acetate} + \text{ATP} \longrightarrow \text{Acetyl phosphate} + \text{ADP}$$

$$\text{Acetyl phosphate} + \text{CoA} \longrightarrow \text{Acetyl CoA} + HPO_4$$

$$\text{Acetyl CoA} + \text{glyoxylate} \longrightarrow \text{Malic acid} + \text{CoA}$$

These reactions may explain how bacterial cells can grow on a two carbon source such as acetate by forming malate which in turn will be oxidized to oxalacetate. The latter compound will act as the two carbon acceptor in the normal tricarboxylic acid cycle. The synthetase has been shown to be present in *A. aerogenes*, *Corynebacterium creatinovorans*, *P. flourescens* and *E. coli*. In all these organisms the malate synthetase appears to be adaptive and is only formed when the cells are grown on acetate.

This recent work with isocitratase and malate synthetase in bacteria may resolve some of the difficulties encountered in explaining the results of earlier work on terminal respiration. It may be well to re-evaluate much of the work which postulated a dicarboxylic acid cycle in light of this new knowledge.

The tricarboxylic acid cycle has also been demonstrated or postulated to be present in such organisms as *C. creatinovorans* (Fukui and Vandermark, 1952), *Mycobacterium smegmatus*, *M. tuberculosis* (Edson, 1951), *M. phlei* (Blakley, 1952), *P. aeruginosa* (Campbell and Smith, 1956) and *Shigella flexneri* (Pan *et al.* 1957).

Other than the work previously cited (LePage and Umbreit, 1943b) in which the effects of certain di- and tricarboxylic acids were noted nothing is known of the terminal oxidation in the thiobacilli although it has been postulated by many workers that such oxidation is probably similar to that of heterotrophic microoganisms.

GENERAL METHODS

The methods described cover only the general procedures used in this study. Specific methods for the various determinations are given in the appropriate sections.

The culture of <u>Thiobacillus</u> <u>thioparus</u> used in this study was obtained from the American type culture collection and is cataloged as number 8158. The organism is a motile gram negative rod which grows in media containing only inorganic salts. It will not grow in nutrient broth, nutrient agar or other common laboratory media.

The medium used for growing this organism was that formulated by Starkey (1934) with the phosphate buffer adjusted to give a pH of 7.0. This medium contained the following ingredients:

(1)	$Na_2S_2O_3 \cdot 5H_2O$	10.0g
	$MgSO_4 \cdot 7H_2O$	0.1g
	$CaCl_2 \cdot 2H_2O$	0.01g
	$(NH_4)_2SO_4$	0.1g
	Water	896 ml
(2)	$FeCl_3 \cdot 6H_2O$	0.02g
(3)	$MnSO_4 \cdot 2H_2O$	0.02g

(4)	KH_2PO_4	2.5g
	K_2HPO_4	5.0g
	Water	100 ml

final pH 7.0

Parts one and four were autoclaved separately and parts two and three were filtered through a Seitz filter. The $FeCl_3 \cdot 6H_2O$ was made up as a one percent solution in water. Two ml of each per liter were added aseptically to part one and part three was then aseptically added to complete the medium.

In the latter part of this study another medium formulation, described in The Manual of Microbiological Methods (1957), was used. This medium was prepared as follows:

(1)	K_2HPO_4	2.0g
	$CaCl_2 \cdot 2H_2O$	0.1g
	$MgSO_4 \cdot 7H_2O$	0.1g
	$MnSO_4 \cdot 2H_2O$	trace
	$FeSO_4 \cdot 7H_2O$	trace
	Water	900 ml
(2)	$Na_2S_2O_3 \cdot 5H_2O$	10g
	Water	50 ml
(3)	$(NH_4)_2SO_4$	0.1g
	Water	50 ml

The three parts were autoclaved separately and mixed aseptically after cooling. The trace amounts of $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 2H_2O$ were measured as 0.2 ml of one percent solutions.

In both media mentioned the original formulations call for tap water. In this case distilled water was used.

The second medium was used because it was simpler to prepare and gave the same yield of cells as Starkey's medium.

Stock cultures of T. thioparus were maintained on solid medium at 30C using Starkey's formulation with two percent agar added. Transfers were made every two days as it was found that the organisms would not survive more than three to four days on agar slants of this material.

To obtain a large number of cells for the various determinations made in this study, a New Brunswick fermentor was employed. This machine provides both aeration and agitation for large volumes. Three liters of medium were seeded with bacteria which had been washed from two day old agar slants. The fermentor was set at 30C, an aeration rate of six liters per minute and an agitation speed of 350 R.P.M. If necessary, the pH was adjusted to seven using sodium carbonate. The bacteria were allowed to grow until there was no thiosulfate remaining as determined by iodine titration, a period of about 40 hours.

At the end of the growing period the cells were harvested in a Sharples continuous centrifuge. The resultant material was mostly elemental sulfur with a small amount of cells. This sulfur-cell

mixture was washed in 0.1M tris buffer, pH 7.4, and centrifuged in the cold, 5C, at 20,000 G's for 15 minutes. The sulfur packed on the bottom and the reddish brown cells on top of the sulfur. These cells were carefully removed using a small spatula. This material was again suspended in tris buffer and centrifuged in the cold.

If fresh whole cells were not required, then the cells were acetone dried using the method of Burris (1957). Water was removed from the acetone with sodium sulfate and the dried acetone cooled to 0C. The cells were suspended in enough 0.1M tris buffer, pH 7.4, to make a thin paste and added drop-wise with constant stirring to ten times its volume of the cold acetone. This mixture was allowed to stand for 10 minutes by which time the suspended material had settled to the bottom as a loose precipitate. The supernatant was poured off and the precipitated material vacuum filtered through a Buchner funnel using Whatman #50 filter paper. The precipitate was washed with fresh cold acetone and allowed to remain on the filter paper until dry. The resulting powder was collected and dried for 12 hours in a vacuum desiccator at 5C. The final product was stored in glass stoppered vials at minus 15C until ready for use.

The majority of this work was done with cell free extracts. These were obtained by suspending the acetone powder in 15 ml of cold 0.1M tris buffer, pH 7.4, and exposing it to sonic oscillation for 15 minutes at 0C in a Raytheon sonic oscillator. This material

was collected and centrifuged in the cold for one hour at 20,000 X G. The supernatant was poured off and used as the crude cell extract. The extract was clear and light red in color.

Protein determinations were made using the method of Lowery _et al._ (1951). With this procedure protein is determined colormetrically by the action of alkaline copper and Folin-Ciocalteu phenol reagent with protein to give a blue color. Casein was used as the standard protein solution against which the determinations were compared.

Measurements of oxygen uptake were carried out using standard Warburg procedures.

Procedures requiring the measurement of ultraviolet absorption were carried out in the Beckman D.U. spectrophotometer using quartz cells.

All unstable chemicals such as the various tricarboxylic acid cycle intermediates were stored at minus 15C.

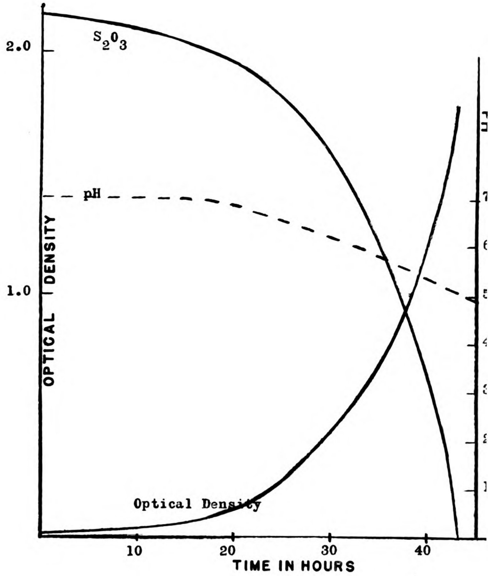
RESULTS

SECTION I: GROWTH CHARACTERISTICS

Typical results of the growth of *T. thioparus* in the New Brunswick fermentor are shown in FIGURE 2. This is a typical picture of the changes which take place in the medium over a period of 40 hours. The pH of the culture remains in a workable range during this period of growth due to the buffer capacity of the medium. The removal of titratable thiosulfate runs parallel to the increase in optical density and simulates a typical growth curve with a lag period and an accelerated growth period. In this case the optical density is only a relative measure of cell concentration since considerable amounts of elemental sulfur, produced by the organism, are dispersed in the medium. At the end of 40 hours the viable cell count is approximately 56 X 10^6 organisms per ml as measured by the drop plate technique and which amounts to approximately 15 to 20 mg of protein (as casein). Allowing the growth to continue on after the thiosulfate had disappeared, and adjusting the pH to 7.0 did not give an increase in viable cells. The counts remained surprisingly steady for periods up to 60 hours. Apparently by this time the organism was reaching a stationary phase of growth in which the production of new cells equaled the death of

FIGURE 2. CULTURE CHANGES OCCURRING DURING THE GROWTH

<u>THIOBACILLUS</u> <u>THIOPARUS</u>.



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old cells. Although the thiosulfate content was zero this static condition was most probably maintained by the utilization of the elemental sulfur present.

During the course of this study a few attempts were made to increase the cell yield. The effect of a number of ions was investigated. These were Al^{+++} ($AlCl_3$), Zn^{++} ($ZnSO_4$), Cu^{++} ($CuSO_4$) and Co^{++} ($CoCl_2$). Also included was Si ($Na_2Si_4O_9$). These were used in all combinations at a concentration of 0.002 percent. The experiment was run in tubes containing 10 ml of medium and incubated for six days at 30C.

The results indicated that the presence of Co^{++} or Cu^{++} at the concentration used inhibits growth completely. The other elements had varying affects as compared to the control. Al^{+++} seemed to have no affect at all, Zn^{++} inhibited somewhat and Si also seemed to affect the growth. From these results it was noted that these ions either reduced or had no affect on growth.

The effect of a surface agent, tween 80, on growing cultures of <u>T.thioparus</u> was also investigated. The results indicated no difference in growth characteristics between cultures in which tween was added and the controls (no tween added).

During the course of this work the stock culture which was maintained on solid medium using agar as the solidifying agent grew much faster (two days) than in liquid medium (six to seven days). The only difference in the ingredients of these media was the agar. The possibility existed, therefore, that some

stimulatory component might be found in the agar being used. Water extracts were made of this agar and added to the medium. Growth under these conditions again was the same as the control. Apparently conditions as found on the surface of a solid material are better for growth than in liquid culture. It may be just a matter of oxygen tension.

Since these particular experiments were secondary to the main problem of this work, the affects of these various agents on growth of *T. thioparus* were not carried out in great detail. These were only survey examinations to determine any possible stimulation of growth which would give better cell yields for future studies.

Later it was found that by increasing the size of inoculum the time of growth could be reduced considerably. Large volumes, as much as one liter, of cells grown in a previous run were added to two liters of fresh medium. This decreased the thiosulfate content to zero in 24 hours. Although this decreased the time of cell harvest, it did not increase the overall cell yield to a significant degree.

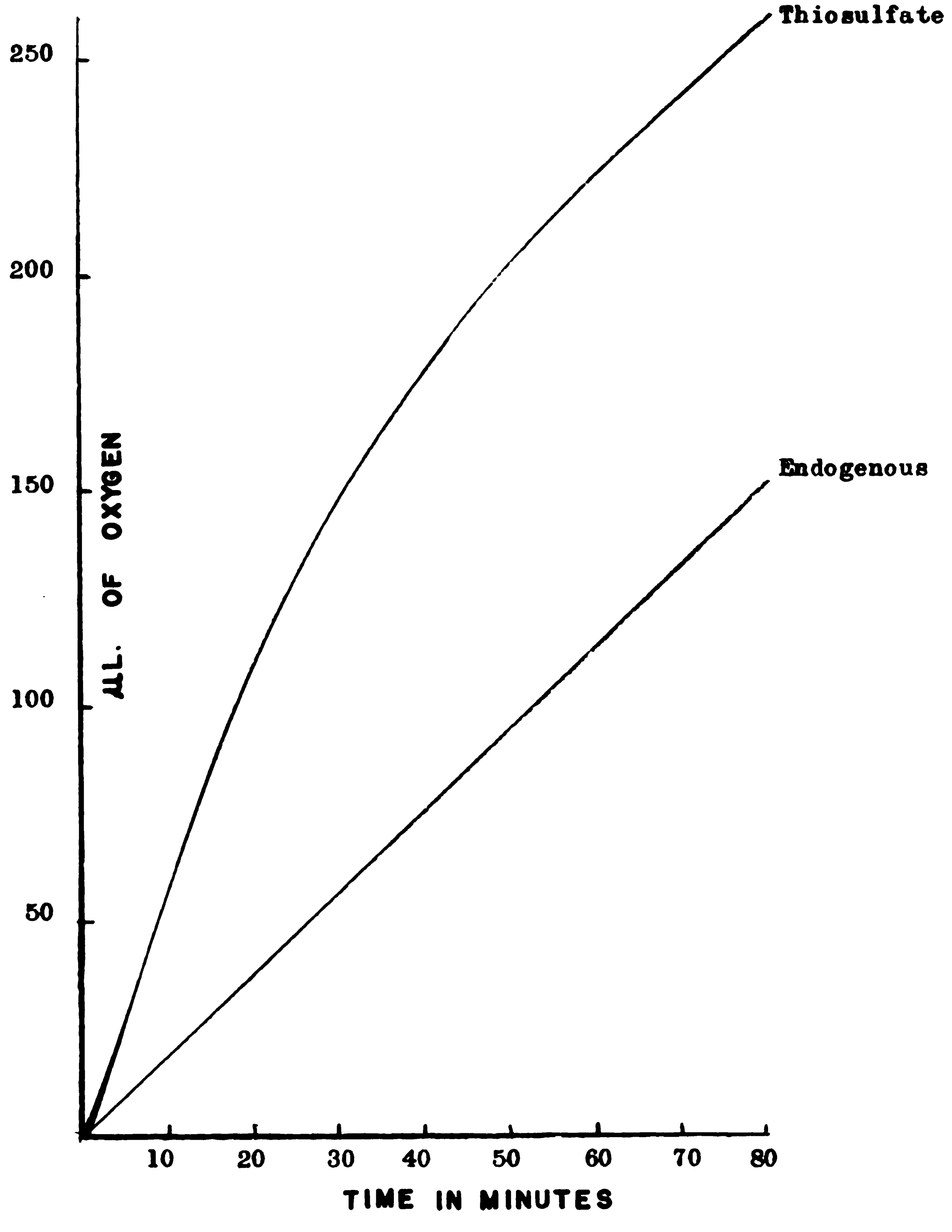
SECTION II: RESPIRATION STUDIES

The oxygen uptake by *T. thioparus* in the presence of thiosulfate and various intermediates of the tricarboxylic acid cycle was investigated using both whole cells and cell free extracts. It was thought that the oxidative activity of these organisms on thiosulfate should be determined in order to be sure that the organism used in these studies would actively oxidize thiosulfate and to obtain some measure of the affect of sonic oscillation on this oxidative activity.

Standard Warburg techniques were employed in these experiments. For whole cells each Warburg flask contained 0.1 ml of 0.1M $Na_2S_2O_3$, 1 ml of 0.05M phosphate buffer, pH 7.0, 1 ml of a washed cell suspension, 0.2 ml of 20 percent KOH in the center well and the volume made up to 3 ml with distilled water. When cell free extracts were used, the above method was followed with the exception that 0.1M tris buffer, pH 7.4, and 1 ml of cell free extract were used.

FIGURE 3 illustrates graphically the activity of whole cells on 10μ moles of thiosulfate. This organism rapidly oxidizes thiosulfate as would be expected of *T. thioparus*. Assuming that the equation: $5Na_2S_2O_3+H_2O+4O_2 \longrightarrow 5Na_2SO_4+H_2SO_4+4S$, describes the reaction taking place, then the theoretical oxygen uptake in the

FIGURE 3. OXYGEN UPTAKE BY THIOBACILLUS THIOPARUS IN THE PRESENCE OF THIOSULFATE.



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presence of 10μ moles of substrate should be 179μ liters. After subtracting the endogenous it can be calculated that about 61 percent of the theoretical oxygen uptake was completed in 80 minutes. However if the endogenous is not subtracted, then the point at which the oxidation of thiosulfate begins to parallel the rate of the endogenous is equivalent to approximately 170 to 180μ liters.

Particular note should be taken of the endogenous respiration. The elemental sulfur that is always associated with cultures of this organism is very difficult to remove completely. This carry over of sulfur is responsible for the high endogenous rate. This high rate probably distorts the true picture of $S_2O_3^=$ oxidation. The problem of ascertaining theoretical rates is complicated by the high endogenous rate since one does not know if the difference between the endogenous oxygen uptake and that in the presence of substrate is actually equal to the affect of substrate alone. In other words, it is not known if the same endogenous rate goes on in the cell both in the presence and absence of substrate. There is no question however that there is a significant difference between the endogenous and the oxygen uptake in the presence of thiosulfate. The rate of oxygen uptake at zero time is about three times that of the endogenous.

Cells of T. thioparus were broken up in the sonic oscillator and the residual sulfur and cell debris removed by centrifugation. Oxygen uptake in the presence of the cell free extract is shown in FIGURE 4. The extract produced about one-tenth the total oxygen

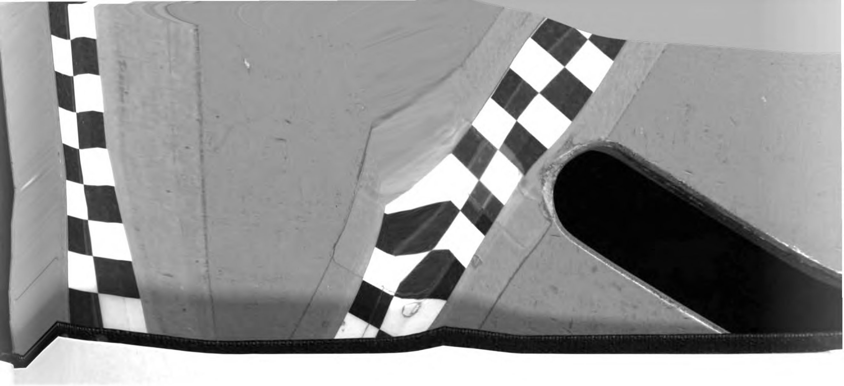
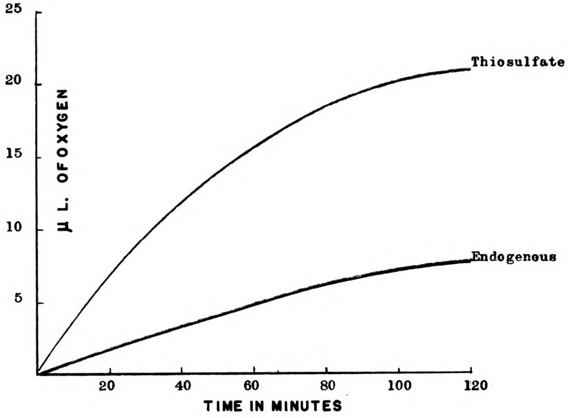


FIGURE 4. OXYGEN UPTAKE BY CELL FREE EXTRACTS OF *THIOBACILLUS THIOPARUS* IN THE PRESENCE OF THIOSULFATE.



uptake of whole cells in the presence of thiosulfate. This reduction in activity is most probably due to a combination of factors which include the removal of residual sulfur, the denaturation of protein, the removal of necessary co-factors and dilution of important constituents. The oxygen uptake of cell free extracts in the presence of substrate is about five times greater than the corresponding endogenous. This is a greater difference than that seen with whole cells and may reflect the removal of residual sulfur. The main thing to be brought out here is that breaking up the cell causes a considerable reduction by thiosulfate oxidation.

The determination of oxygen uptake by *T. thioparus* in the presence of various intermediates of the tricarboxylic acid cycle was carried out in a manner similar to that done in the determination of thiosulfate oxidation. When whole cells were used, each Warburg vessel contained 10μ moles of substrate, 1 ml of cell suspension, 0.1M phosphate buffer, pH 7.4, and 20 percent KOH in the center well. The total volume was brought to 3 ml with the buffer. When cell free extracts were used each Warburg vessel contained 1 ml of cell free extract, 0.1M tris buffer, pH 7.4, 10μ moles of substrate, 0.3μ moles of Mn^{++} and 20 percent KOH in the center well. The total volume was brought to 3 ml with the buffer.

The oxidative activity of whole cells on the tricarboxylic acid cycle intermediates appeared to be in direct proportion to the endogenous rate of cell suspension. The ratio between thiosulfate oxidation and endogenous oxygen uptake, however, tended to become smaller with increasing endogenous activity. The ratios between the endogenous and the substrate oxidation are tabulated for a number of determinations in TABLE I. These ratios indicate an average oxygen uptake in the presence of these organic intermediates of about 1.25 times the endogenous rate. It can be implied from these results that the oxidation of these compounds is, in whole cells, dependent on the rate of endogenous respiration. For example, when the endogenous oxygen utilization is about 300μ liters of oxygen then the average oxygen uptake in the presence of these organic intermediates would be approximately 75μ liters of oxygen over the endogenous. When the cells were carefully washed and much of the residual sulfur removed, the endogenous was as low as 15μ liters of oxygen uptake in 90 minutes and the average oxygen uptake in the presence of these intermediates was only 3.75μ liters of oxygen over the endogenous rate. In comparison the thiosulfate oxidation has been recorded to be as much as 20 times that of the endogenous when the latter was low and as small as 1.2 times the endogenous when the latter was high.

A number of determinations were made with cell free extracts from both acetone powders and newly harvested cells. The results of these experiments were negative since oxidative activity could not be shown against the tricarboxylic acid cycle intermediates with these extracts. These negative results could be dues to a number of factors as described for cell free activity against thiosulfate, that is, denaturation of enzymes, loss of co-factors,

TABLE I

RATIO OF SUBSTRATE OXIDATION / ENDOGENOUS RESPIRATION IN THE PRESENCE OF VARIOUS TRICARBOXYLIC ACIDS

Substrate	Substrate / Endogenous			Average
	Trial 1	Trial 2	Trial 3	
Pyruvate	1.33	1.39	1.39	1.37
Isocitrate	1.11	1.19	——	1.15
Alpha-keto-glutarate	1.24	1.28	1.08	1.20
Succinate	0.97	1.50	1.23	1.23
Fumarate	1.31	1.73	1.06	1.37
Malate	1.17	0.89	——	1.03
Citrate	1.27	1.36	1.39	1.34
Oxalacetate	1.15	1.60	1.16	1.30

dilution of co-factors and possibly the loss of order which may exist in the whole cell.

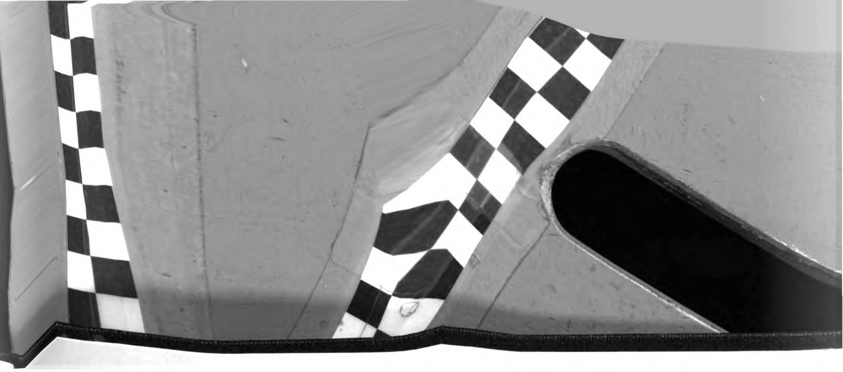
SECTION III: TRICARBOXYLIC ACID CYCLE REACTIONS

This section deals with the determination of the presence of specific tricarboxylic acid cycle reactions in cells of *T. thioparus*.

PART A

To demonstrate the enzymatic activity of *T. thioparus* on citrate, cis-aconitate and isocitrate, experiments based on the reduction of TPN in the presence of isocitric acid and the enzyme, isocitric dehydrogenase, were carried out. Triphosphopyridine nucleotide reduction was measured by the method of Ochoa (1955) in which the reduction is followed by measuring the absorption of ultraviolet light at 340mμ by reduced TPN. The reactions were carried out at room temperature in the Beckman DU spectrophotometer. Each curvett contained 0.1M tris buffer, pH 7.4, 0.5μM TPN, 0.1μM Mn^{++}, 1.0 ml of cell free extract (3 mg protein per ml) and 5μM citrate, cis-aconitate or isocitrate depending on the substrate being tested. The total volume was brought to 3 ml with the buffer. The cell extract was prepared by the sonic oscillation of acetone powders.

The activity of cell free extracts of *T. thioparus* on citrate,



cis-aconitate and isocitrate as measured by TPN reduction is shown in FIGURE 5. It is readily noted that the amount and rate of TPN reduction becomes increasingly smaller with different substrates. The smallest activity was in the presence of citrate followed by cis-aconitate and isocitrate. In order to obtain a better indication of the rate of TPN reduction the results were tabulated using the change in optical density per unit time as the ordinant and time as the abscissa. The best straight line was calculated by the method of least squares and the ordinant intercept thus calculated taken as the rate of TPN reduction at zero time. These rates may be tabulated as follows:

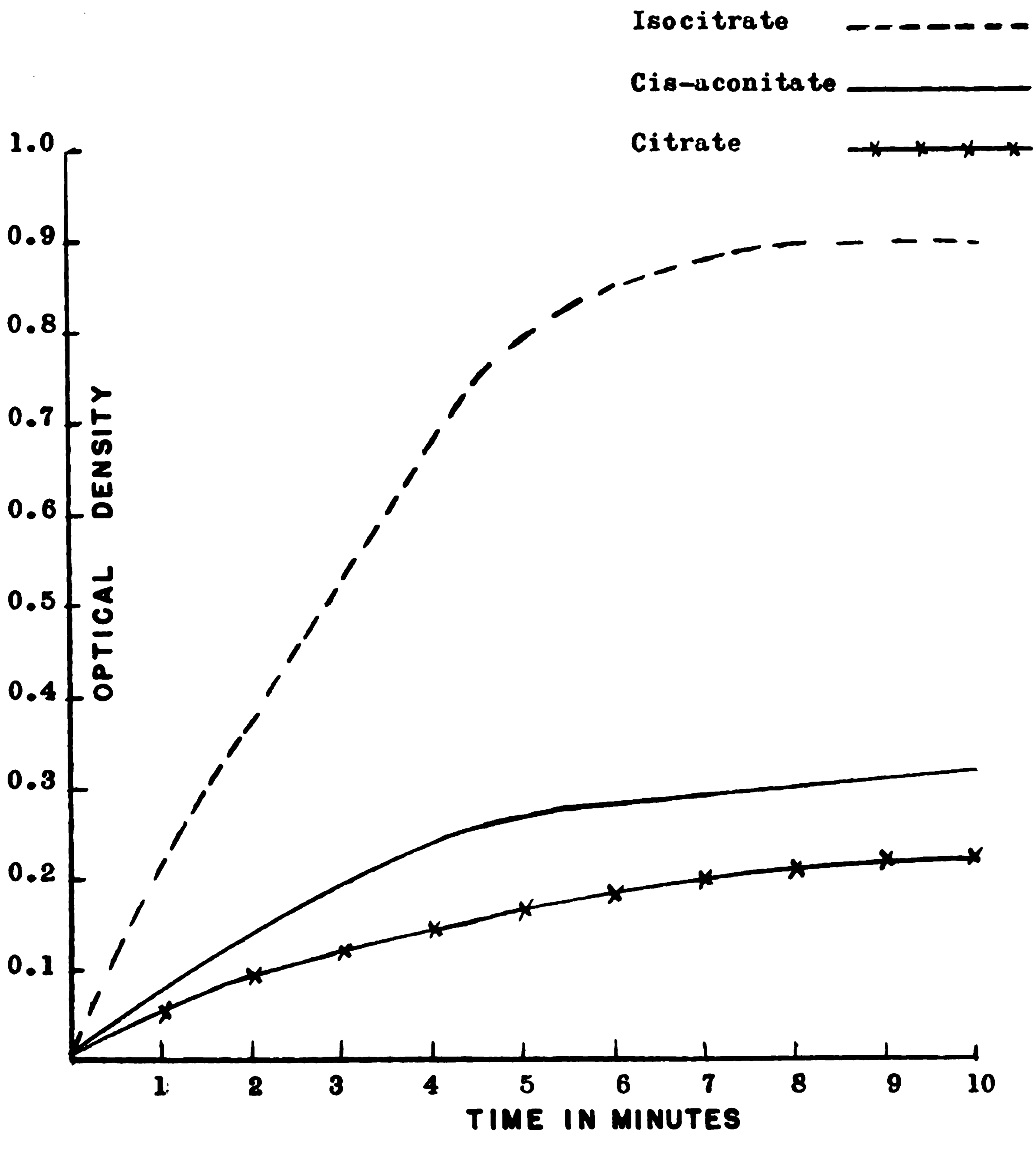
SUBSTRATE	CHANGE IN OPTICAL DENSITY PER MIN. AT ZERO TIME	MOLES OF TPN** REDUCED PER MIN. AT ZERO TIME
Citrate	0.052	0.025
Cis-aconitate	0.081	0.039
Isocitrate	0.223	0.107

** Calculated using the extinction co-efficient of TPN reduced as $6.22 \times 10^{6}\ \mathrm{Cm}^{2} \times \mathrm{moles}^{-1}$

These results indicate the presence of isocitric dehydrogenase in cell free extracts of T. thioparus. The established pathway for these reactions in other organisms is:

$$\text{citrate} \xrightleftharpoons{\text{aconitase}} \text{cis-aconitate} \xrightleftharpoons{\text{aconitase}} \text{isocitrate}$$

FIGURE 5. REDUCTION OF TPN BY CELL FREE EXTRACTS OF *THIOBACILLUS THIOPARUS* IN THE PRESENCE OF CITRATE, CIS-ACONITATE AND ISOCITRATE.



$$\text{isocitrate} \xrightleftharpoons[\text{dehydrogenase}]{\text{isocitric}} \text{alpha-ketoglutarate}$$

The equilibrium of these reactions is in favor of citric acid. If such a system existed in _T. thioparus_, one would expect that isocitrate would show a greater dehydrogenase activity than citrate or cis-aconitate. From the data such seems to be the case since the rate of TPN reduction is approximately four times as great as that in the presence of citrate and three times as great as in the presence of cis-aconitate. These results may also be expressed in terms of the percent of TPN reduced. The cell free extract reduced 27 percent of the TPN in the presence of citrate, 31 percent in the presence of cis-aconitate and 86.4 percent in the presence of isocitrate. This latter result shows that TPN was not limiting the reaction.

This isocitric dehydrogenase is TPN specific. No activity was seen when diphosphopyridine nucleotide (DPN) was used.

A number of attempts were made to demonstrate aconitase activity. The method used was that of Racker (1950) in which the production or removal of cis-aconitate is measured by a change in optical density at 240mμ, the wave length most highly absorbed by cis-aconitate. No activity could be demonstrated either from citrate or isocitrate to cis-aconitate or from the aconitate to the other compounds. The extracts were also supplemented with 2μ moles of Fe^{++} since this is a known co-factor for aconitase in other

organisms. Again no aconitase activity was seen but a change in optical density was noted in the presence of the iron, forming some substance which has a high absorption at 300mμ. The wave length used for the determination of cis-aconitate, 240mμ, is also absorbed by cell free extracts of T. thioparus due, probably, to the protein and nucleic acid content of these extracts. If the aconitase activity was very low this absorption at 240mμ might be masked by the high protein and nucleic acid content. This may be the reason for the negative results. However, since TPN is reduced in the presence of both citrate and cis-aconitate it would seem reasonable that aconitase is present.

PART B

The purpose of the following experiments was to demonstrate the production of alpha-ketoglutarate by T. thioparus in the presence of isocitrate and to find evidence for or against the presence of isocitratase.

The reactions were carried out in the following manner. To test for the production of alpha-ketoglutarate from isocitrate, one ml of cell free extract was added to a Warburg vessel containing 10μ moles of isocitrate, 2μ moles TPN, 0.1μ moles Mn^{++}, 0.1M tris buffer, pH 7.4, 3μ moles of arsenite. The total volume was brought to 3 ml with the buffer. The reaction was carried out at 30C for

100 minutes. Arsenite was added to block the conversion of alpha-ketoglutarate to succinate and allow the accumulation of the former.

To test for the presence of isocitratase, one ml of cell extract was added to a Warburg flask containing 10μ moles of isocitrate and 0.1M tris buffer, pH 7.4. The total volume was 3 ml. This reaction was carried out in an atmosphere of nitrogen in order to inhibit isocitric dehydrogenase and shunt the reaction to glyoxalate and succinate via the citratase reaction, if such were present. This reaction was carried out at 30C for 100 minutes.

The reactions were stopped by adding 0.5 ml of 10 percent phosphotungstic acid in 0.5M HCL. The precipitate formed was filtered through Whatman #50 and the clear filtrate tested for alpha-ketoglutarate and glyoxylate by the method of Cavallini _et al._ (1953). This method consists of converting any of the keto acids present inte the corresponding dinitrophenylhydrazone derivative by adding 1 ml of a 0.1 percent solution of 2,4-denitrophenylhydrazone to the reaction mixture. The resulting hydrazones are extracted with ether and dried over-night in a vacuum desiccator. The residue is then dissolved in 1 ml of 1.0N NH_4OH and 1 ml of $CHCl_3$ until completely dissolved. One hundred lambda of the NH_4OH layer was chromatographed on Whatman #1 paper using the ascending technique.

The results of these experiments are tabulated in TABLE II. These data give support to the presence of isocitric dehydrogenase in _T. thioparus_ since alpha-ketoglutarate appears as the product of

TABLE II

FORMATION OF ALPHA- KETOGLUTARATE BY THE ACTION OF CELL FREE EXTRACTS OF *T. THIOPARUS* ON ISOCITRIC ACID

Standards	Rf Values of the 2,4-Dinitrophenyhydrazones	
Alpha-ketoglutarate	0.33	
Glyoxylate	0.40	0.58
Reactions with:		
Isocitrate (aerobic)		
test	0.31	0.69
control	----	0.65
Isocitrate (anaerobic)		
test	----	0.66
control	----	0.66

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the reaction of isocitrate, TPN and cell free extract. This is indicated by the production of a spot which has the same Rf as known alpha-ketoglutarate. No glyoxylate was found under aerobic or anaerobic conditions which would indicate that isocitratase was not present. The spots giving an Rf value in the area of 66 are due to excess phenylhydrazone.

PART C

The purpose of these experiments was to demonstrate succinic dehydrogenase activity in *T. thioparus*. Two methods were used both based on the reduction of methylene blue in the presence of cells and succinate. These methods were described by Burris (1945). The first employs the Thunberg technique. The Thunberg tube contained 2 ml of 0.04M succinate, 0.1 ml of 1-1000 methylene blue, 2 ml of 0.1M phosphate buffer, pH 7.4, and one ml of cells. The cells were washed from 48 hour agar slants. The tube was evacuated for 10 minutes and incubated at 30C until the dye was reduced. The second method was carried out in agar. This medium contained 0.5 ml of 0.004M succinate, 0.5 ml of 1-1000 methylene blue, 2 ml of 2 percent agar in 0.1M phosphate buffer at pH 7.4. The agar was melted and cooled to about 45C and added to a four inch test tube containing the other ingredients plus one ml of cells and allowed to solidify. The tube was incubated at 30C until the leuco form appeared.

These tests were quite qualitative since whole cells had to be used. Whole cell suspensions contain so much sulfur that photometric measurements are impossible. In the Thunberg tube the cells took 45 minutes to completely reduce the methylene blue while the control without succinate was reduced in 135 minutes. The same results were obtained using the agar method except that the endogenous never reached the complete leuco form.

These results indicate the presence of succinic dehydrogenase in these cells since the dye is reduced considerably faster in the presence of succinate. The endogenous reduction is most probably due to the presence of colloidal sulfur which is being oxidized and in turn reducing the methylene blue.

These same tests were carried out using acetone powders and cell free extracts. The results were all negative. In most organisms the enzyme succinic dehydrogenase is associated with the cell wall. This would also seem to be the case with *T. thioparus* because of the negative results with acetone powders and cell free extracts. Acetone drying appears to have a drastic effect on cell permeability which would indicate an effect on the cell wall. It would probably follow then that enzymes associated with the cell wall would be affected as well.

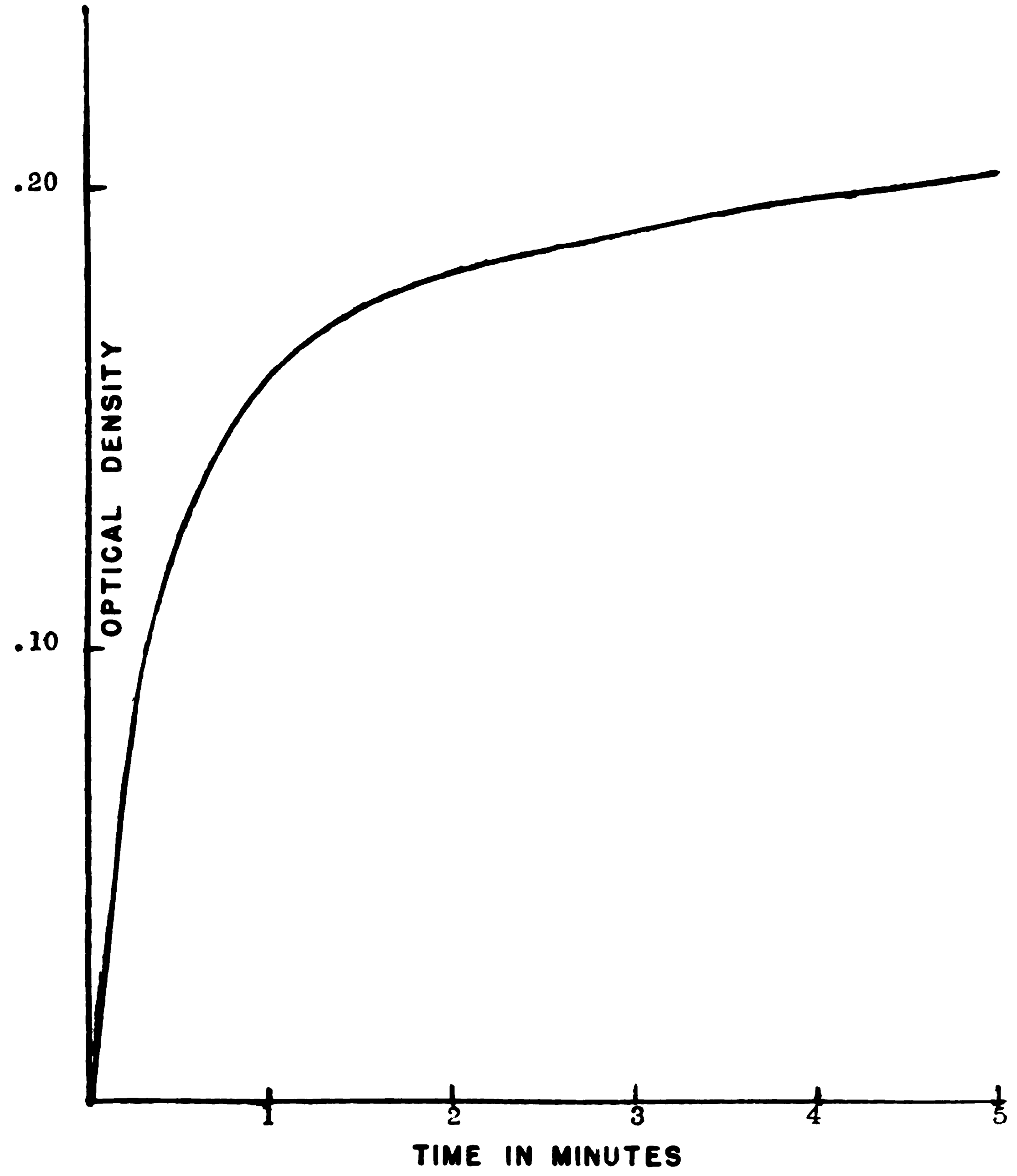
PART D

Malic dehydrogenase determinations were made using the method of Mehler *et al.* (1948). This method is based on the absorption of ultraviolet light at 340mμ by reduced DPN. One ml of cell free extract (1 mg protein per ml) was added to a quartz curvett containing 0.1M tris buffer, pH 7.4, 10μ moles L-malate and 1.5μ moles of DPN oxidized. The total volume was brought to 3 ml with buffer. The reaction was carried out at room temperature and the change in optical density with time was recorded.

The results of these experiments are illustrated in FIGURE 6. The amount of DPN reduction was quite small; a total of 0.108μ moles in five minutes. The overall reaction was relatively rapid reaching the peak of activity in two minutes. The reaction rate at zero time was approximately a change in optical density of 0.4 per minute or 0.19μ moles DPN reduced per minute. However the fact that activity was present and not the rate or amount of DPN reduction is the important thing here. No activity was obtained with this system in the presence of TPN indicating that the malic dehydrogenase of these cells is DPN specific.

A number of attempts were made to demonstrate fumarase activity in this organism. The method of Racker (1950) was employed in which the production of fumarate from L-malate is followed by measuring the absorption of ultraviolet light between 240 to 300mμ. Fumarate absorbs ultraviolet light at this wave length in proportion to its molar concentration. One ml of cell free extract was added to a curvett containing 10μ moles of L-malate and 0.1M tris

FIGURE 6. THE REDUCTION OF DPN BY CELL FREE EXTRACTS OF <u>THIOBACILLUS</u> <u>THIOPARUS</u> IN THE PRESENCE OF L-MALATE.



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buffer, pH 7.4, with a final volume brought to 3 ml with the buffer.

In all these experiments no activity was observed. Since phosphate is a known activator of fumarase, the tris buffer used above was replaced with 0.1M phosphate buffer, pH 7.4, but the results were still negative.

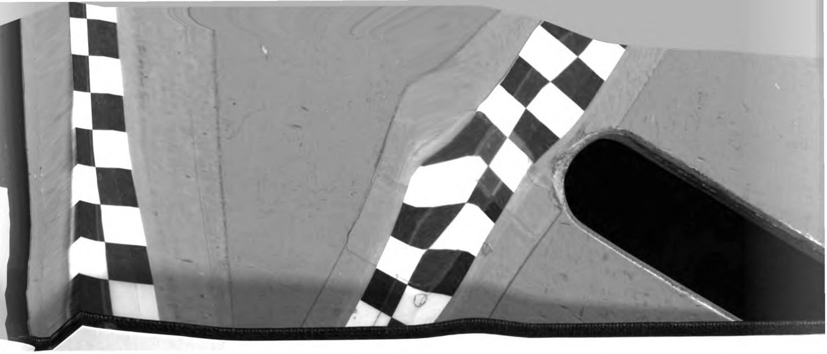
The wave length used with this method presents the same problem as was encountered with attempts to demonstrate aconitase activity. The cell free extracts of _T_. _thioparus_ absorb highly at these wave lengths and this absorption might mask fumarase activity if the activity were very small.

PART E

A series of experiments was carried out in order to find evidence for the production of citric acid from acetate, pyruvate and oxalacetate. One ml of cell free extract was added to Warburg vessels containing 0.1M tris buffer, pH 7.4, and 10 moles of substrate. The total volume was brought to 3 ml with buffer. The reactions were carried out with shaking at 30C for 60 minutes. The reaction was stopped by the addition of 0.5 ml of 10 percent phosphotungstic acid in 0.5M HCL. The precipitate formed was filtered off using Whatman #50 paper and the resultant filtrate tested for citric acid. The method employed for the determination of citric acid was that of Ettinger _et_ _al_. (1952) in which any citrate present is converted to the penta bromoacetone by the addition of $KMnO_4$ and KBr. The excess $KMnO_4$ was removed with H_2O_2 and the bromoketone extracted with N-heptane. The heptane layer was then added to a pyridine-KOH mixture and heated to 80C for four minutes and immediately cooled to 0C. This last step results in the development of a red color in the pyridine layer, the intensity of which is proportional to the concentration of citrate present. The entire procedure with the exception of heating the pyridine mixture was carried out at 5C.

These experiments indicated that citrate was formed by cell free extracts of *T. thioparus* in the presence of pyruvate and oxalacetate but never in the presence of acetate alone. Attempts to show the presence of the condensing enzyme, citrogenase, by demonstrating an increase in citrate production when pyruvate or acetate plus oxalacetate were added to the cell free extract were uncertain in that quantitative results varied from one run to another. Citrate was produced in all cases; however, in one instance a mixture of pyruvate and oxalacetate or acetate and oxalacetate would produce more citrate than pyruvate or oxalacetate alone. In the majority of cases the citrate produced in the presence of the afore mentioned compounds would only slightly exceed, or not exceed at all, that of oxalacetate alone. The over-all trend of these reactions indicate that these cells produce as much citrate from oxalacetate as they do from the combination of acetate and oxalacetate or pyruvate and oxalacetate. It should be reiterated, however, that these results were erratic and that the only definite information obtained was that these cells can produce citrate in all cases except in the presence of acetate.

This method is supposedly specific for citrate; however during the course of these experiments the pyruvate and oxalacetate controls consistently developed a red color some times more intense than others. These non-specific reactions may be the cause of the discrepancies in the results obtained.



DISCUSSION AND CONCLUSION

Using standard Warburg techniques it was shown that the organism used in this study rapidly oxidizes thiosulfate. The endogenous respiration rate, which is often quite high in whole cell suspensions due to residual sulfur, may confuse the picture of oxygen uptake and give misleading results. This same situation has been discussed rather thoroughly by Baalsrud and Baalsrud (1954) working with Thiobacillus denitrificans. When cell free extracts are employed,the oxidative activity is reduced to a considerable degree, about one-tenth the activity of whole cells as measured by thiosulfate oxidation.

Respiration studies carried out in the presence of tricarboxylic acid cycle intermediates showed some interesting results when whole cells were used. The rate of oxygen uptake was by no means equal to that in the presence of thiosulfate but under certain conditions a considerable amount of oxygen was removed by T. thioparus in the presence of these intermediates. Again as in thiosulfate oxidation studies, there was a high endogenous rate due to the carry-over of sulfur. It appeared that the oxygen uptake was directly related to the amount of oxygen removed by endogenous respiration. The higher the endogenous rate the greater the oxygen uptake. However, the ratio of oxygen uptake in the

presence of substrate to the endogenous oxygen uptake appeared to be a relatively constant ratio. Thiosulfate oxidation, on the other hand, indicated more independence from the endogenous rate in that the ratio of substrate oxidation to endogenous activity became smaller as the endogenous rate became higher.

This relationship of endogenous respiration to substrate oxidation may be speculated upon in the following manner. This organism being an autotroph normally does not metabolize organic materials. However, Vogler *et al.* (1942) have presented some evidence that the addition of C_4 dicarboxylic acids enhances the respiration rate of *T. thiooxidans*. It may be possible that a truly resting cell can only transfer the compound across the cell wall very slowly but if an active oxidation such as a high endogenous rate is being carried out the "permease system" is activated and the compounds gain access to the cell interior more readily. An analogous situation is that reported by Gale (1948) in which glutamic acid could not enter into cells of staphlococci unless glucose was also present.

Cell free extracts of *T. thioparus* did not possess any activity against these organic intermediates. This result does not necessarily mean that the cells are incapable of utilizing these compounds. A number of reasons for this inactivity may be as follows: the activity as measured with thiosulfate oxidation is reduced about ten-fold, there is a large dilution and loss of order, a loss of required co-factors and probably denaturation of some of protein.

The only conclusion to be drawn at this point is that whole cells show an increase in oxygen uptake in the presence of tricarboxylic acid cycle intermediates and that this uptake appears to be dependent on the endogenous rate.

The respiration studies gave no evidence for the presence or absence of a tricarboxylic acid cycle in this bacterium. Therefore, a series of experiments was carried out to obtain evidence for or against the presence of certain reactions common to the afore mentioned cycle.

Isocitric dehydrogenase determinations were quite successful. It was by far the most active enzyme determined. Aconitase could not be demonstrated directly but it was shown that TPN is reduced, at different rates, in the presence of citrate and cis-aconitate as well as isocitrate. These reactions were all TPN specific.

More evidence for the presence of isocitric dehydrogenase was found by the demonstration that alpha-ketoglutarate is the product of this dehydrogenase activity and also that alpha-ketoglutarate is an intermediate in these reactions.

From the experiments performed it would appear that the enzyme, isocitratase, does not play a role here since glyoxylate could not be demonstrated when isocitrate and cell free extracts were reacted together under anaerobic conditions. Obviously these negative results are not conclusive since there may be certain

co-factors or conditions necessary in this particular organism about which we are unaware.

Succinic dehydrogenase activity was measured qualitatively by methylene blue reduction and it would be reasonable to assume that succinate arises from alpha-ketoglutarate although this latter reaction was not measured. In the typical tricarboxylic acid cycle succinic dehydrogenase activity results in the formation of fumarate. However, fumarase activity could not be demonstrated in cell free extracts of this organism yet malic dehydrogenase activity could be shown. This latter reaction was not very strong since only 0.1μ mole of DPN was reduced. However the reaction was positive and was specific for DPN. From this experiment one would assume fumarate to be an intermediate since both succinate and malate are oxidized by the cell extracts and fumarate is normally the bridge compound between these two.

Attempts to show the presence of the condensing enzyme were disappointing. Citrate was produced by cell free extracts in the presence of pyruvate and oxalacetate but not in the presence of acetate. Attempts to show the citrogenase enzyme by demonstrating an increase in citrate production in the presence of pyruvate and oxalacetate or acetate and oxalacetate were not conclusive since the results were erratic. In the majority of cases it seems that as much citrate is produced from oxalacetate and cell free extract as when oxalacetate is in combination with pyruvate or acetate. These results indicate that citrate is apparently an intermediate

in these cells and that oxalacetate alone or pyruvate alone may have to be in some active state before it will react to form citrate. Whether the citrate is formed by a typical condensation reaction of an active acetate and oxalacetate or by the condensation of two pyruvate molecules as reported in *Aspergillus niger* (Cleland *et al.* 1954) or by some other mechanism remains to be seen.

From these results it seems reasonable to conclude that these organisms have a terminal respiration which is very similar to that found in heterotrophic microorganisms. The data obtained appear to agree, in most particulars, with the well established tricarboxylic acid cycle. The weakest points in this conclusion are to be found in the lack of direct evidence for the enzymes aconitase and fumarase and in the inconclusive results in determining the condensing enzyme, citrogenase. More work will be required to clarify these points.

This organism shows some respiratory activity on these compounds. The activity appears to be related to the endogenous respiration rate and may indicate a relationship between cell permeability and state of activity or presence of an essential substrate.

SUMMARY

Thiobacillus thioparus grew quite slowly and all attempts to stimulate the growth were negative. The rate of growth was dependent on the size of the original inoculum.

Whole cells of this organism rapidly oxidized thiosulfate as measured by the standard Warburg technique. Cell free extracts possessed a diminution of oxidative activity of about one-tenth that found in whole cells.

Whole cell suspensions showed some oxygen uptake in the presence of tricarboxylic acid cycle intermediates. This uptake appeared to be directly proportional to the endogenous rate. Cell free extracts contained no activity against these organic intermediates.

Evidence was presented for the occurrence of various enzymes of the tricarboxylic acid cycle. No direct evidence could be obtained for the presence of cis-aconitase or fumarase. Tests for the condensing enzyme, citrogenase, were inconclusive. Citrate was shown to be active in the metabolism of T. thioparus but the actual mechanism involved in its formation was not clear. The enzyme, citratase, appeared to be absent.

From these results it appears that T. thioparus has a terminal respiration similar to that found in many heterotrophic microorganisms. This respiration is the same as or closely related to the conventional tricarboxylic acid cycle of Krebs.

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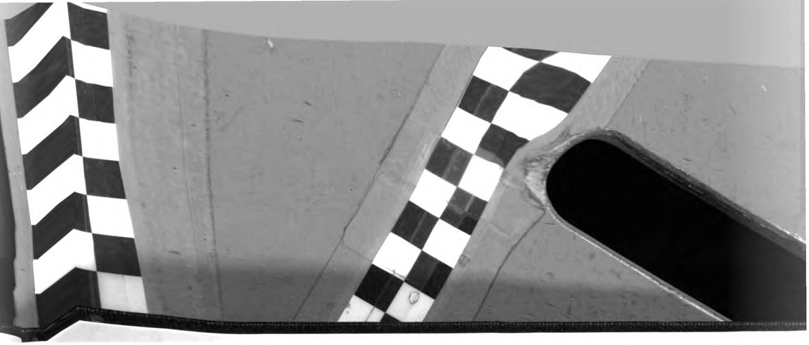

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