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An investigation of the intermediary metabolism of L-glutamic acid by Brucella abortus, strain 19, was carried out. L-glutamic acid is oxidized by this organism more rapidly than any other compound and for this reason elucidation of the pathway by which it is oxidized is of considerable interest. This strain of Brucella abortus is well suited for laboratory investigation because of its low virulence.

Measurement of the respiratory gaseous exchange of resting cell suspensions was made by the use of conventional Warburg techniques.

Study of the adaptive enzyme patterns in this organism showed that the enzymes for the oxidation of L-glutamate and L-asparagine oxidation may be adaptive, but that those for the oxidation of alpha-ketoglutarate, succinate and acetate are not. Rates of oxidation of members of the tricarboxylic acid cycle were greatly increased by lowering the pH of the reaction flask from 6.8 to 5.5. Alpha-ketoglutarate, succinate and malate were oxidized more rapidly than L-glutamate. Fumarate, pyruvate, acetate and oxaloacetate were oxidized 94,80, 77 and 51 percent respectively, as fast as glutamate. Citrate was not oxidized. An increase in the substrate concentration brought about marked stimulation in the rate of oxidation of alpha-ketoglutarate, succinate, aspartate and glutamine, while the effect on the rate of glutamate oxidation was only moderate. Apparent oxidation of citrate occurred at 0.0555M with a Q_{O_2} (N) of 108(87). These results indicate that permeability was formerly the limiting factor in the oxidation rate of TCA cycle intermediates, and that the cell is more permeable to the unionized molecule.

Cell suspensions which were frozen and thawed ten times in an attempt to remove the permeability barrier oxidized all members of the TCA cycle except citrate. The activity of these

preparations toward glutamate proved to be very low. A cell free preparation made by grinding a cell paste with alumina yielded the following Q_{O_2}(N) rates; succinate 114(19), alpha-ketoglutarate 46(19) and citrate 28(19). The preparation was very unstable and showed no activity with a glutamate substrate. Attempts to demonstrate citrate formation in a resting cell suspension gave results which although positive, could not be definitely considered significant.

The use of 2,4-dinitrophenol as an assimilation inhibitor resulted in oxygen uptake and carbon dioxide evolution which indicated complete oxidation of succinate and acetate. The oxidation of alpha-ketoglutarate was 78 percent, and glutamate 57 percent of the complete theoretical utilization value.

The use of malonate with a 10:1 inhibitor substrate ratio resulted in a 26 percent inhibition of succinate at pH 5.5 and 59 percent inhibition using frozen cells. Arsenious oxide, an inhibitor of alpha-keto acid oxidation, markedly inhibited the oxidation of succinate, alpha-ketoglutarate and glutamate, even when used in very low concentrations. Trans-1,2-Cyclopentane-dicarboxylic acid increased the rate of L-asparagine oxidation at pH 6.8, while at pH 5.5 it inhibited the oxidation of all substrates tested. Formaldehyde, although oxidized slowly by brucellae, acted as an inhibitor of alpha-ketoglutarate and succinate oxidation at pH 5.5.

A survey of the results obtained showed that all tested TCA cycle intermediates, including citrate, were oxidized and that alpha-ketoglutarate and succinate oxidases are constituitive. Both these points indicate the presence of a citric acid cycle in brucellae. No evidence against the presence of a citric acid cycle was obtained. Inhibitor studies show that glutamate is probably oxidized via alpha-ketoglutarate and succinate.

THE OXIDATION OF GLUTAMIC ACID AND RELATED COMPOUNDS BY BRUCELLA ABORTUS

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THE OXIDATION OF GLUTAMIC ACID AND RELATED COMPOUNDS BY *BRUCELLA ABORTUS*

INTRODUCTION

The genus *Brucella* embodies certain pathogenic bacteria which are the causative agents of undulant fever in man and contagious abortion in cattle and other domestic animals. In this work the relatively avirulent strain 19 of *Brucella abortus* has been used because it is comparatively safe for laboratory investigations without differing to any significant extent physiologically, from the more virulent members of the genus. This thesis represents an attempt to elucidate the mechanism of the oxidative disimilation of glutamic acid and related compounds by the use of techniques adapted to the Warburg constant volume respirometer.

HISTORICAL

Until quite recently, research on the physiology of the brucellae dealt largely with the nutritional requirements of the group. Few studies of a fundamental nature had been conducted on the metabolism of these organisms. This lack of experimentation may be attributed partially to the difficulties encountered in the use of a pathogenic bacterium. The isolation of the relatively non-pathogenic strain 19 of Brucella abortus has afforded a tool of which many investigators are now availing themselves. In addition, the development of chemically defined media (18,p. 22; 20,p. 777; 31, p. 311) has permitted testing of these organisms under more controlled conditions.

Most of the early knowledge concerning the metabolism of the brucellae was acquired as secondary information in developing a physiological basis for identification and differentiation of species (29,p. 488; 43,p. 376). This information gave only the beginning and end products of metabolism, with no hint of the intermediate steps. Recently, however, studies on the intermediary metabolism of the brucellae have appeared in the literature.

In this respect, Kien-Hun and Kan (24,p. 401) found that B. abortus and Brucella melitensis could dehydrogenate several organic compounds using methylene blue as a

hydrogen acceptor. Among the amino acids examined, alanine, glycine, asparagine and cystine acted as hydrogen donors.

Probably the first work using the Warburg technique with brucellae was done by Mannozi-Torinini and Vendramini (27, 28). This work has supplied a starting point for other investigators. These workers used resting cell suspensions of two strains of B. abortus. Although, the Warburg microrespirometer was used to determine O_2 uptake, the data obtained was not considered quantitative. Carbon dioxide and ammonia were determined by titration. The results were expressed as a percentage increase over the control values. Fourteen amino acids were tested, of which, only four (alanine, cysteine, glutamic acid and asparagine) caused an appreciable increase in oxygen uptake with both strains. Carbon dioxide was produced in the presence of glutamic acid, alanine and asparagine. Deamination was shown to occur with glutamic acid, alanine and asparagine but not cysteine or any of the other 10 amino acids. The observation that pyruvate was oxidized rapidly coupled with the above data led Mannozi-Torini and Vendramini to conclude that oxidative deamination occured with subsequent oxidation of the keto acids formed. Cysteine was reported as being hydrolyzed to serine and hydrogen sulfide.

Attimonelli (8) reported that, with the exception of

formic acid, monocarboxylic acids were oxidized readily. D'Ambrosio (14,;. 247) noted B. abortus could oxidize pentose and hexose sugars. Steinbach (39) tabulated the oxidizing activity of several bacteria. Using the Warburg technique, B. abortus was reported to oxidize alanine, glutamic acid, asparagine, glucose, lactate, pyruvate, succinate, malate, oxalacetate, glycerophosphate and acetaldehyde. Leucine, formaldehyde, malonate, citrate, maleinate and fumarate were used to a variable or slight degree. Considerable variation was incurred in these determinations.

In a similar connection, Caselli (13) demonstrated that B. abortus is capable of desaminating alanine aerobically and anaerobically. However, the anaerobic desaminase appeared less active. Lactic acid, pyruvic acid and alanine all increased the respiration rate to some extent. These data differ from that of Gerhardt (20,p. 463), who showed that ammonia is not produced in appreciable quantities anaerobically from glutamic acid, asparagine or alanine.

The present study is essentially a continuation of the investigations of Gerhardt (19,p. 55-97) and, for that reason, his work is considered here in some detail. Twenty-seven amino acids and related compounds were tested by Gerhardt to determine relative rates of oxidation and amounts of ammonia produced by strain 19 of Brucella abortus.

L-glutamic acid was oxidized more rapidly than any other substance tested; glucose ranked second, L-asparagine, third, and DL-alanine, fourth. The remaining compounds displayed slight activity (19,p. 66). Cysteine was shown to be auto-oxidized, (probably to cystine), in the absence of bacterial cells. In addition, Gerhardt found a negligible oxidation of glutamine and aspartic acid in comparison with glutamic acid and asparagine. Ammonia production paralleled oxygen uptake. Examination of stereospecificity of glutamate, asparagine and alanine showed that D-glutamate and D-asparagine not only were not oxidized but actually inhibited the oxidation of the L-forms. D-alanine was oxidized, but not as rapidly as L-alanine. Simultaneous oxidation of L-asparagine and L-glutamate indicated that perhaps these compounds were oxidized by two closely allied systems. Determination of the stoichiometric ratios of the gaseous exchange resulting from the disimilation of glutamic acid and asparagine varied and did not give clear cut evidence of any definite end products, probably due to the assimilation of these substrates. The determination of oxidation rates of possible intermediates, such as, alpha ketoglutarate and succinate, gave results which were too low to admit them as intermediates. Compounds in the tricarboxylic acid cycle showed only slight uptake; except citrate, which reacted negatively; cis-aconitate

and oxalosuccinate were not tested. Use of competitive inhibitors was also relatively unfruitful. Malonate did not inhibit succinate oxidation under several experimental conditions.

Work by other investigators has shown that the presence of the tricarboxylic acid cycle is by no means universal among bacteria. Karllson and Barker (23,p. 86) state that there is no tricarboxylic acid cycle in Azotobacter agilis while Stern and Ochoa (40,pp. 234-235) demonstrated the existence of an oxalacetate-acetate condensing enzyme by disrupting the cell wall. Ajl (1,p. 506) (2,p.143) (4,pp. 850-854), working with Escherichia coli, reported evidence in favor of a Thunberg-Knoop cycle using isotope and simultaneous adaption techniques. The fact that citrate is synthesized by E. coli cannot be taken as a criterion of the presence of a Krebs cycle (32,pp. 226-227). The probable operation of a tricarboxylic acid cycle in Micrococcus lysodeikticus (5,pp.848-854), in citrate grown Aerobacter aerogenes (3,p. 386) and in Pseudomonas aeruginosa (12,p. 858), has been demonstrated. On the other hand, with E. coli (1,p. 506) (2,p. 144) (4,pp.854-855), Corynebacterium creatinovorans (9,pp. 149,153), and acetate grown A. aerogenes (3,p. 386), a Thunberg-Knoop cycle is probably the main oxidative mechanism. The establishment of the pathway of aerobic disimilation in bacteria has been complicated by the fact the cell permeability effects

interfere in experiments using whole cells, while cell extracts usually have little activity under the reported experimental conditions, presumably because of enzyme lability.

The possibility of the existence of a tricarboxylic acid cycle in Brucella abortus has been shown by Altenbern and co-workers (6,pp. 102-104). These workers incubated a suspension of B. abortus with various tricarboxylic acid cycle intermediates under vigorous aeration, then filtered out the bacteria and added to the filtrate L-asparagine and a fresh suspension of cells. After a further anaerobic incubation, the solution was analyzed chromatographically for glutamic acid and alanine. Since active transaminases had been demonstrated it was postulated that glutamic acid and alanine would arise from alpha-ketoglutarate and pyruvate. Citrate and cis-aconitate gave rise to considerable glutamate, whereas isocitrate produced only a trace. Succinate, fumarate, and L-malate substrates did not produce citrate but did give rise to a trace of alanine, as did the higher members of the cycle. These results indicated the oxidation of tricarboxylic acid cycle intermediates but no noticeable C_2 to C_4 condensation under the conditions used. The authors suggest that the data presented is evidence for the presence of a Kreb's cycle.

EXPERIMENTAL METHODS

Cultures & Cell Preparations

Strain 19 of Brucella abortus was used throughout this work and all experimental data and discussion refers to this culture. The original culture was sent to the University of Wisconsin by the Bureau of Animal Industry, United States Department of Agriculture. This culture was descended directly from the original isolate. A fifth generation transfer of the culture originally received from the U.S.D.A. was lyophilized in milk and held under refrigeration as the permanent stock culture. A number of vials of the lyophilized organism were brought from Wisconsin by Dr. Philipp Gerhardt to serve as stock culture for this work. The culture was at all times handled in a manner designed to minimize dissociation. At bimonthly intervals a lyophilized vial was opened and transferred to an agar slant. A number of agar slant subcultures prepared from this culture then served as stock organism. All cultures were grown either in 18 mm screw cap pyrex test tubes or in eight ounce prescription bottles. These contained 50 ml of agar, slanted to give maximum surface. Albimi Brucella medium with additional agar to make a final concentration of two percent was used for all stock cultures and for experimental preparations unless otherwise stated.

In the preparation of resting cell suspensions, for experimental work, transfers were made to a number of prescription bottle agar slants. The inoculum was spread evenly over the agar surface with a sterrile bent glass rod. The cultures were then incubated at 35-37°C for 24 hours. The resulting growth was washed off with 0.85 percent sodium chloride in pH 6.8, 0,067M phosphate buffer. This suspension was made up to approximately 50 ml and centrifuged (at maximum speed for one minute in a Serval angle centrifuge), the supernatant decanted and the cells resuspended in the above diluent. A second centrifugation and resuspension followed. The cells were then suspended in the buffered saline to a turbidity such that a 1:11 dilution in matched 20 mm Pyrex tubes gave a reading of 21 percent light transmittance using a Beckman Model B spectrophotemeter at 660 mμ. One ml of the undiluted suspension containing approximately 400 μg nitrogen was used in each Warburg flask. This suspension could be safely stored for one week at 3-4°C.

Manometric Techniques

Measurement of the respiratory gaseous exchange of resting cell suspensions was made by the use of conventional manometric techniques as detailed by Umbreit, Burris and Stauffer (41, pp. 1-37) and will not be repeated except where modifications in method were made.

A standard Warburg constant volume microrespirometer was used throughout the work. The water bath was maintained at 34±0.1°C. Flasks were shaken at the rate of 60 complete strokes per minute through a distance of 3.2 cm. In a typical experiment to determine the rate of oxygen consumption, each flask contained 1.0 ml of cell suspension and 1.5 ml of 0.067M phosphate buffer, the sidearm contained 0.5 ml substrate in buffer and the center well contained 0.2 ml of 20 percent potassium hydroxide to give a total flask content of 3.2 ml. All experiments were carried out in an air atmosphere. After being placed in the bath, the flasks were allowed to equilibrate for 15-20 minutes. Substrate concentration was 0.0033M unless otherwise noted. The substrate was tipped immediately after closing the stopcocks and taking a reading. Respiration was allowed to continue for at least 60 minutes with readings at ten minute intervals. The rate of oxygen consumptions was expressed as $Q_{O2}(N)$, that is the microliters of oxygen consumed per mg of cell nitrogen per hour. $Q_{CO2}(N)$ refers similiarly to carbon dioside evolved. In calculation of moles of gas exchange per mole of substrate utilized, the endogenous respiration was subtracted from the exogenous. Where a figure in brackets follows the $Q_{O2}(N)$ it represents the endogenous respiration which has not been subtracted.

Carbon dioxide was calculated by replacing the potassium hydroxide with buffer in one of a pair of flasks and calculating carbon dioxide by difference (41,pp. 17-20). Allowance was made for retention of carbon dioxide in the buffer at the pH of the reaction.

Nitrogen Analysis

Cell suspensions used for manometric work were analyzed for total nitrogen by a modification of the method described by Umbreit (41, p. 161). Nessler's reagent was prepared by the method of Johnson (22, p. 576). Two ml of the digestion reagent was added to & one ml of the 1:11 dilution of cell suspension and the mixture digested overnight in Pyrex tubes on a sand bath at approximately 160°C. After cooling, two drops of 30 percent hydrogen peroxide were added and the mixture heated rapidly until fuming ceased and the sulfuric acid began condensing five cm up the side of the tube. After cooling, the following reagents were added rapidly (in order): ten ml distilled water, two ml of Nessler's reagent and five ml 2N potassium hydroxide. The color was allowed to develop for 20 minutes. Then the solution was transfered to 20 mm matched pyrex tubes and read 490 mμ. Appropriate controls were included. Optical density was read directly. Controls followed Beer's Law from 0 to 60 γg of nitrogen per tube.

Citrate Analysis

Citric acid formation by resting cell suspensions from various substrates was tested. Two ml of a suspension of organisms, (containing approximately one mg of cell nitrogen per ml), and 0.5 ml of 0.1M substrate were incubated in Warburg flasks with shaking for 1.5 hours at 34°C. No attempt was made to measure gas exchange and the stopcocks were left open. Control tubes contained cells which had been immersed for five minutes in boiling water in a small test tube. Final concentration of the substrate was 0.0167M. Where two substrates were combined 0.25 ml of a 0.1M solution of each was used. All reactions were carried out at pH 6.8 in 0.067M phosphate buffer. After incubation 0.5 ml of 25 percent trichloracetic acid was added to each flask and the flasks were then placed in the refrigerator for at least one hour. The cells were then removed by centrifugation and 0.5 or 1.0 ml aliquots of the supernatant were used for analysis of citric acid. Citric acid was determined by a modification of the method of Dickman and Cloutier (15, pp. 379-380) as follows: Instead of glass stoppered volumetric flasks, 18 mm screw cap test tubes were used. An aliquot of 0.5 or 1.0 ml of protein free solution was placed in each tube and 0.1 ml of each of the following were added in order; 0.4M KBr, 18N H_2SO_4 and 0.3M $KMnO_4$. The tubes were then placed upright in a rack and gently shaken for

ten minutes on a Kahn shaker. Excess $KMnO_4$ was destroyed by the addition of 0.1 ml of 1.5M $NaNO_2$ and the mixture gently agitated. The solution was observed to be cloudy if more than two micromoles of citric acid were present. Next, 0.1 ml of 2M urea was added and the upright tubes shaken vigorously (275 spm) until the reaction subsided (generally four to five minutes). Then one ml of purified heptane (prepared from Skellysolve L) was added to each flask and the flasks were shaken for ten minutes. They were then centrifuged at 2500 rpm to break the emulsion, and the aqueous layer was removed and discarded. Following the latter step 0.5 ml of distilled water was added to each tube and the tubes shaken for 30 seconds, centrifuged, and the aqueous layer discarded. After a second washing 1.5 ml of four percent thiourea in saturated borax solution was added to the heptane and the mixture shaken for ten minutes. The contents were poured into optically standardized 10 mm Leitz spectrophotometer tubes and centrifuged. The absorbency of the aqueous layer at 435 mμ was determined with a Beckman Model B spectrophotometer. A standard curve followed Beer's Law up to 0.4 micromoles of citric acid.

Adaptive Enzyme Methods

Several synthetic culture media were tested for capacity to support growth of reasonable quantities of

brucellae, which would permit respiration studies using simultaneous adaption techniques (37, pp. 339-347).

The basal medium given below was the same as the "C-basal" described by Gerhardt (17, p. 26) except that lactate and glycerol were omitted. The modifications

NaCl	0.75 percent
K_2HPO_4	1.00 "
$Na_2S_2O_3 \cdot 5H_2O$	0.01 "
Mg (as $MgSO_4 \cdot 7H_2O$)	10.00 µg/ml
Fe (as $FeSO_4 \cdot 7H_2O$)	0.01 "
Mn (as $MnSO_4 \cdot 4H_2O$)	0.10 "
Thiamin HCl	0.20 "
Nicotinic acid	0.20 "
Calcium pantothenate	0.04 "
Biotin	0.001 "

were made up by adding various carbon and nitrogen sources to the basal medium listed above. The pH was adjusted to 7.0 before autoclaving.

Modification	Addition to medium
A	0.5 percent DL-alanine
B	0.5 percent L-glutamic acid
C	0.05 percent ammonium sulfate
	0.75 percent lactic acid

Five hundred ml of modifications A and B inoculated with a one percent inoculum containing approximately ten billion cells per ml were used in 2500 ml low form culture flasks. Cultures were grown up to one week on a

shaker at 37°C. Growth was insufficient to provide enough cells to establish enzymatic differences. Cultures did not subculture successfully. Use of the complete Gerhardt and Wilson medium gave more cells but their respiratory activity was very weak.

To conveniently use larger quantities of liquid medium, and appartus was set up in which the cells were grown inside a semi-permeable cellophane tube containing approximately 100 ml. The tube was immersed in 3500 ml of medium in a 4000 ml erylenmeyer flask. The interior of the tube was aerated, with air passed through six inches of sterile cotton, from a sintered glass sparger. By this method the cells were grown in a small volume, (eliminating centrifugation of large amounts of material) while receiving the benefit of the nutrients contained in a large volume of media. Media used were modification A and B. Growth occured only in flasks medium B. Exposure of a heavy concentration of cells to medium B for four days at 37°C with shaking did not result in an adaptive curve for L-asparagine or DL-alanine.

Use of media B and C solidified with two percent agar gave sparse growth which could be subcultured and produced a small quantity of cells in three to five days. Testing by the method of Levine and Garber (25, p. 508) showed no dissociation to the rough form in successive cultures on

these media.

Freezing and Thawing

The most practical method of eliminating permeability effects is by rupturing the cell wall. This treatment produces unavoidable alteration in the enzymatic activity of the cell but is, nevertheless, a useful tool. Freezing and thawing with consequent rupture of the cell wall probably involves the fewest undesirable changes. Cells were grown, harvested, washed and suspended in a small amount of buffered saline. This suspension was poured into the top of a two quart stainless steel double boiler, forming a layer from 0.25 to 0.50 cm deep. The top section of the boiler was then placed in the bottom, which contained a mixture of dry ice and acetone at a temperaature of approximately -70°C. The suspension froze solid in one minute. The top of the boiler was then placed in water at 10°C until thawing was just completed. This process was repeated a number of times (ten was found to be the most satisfactory). After freezing and thawing, the suspension was made up with buffered saline, to a volume which showed a light transmittance of 15 percent (1:11 dilution) and contained roughly 400 γg of nitrogen per ml. The nitrogen content was later determined as described previously.

Cell Free Preparations

Preparation of a cell free extract by grinding with alumina was carried out using the method of McIlwain (31, pp. 288-291) as modified and suggested by Stanier (38). Norton levigated alumina (manufactured by Norton Company, Worcester, Mass.) was washed twice with distilled water and dried at 100°C. First the cells were harvested and washed once. For the second washing, the suspension was centrifuged in a tared heavy glass centrifuge tube. After centrifugation, the supernatant was decanted as completely as possible and the tube plus wet cells weighed. Grinding compound 2.5 times the weight of cells was added to the tube and mixed with the cells. Then the mixture was transferred to an ice cold mortar, in and ice and water bath, and ground for ten minutes. More alumina was added during grinding if the mix seemed too moist. After grinding, the mix showed a greyish color and a paste-like consistency. The mass was transferred to an ice cold heavy glass centrifuge tube and residue in the mortar was washed into the tube with 12 ml ice cold buffered saline. The tube contents were mixed to a homogenous suspension and then centrifuged at 12,000 rpm for ten minutes, the centrifuge head having been previously chilled in a deep freeze. Centrifugation first brought down the alumina in a compact layer, on top of this was a layer of cell

debris of a light orange or brown color which was about 0.3 cm deep with a rather indefinite upper boundary. The supernatant was viscous, opalescent and gave a precipitate with trichloracetic acid. This solution was pipeted off into another tube held in an ice and water bath, and one ml distributed from there into previously prepared Warburg flasks. The flasks were immediately placed on the manometers and into the water bath. After five minutes equilibration, the stopcocks were closed and the flasks tipped. It was found to be of the utmost importance to keep the preparation ice cold at all times and to work as rapidly as possible. The minimum time in which the operation could be performed by two people from commencement of grinding to the time of first reading was forty minutes, using eight flasks. This method of preparation of cell free material involves rather intimate contact with the organism and is not readily adapted to use with virulent pathogens.

EXPERIMENTAL RESULTS

Adaptive Enzymes: Attempts to show adaptive trends on various media have given results which are difficult to interpret. The cells for Figure 1 were grown in liquid medium B inside a semi-permeable sack. The rate of oxygen uptake with L-asparagine gradually increased for 160 minutes then became constant with a $Q_{O2}(N)$ of 179 (17). As far as can be ascertained any adaptive effect is delayed. The curves shown in Figure 2 were obtained with cells grown on solid medium C. The trend in oxygen uptake for glutamate is possibly adaptive. On the other hand, the curves for alpha-ketoglutarate and succinate are not adaptive. In this instance, 0.0166M substrates were used to see if the increase in concentration would accelerate adaption. Subsequent work showed that this concentration of alpha-ketoglutarate and succinate were oxidized as fast as glutamate by cells grown on the complex medium. Since, in this experiment these substrates were oxidized more slowly; the addition of a nitrogen source probably stimulates the metabolic activity of the cells. Reference to Figure 3 shows oxidation rates substrates tested with cells grown on medium B. No adaptive curve is shown for alpha-ketoglutarate or succinate and a curve similiar to that in Figure 2 is obtained for glutamate. A consideration of both figures indicates that the oxidation of

alpha-ketoglutarate and succinate is not carried out by adaptive enzymes.

A possible adaptive curve for asparagine is shown in Figure 4. The oxidation rate of glutamate increases but the process does not appear to be adaptive. Acetate oxidation is not adaptive. The trend indicated in Figure 5 shows oxidation rates of cells grown on medium C plus 0.3 per cent L-asparagine. None of the substrates exhibited adaptive oxidation patterns. Aspartic acid was oxidized slowly. The apparent increase in oxidation rate at the end of the experiment is probably an artifact. Final $Q_{O2}(N)$ rates (last 20 minutes) are given in Table 1. It is interesting to note that during the final 30 minutes of the experiment asparagine was oxidized more rapidly by cells previously grown without asparagine. A tabulation of results obtained is given in Table 2.

Table 1

Oxidation rates during final 20 minutes on medium C and medium C plus 0.3 percent L-asparagine.

SUBSTRATE[1]	Oxidation rate	
	Medium C	Medium C plus asparagine
Acetate	81(29)	86(35)
L-asparagine	165(29)	88(35)
L-glutamate	143(29)	111(35)
L-asparate	----	55(35)

Table 2

Simultaneous adaption to various substrates

SUBSTRATE[1]	Adaption of cells previously grown on:					
	Medium C		Medium B		Medium C plus asparagine	
	Actual	Theory	Actual	Theory	Actual	Theory
L-glutamate	-	-	±	-	±	-
alpha-Ketoglutarate	+	-	+	+		-
Succinate	+	-	+	+		+
Acetate	+	+		+	+	+
L-asparagine	-	-	-	-	±	+

[1]Reaction at pH 6.8; substrate concentration 0.0033M.

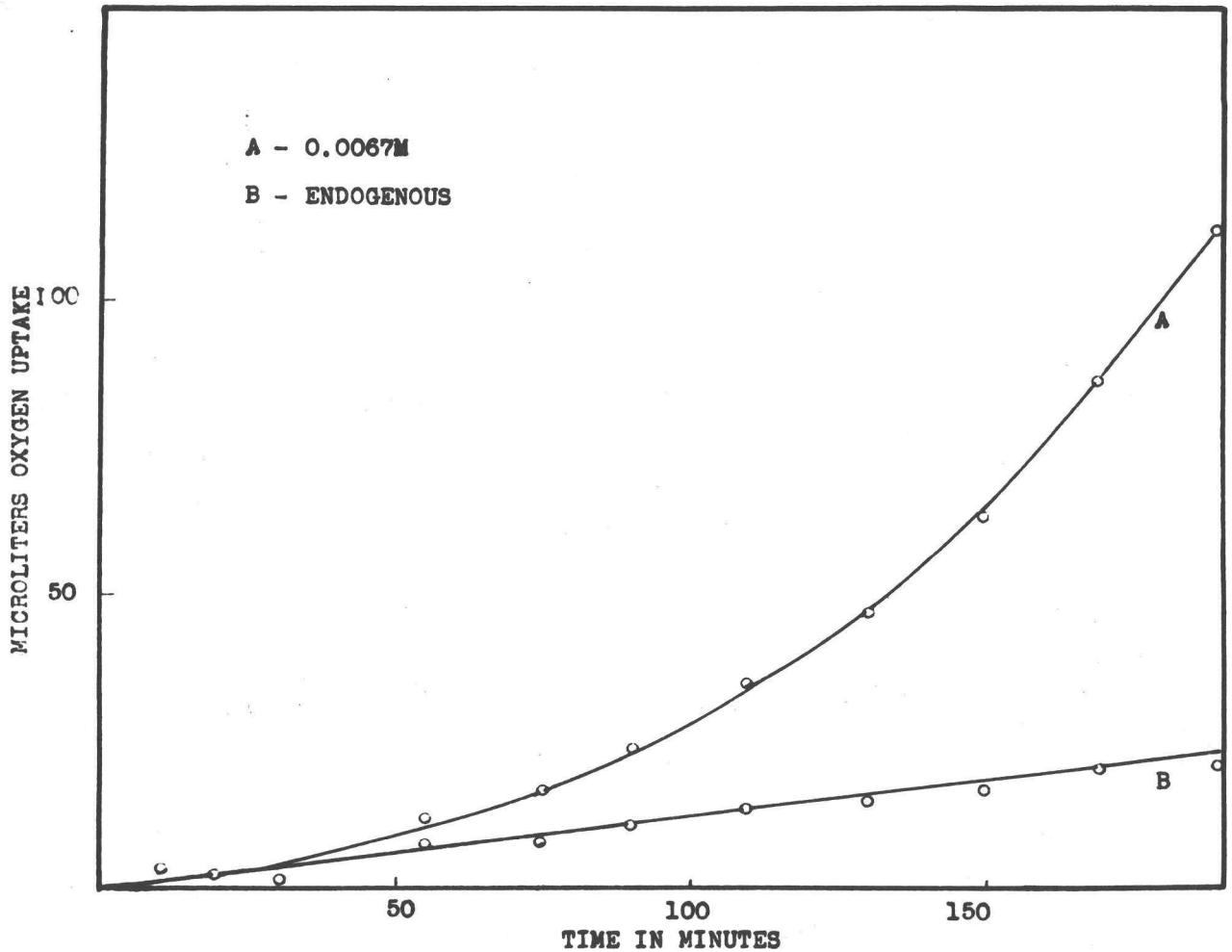

Figure 1. Oxidation of L-asparagine by cells grown on medium B.

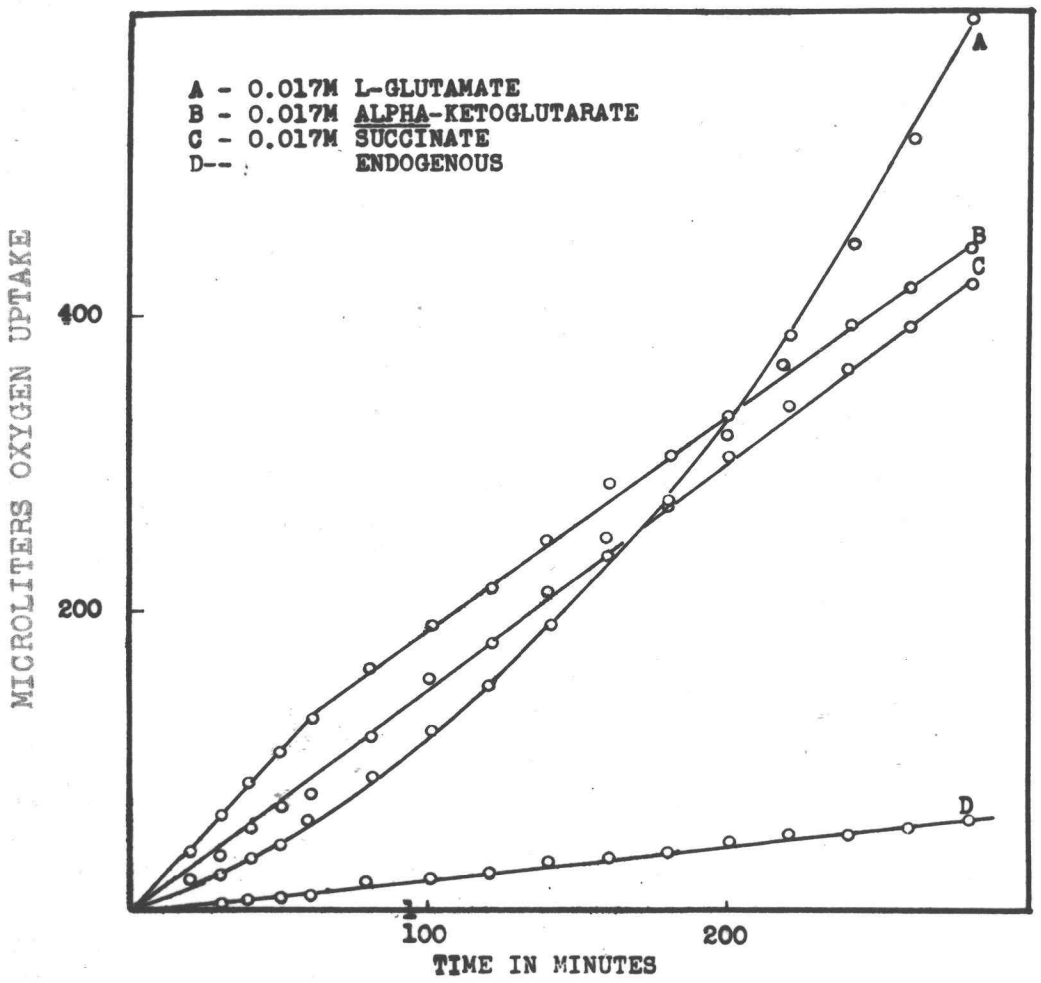

Figure 2. Oxidation of various substrates by cells grown on medium C.

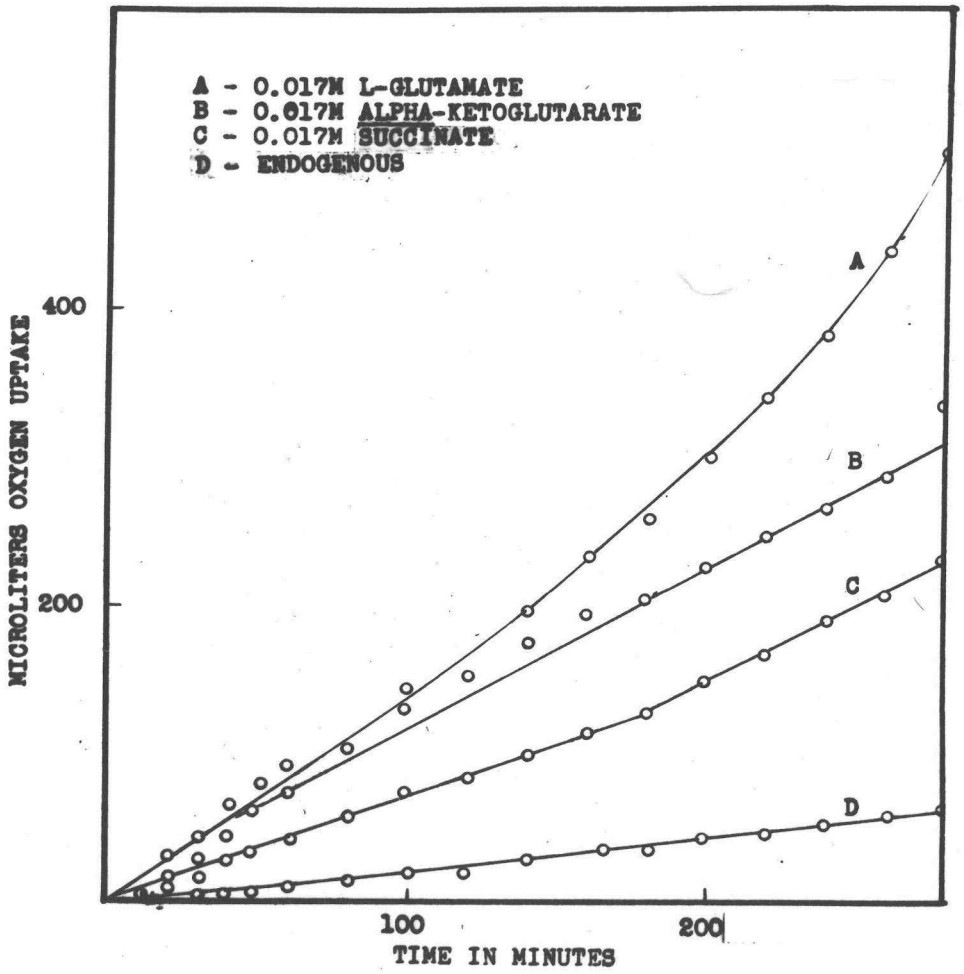

Figure 3. Oxidation of various substrates by cells grown on medium B.

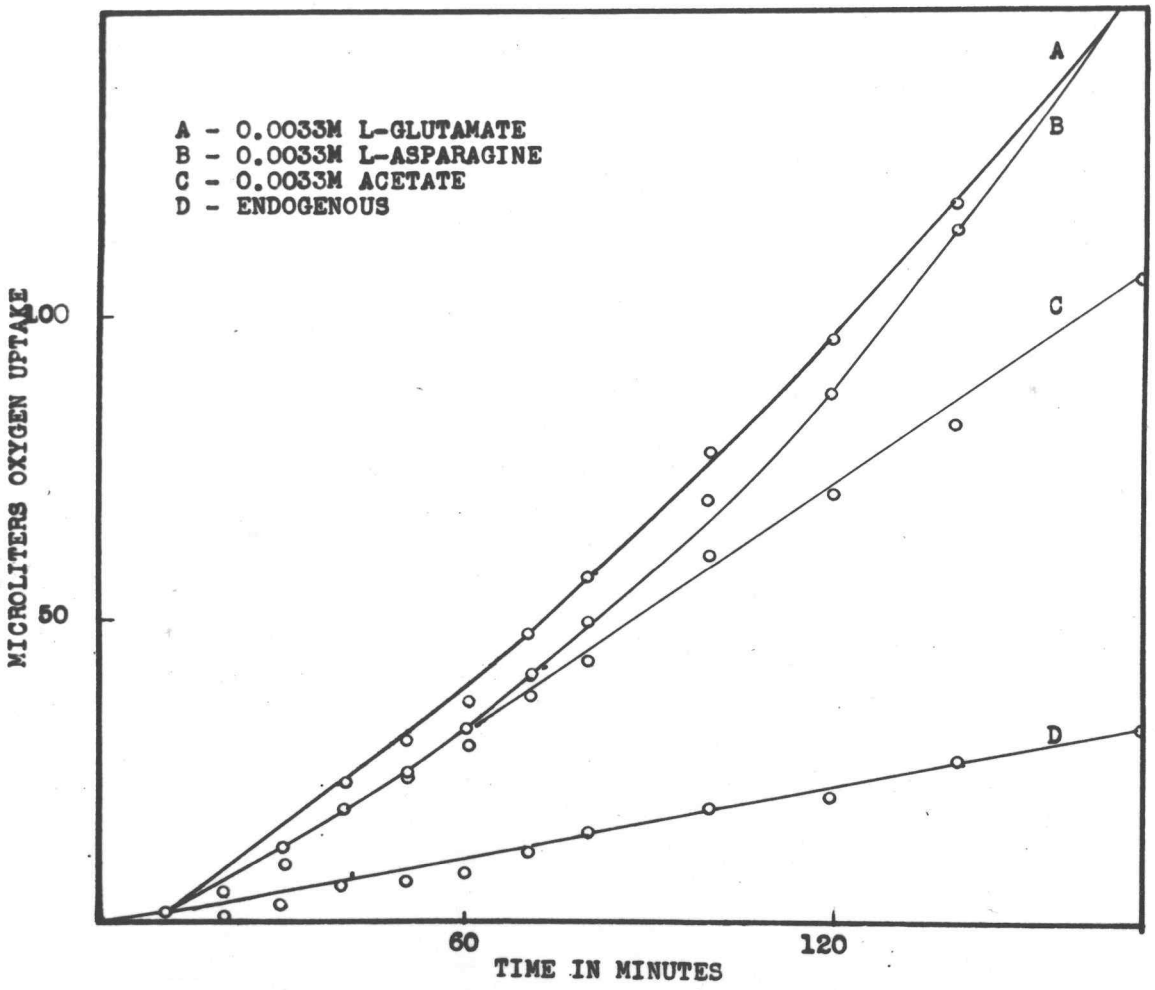

Figure 4. Oxidation of various substrates by cells grown on medium C.

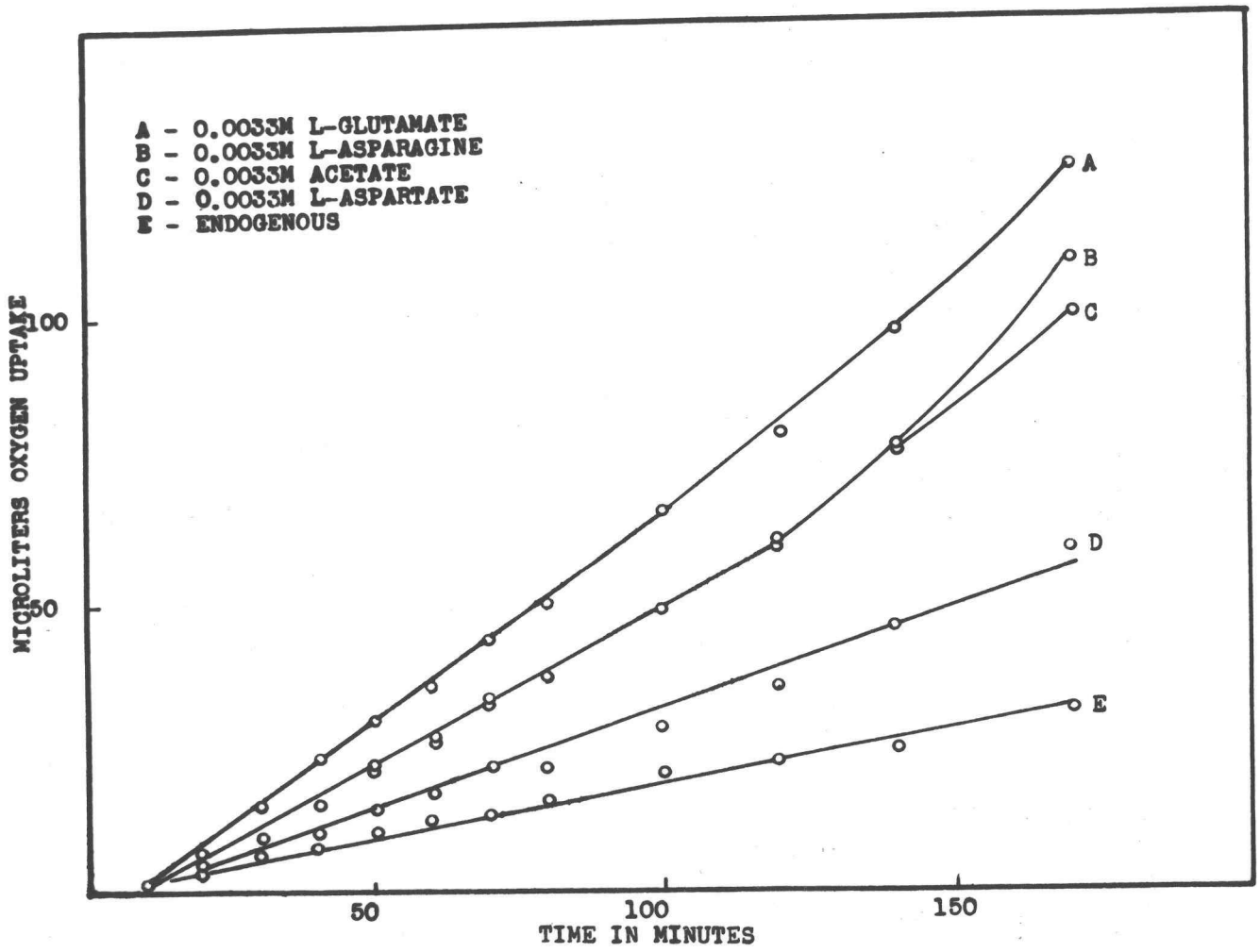

Figure 5. Oxidation of various substrates by cells grown on medium C plus 0.3 percent L-asparagine.

Hydrogen Ion Effects: Other workers have shown that pH may play an important part in cell permeability (11, p. 509). Barron and co-workers (9, p. 141) found that intermediates of the tricarboxylic acid cycle, except citric acid, were oxidized more rapidly by C. creatinovorans when the pH was lowered from 7.0 to 5.5. Testing of the oxidative behavior of brucellae toward TCA cycle members at pH 5.5 has shown that $Q_{O2}(N)$ rates are generally increased markedly. As is indicated in Table 3 succinate and alpha-ketoglutarate are particularly sesitive to pH changes. $Q_{O2}(N)$ values at pH 5.5 were found to vary considerably with succinate, alpha-ketoglutarate and fumarate in successive experiments. However, marked stimulation in oxygen uptake rates occurred in all cases. At the lower pH level alpha-Ketoglutarate, succinate and malate were oxidized more rapidly than glutamate. Pyruvate, acetate and oxalacetate were oxidized 80, 77, and 51 percent respectively, as fast as glutamate. Further testing at other pH values might increase the oxidation rate of these compounds up to that of glutamate. In experiments using the same lot of cells at both pH values glutamate oxidation was depressed slightly at the lower pH. The endogenous respiration was either depressed slightly or not at all. Citrate was not oxidized at a significant rate at either pH level. Preliminary tests at pH 4.5

showed the cells to have no activity since no oxygen uptake occured either with or without substrate.

Oxygen uptake curves at pH 5.5 are shown in Figure 6. The decrease in uptake rates at the end of the experiment is possibly explained by an increase in the pH of the flask contents. In other experiments the pH was found to rise from 5.5 to 5.9 to 6.0 during the course of the experiment.

Table 3

Effect of pH on oxidation of various compounds

SUBSTRATE#	$Q_{O2}(N)$	
	pH 6.8	pH 5.5
Glutamate	552(67)	521(62)
Alpha-ketoglutarate	278(67)	665(62)
Succinate	205(62)	487(62)
Fumarate	96(82)	498(67)
Malate	406(62)	622(98)
Oxalacetate	119(82)	264(98)
Pyruvate	116(79)	415(98)
Acetate	200(79)	402(65)
Citrate	98(98)	98(98)
Aspartate	65(55)	107(48)
Asparagine	180(55)	55(48)

All substrates 0.0033 M concentration.

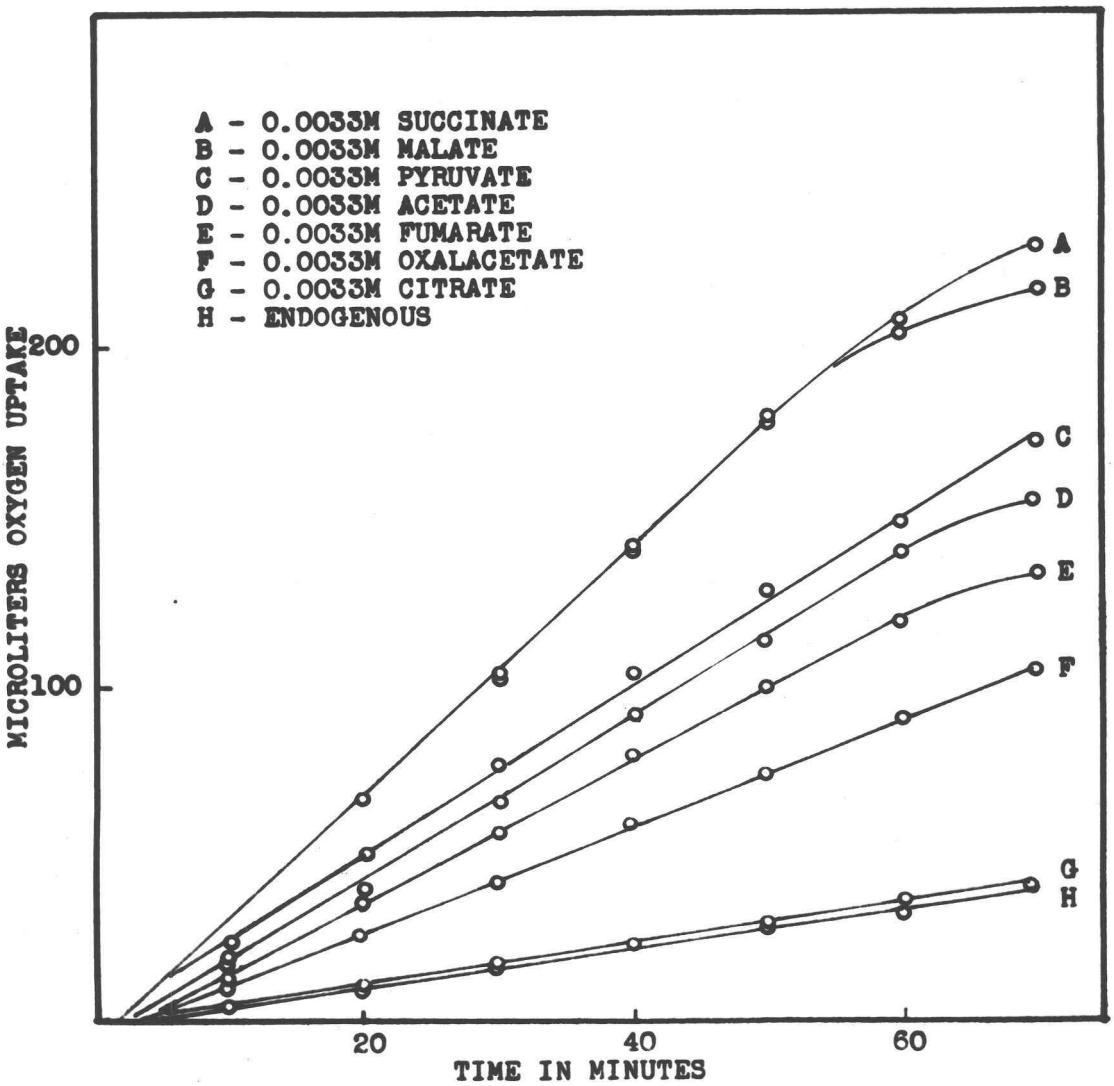

Figure 6. Oxidation rates of citric acid cycle intermediates by resting cells at pH 5.5.

Concentration effects: If the permeability of the cell wall to various acid substrates is a function of the unionized acid concentration then an increase in substrate concentration should have the same effect as an increase in the hydrogen ion concentration. A study of the data in Table 4 shows the effect of concentration on oxygen uptake rates using several substrates. Succinate and alpha-ketoglutarate oxidation rates were markedly increased. The curve of rate versus substrate concentration is shown in Figure 7. Further reference to Table 4 shows an apparent anomaly, in that 0.0333M fumarate is oxidized at almost the same low rate at pH 6.8 and 5.5, while the most rapid oxidation is at 0.0033M and pH 5.5. This reduction in rate of oxygen uptake at higher concentration is probably due to an inhibitory substance present in the fumaric acid used (Eastman Kodak, Practical, 98 percent). Aspartic acid oxidation was stimulated at higher concentrations; this effect was probably due to an increase cell penetration. Glutamate is oxidized somewhat faster at the higher concentrations but the effect is much less marked than with the other compounds.

The effect of concentration on oxygen uptake with glutamine and citrate is shown in Figure 8. Citrate is oxidized slightly at 0.0555M and 0.167M concentrations, with $Q_{O2}(N)$ values of 108(87) and 99(87) respectively. In the same experiment 0.333M citrate was oxidized for the

first twenty minutes, than the rate decreased below the endogenous. This effect was probably due to inhibition by too high a substrate concentration. The curve for glutamine breaks sharply between 20 and 30 minutes. Since the break is sharper at high concentration of the substrate it may be caused by the accumulation of some inhibitory substance. These results with glutamine were obtained with Nutritionsl Biochemical Company lot number 1454. Use of NBC lot number 4010 at 0.167M and 0.0033M concentration gave curves which did not break and had Q_{O2}(N) values of 592(82) and 212(82) respectively. A microbiological assay (7) of lot number 1454 using Leuconstoc mesenteroides P-60 and 17-5 showed that this compound was probably contaminated with glutamic acid.

Table 4

Effect of concentration of substrate on oxidation of various compounds

SUBSTRATES#	$Q_{O2}(N)$					
	0.33M	0.167M	0.033M	0.0167M	0.0033M	0.00033M
Succinate	602(120)	636(120)	591(120)	541(120)	268(120)	122(120)
Alpha-ketoglutarate	757(57)	800(57)	688(57)	578(57)	252(57)	121(57)
Fumarate			246(93)	163(93)		
Fumarate*			218(67)		498(67)	
Aspartate		399(82)			94(82)	
Glutamate		580(82)	502(57)		433(57)	
Alanine				106(48)	99(48)	69(48)

Reaction at pH 6.8

*This reaction only at pH 5.5

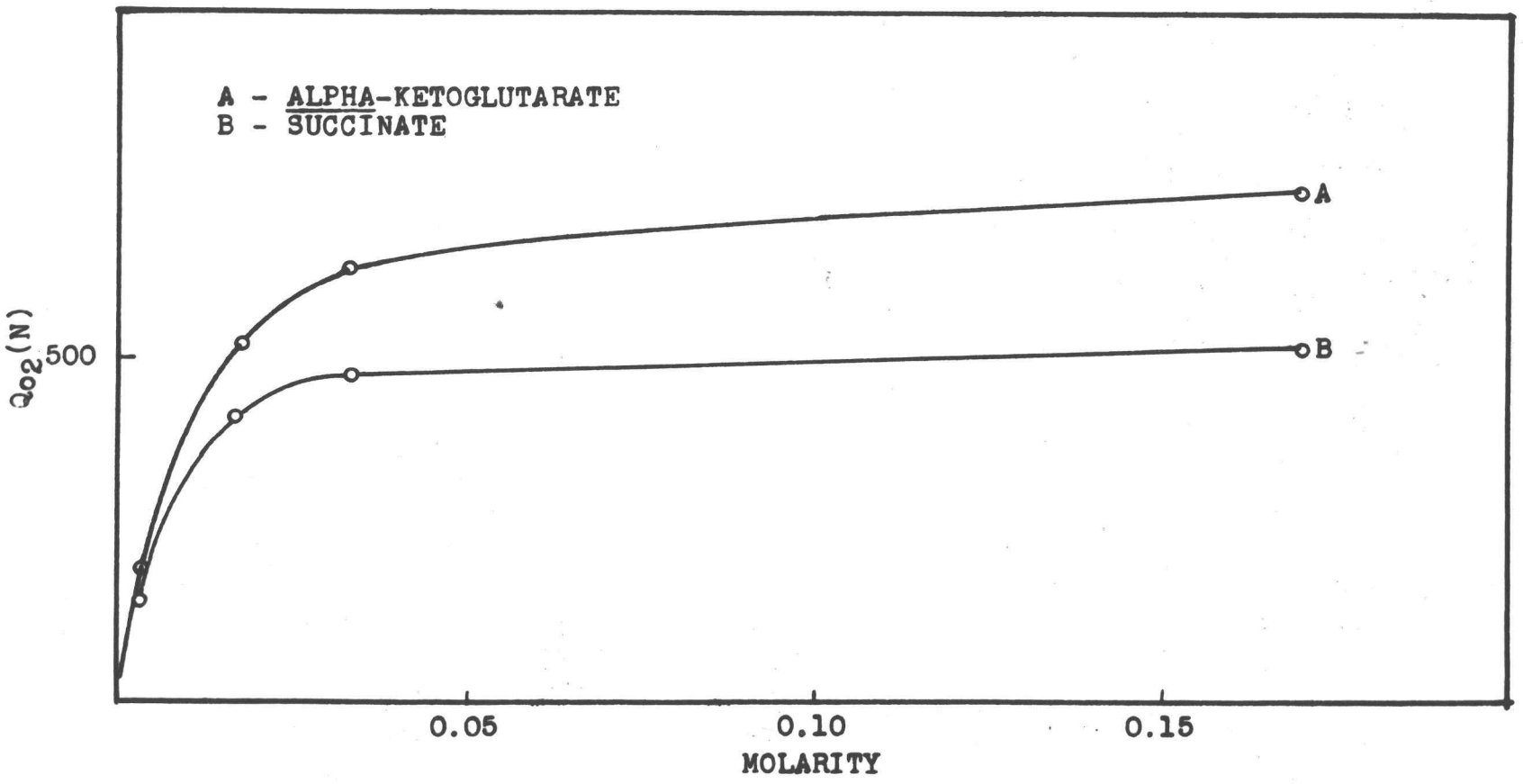

Figure 7. Effect of substrate concentration on rate of oxygen consumption

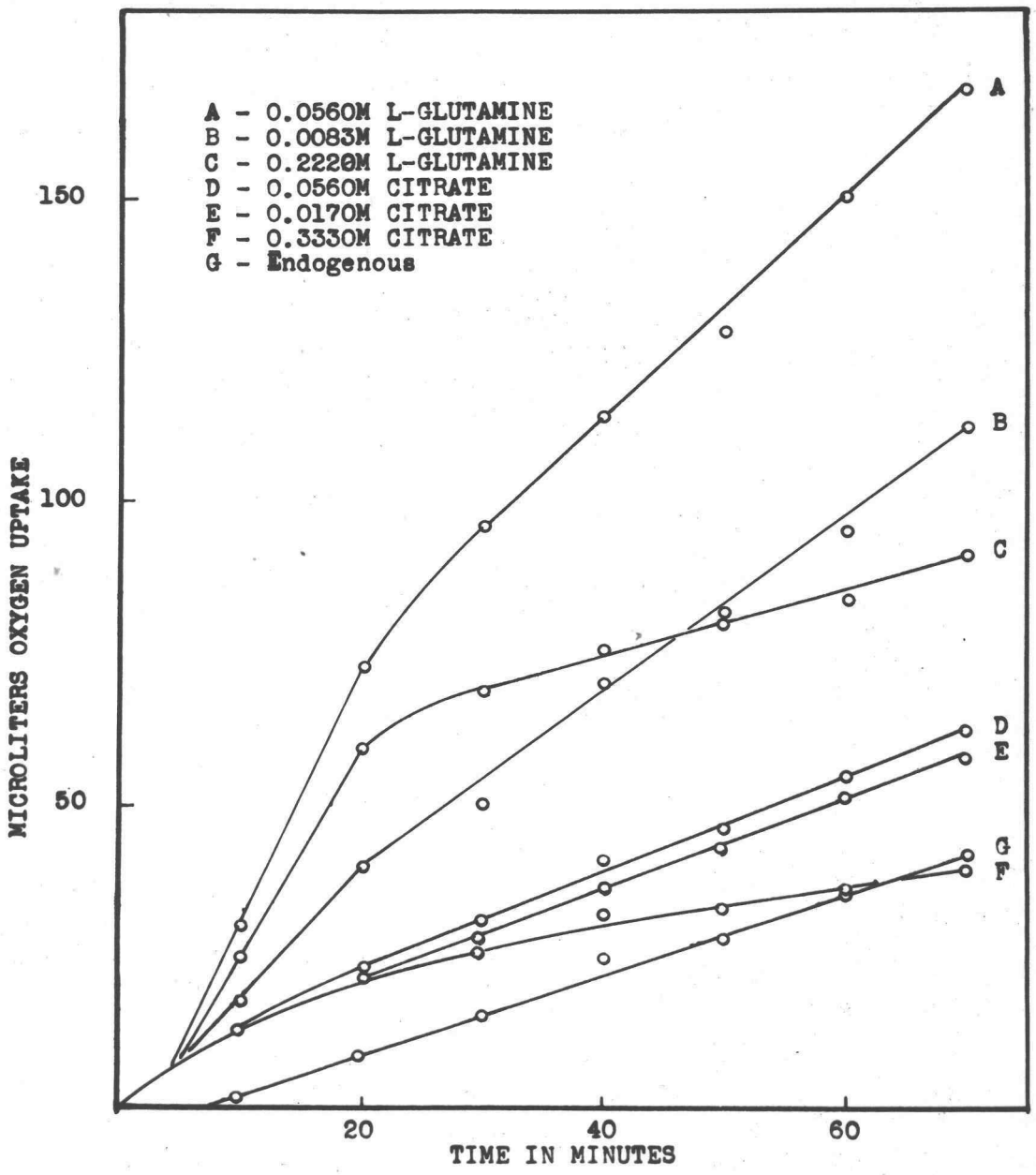

Figure 8. Effect of substrate concentration on the oxidation rate of citrate and L-glutamine.

Inhibitors:Malonate: Malonate is the classic inhibitor of succinate oxidation and, for this reason its effect under various conditions was tested using resting cell suspensions of brucellae. The inhibition of succinate oxidation by different concentrations of malonate at pH 5.5 is shown in Table 5. A 2:1 ratio of malonate to succinate showed no inhibition. However, when the ratio was increased to 10:1 and inhibition of 26 percent resulted. The results obtained using cells frozen ten times with 0.0033M substrate and 0.0167M malonate are shown in Table 6. The oxidative activity of these cells was low. Glutamate, alpha-ketoglutarate and succinate were inhibited to a greater degree than malate; malonate in the concentration used had no effect on the endogenous respiration of the preparation. In another experiment using frozen cells with the same concentration of substrate and 0.0333M malonate an inhibition of 58.6 percent was noted. Gerhardt (19, p. 85) using the same substrate concentration at pH 6.8 was unable to demonstrate any significant inhibition of succinate oxidation even with a 10:1 inhibitor-substrate ratio.

Work of a similar nature done at Wisconsin (43) with toluene treated cells showed inhibition of succinic dehydrogenase activity by malonate using the Thunberg technique. The cells showed no activity using the Warburg microrespirometer.

Table 5

Effect of pH and concentration on malonate inhibition of oxidation of succinate

SUBSTRATE	pH 5.5		pH 6.8	
	$Q_{O_2}(N)$	%Inhibition*	$Q_{O_2}(N)$	% Inhibition*
0.0033M Succinate	483(62)		243(52)	
0.0033M Succinate plus 0.0067M Malonate	483(62)	0	248(52)	-2.6
0.0067M Malonate	62(62)	0	55#(52)	-5.8
0.0033M Succinate	538(50)		243(52)	
0.0033M Succinate plus 0.033M Malonate	398(50)	28.6	230(52)	6.8
0.033M Malonate	50(50)	0	55#(52)	-5.8

* Calculations corrected for endogenous respiration; e.g., 100% inhibition reaches the endogenous rate.

0.0033M Malonate.

Table 6

Inhibition by malonate of oxidation of various compounds with frozen and thawed cell preparation

SUBSTRATE*	$Q_{O_2}(N)$	PERCENTAGE OF INHIBITION #
Glutamate	224(54)	
Glutamate plus 0.017M malonate	212(54)	7.1
Alpha-ketoglutarate	261(54)	
Alpha-ketoglutarate plus 0.017M malonate	242(54)	9.2
Succinate	263(54)	
Succinate plus 0.017M malonate	233(54)	14.3
Malate	235(54)	
Malate plus 0.017M malonate	228(54)	3.9

* Reaction at pH 6.8, substrates 0.0033M concentration.

Calculations corrected for endogenous respiration.

Arsenious oxide: Along with malonate, it seemed possible that the use of other inhibitors might yield fruitful results. One of the more likely of these was arsenious oxide. Testing of various concentrations of As_2O_3 with glutamate, alpha-ketoglutarate and succinate has given the results shown in Table 7. The substrate was tipped twenty minutes after tipping the inhibitor. Of the three substances tested, alpha-Ketoglutarate was inhibited the least, perhaps due to the high concentration of acid. Glutamate may be inhibited at the alpha-ketoglutarate step. Succinate maybe inhibited at the oxalacetate point. Perhaps the inhibition of succinate oxidation indicates inhibition of a cyclic process.

Table 7

Inhibition by arsenious oxide of oxidation of various compounds at pH 6.8 and 5.5

SUBSTRATE	pH	$Q_{O_2}(N)$	PERCENTAGE OF INHIBITION*
0.0033M Glutamate	6.8	670(80)	100
0.0033M Glutamate plus 0.00026M As_2O_3	6.8	80(80)	
0.0033M Glutamate plus 0.0000325M As_2O_3	6.8	264(80)	68.7
0.00026M As_2O_3	6.8	80(80)	0.0
0.0033M Glutamate	5.5	515(62)	
0.0033M Glutamate plus 0.0000325M As_2O_3	5.5	276(62)	52.8
0.0033M Alpha-ketoglutarate	5.5	642(62)	
0.0033M Alpha-ketoglutarate plus 0.0000325M As_2O_3	5.5	452(62)	32.8
0.0033M Succinate	5.5	640(62)	
0.0033M Succinate plus 0.0000325M As_2O_3	5.5	439(62)	34.8
0.0000325M As_2O_3	5.5	62(62)	0.0

*Calculations corrected for endogenous respiration.

Formaldehyde: Berheim (10, pp. 133-134) showed that 0.0062M formaldehyde inhibited succinoxidase of rat liver or kidney homogenates. L-proline oxidase, choline oxidase and sarcosine oxidase were also inhibited, while cytochrome, tryosine, D-methionine and D-alanine oxidases were not, even with concentration of 0.0124M formaldehyde. The extent and amount of inhibition was the same at pH 6.0 and 8.0. On the other hand, it has been observed that brucellae may oxidize formaldehyde to a variable or slight degree (39). The curves depected in Figure 9 show the effect of 0.0124M and 0.0062M formaldehyde on the oxidation of alpha-ketoglutarate and succinate at pH 5.5. Substrate concentration was set at 0.00167M to reduce to concentration effect. The formaldehyde was tipped twenty minutes before the substrate. It is apparent that formaldehyde was oxidized by the organisms at a slow but significant rate (Q_{O2}(N) 117(70)). The fact formaldehyde was oxidized shows that at these concentrations it did not have a general poisoning effect since some enzymes were still active. Formaldehyde caused a 37.5 percent inhibition of alpha-ketoglutarate oxidation at 0.0062M and 53.5 percent at 0.0124M. Succinate was inhibited 36 percent at 0.0062M and 48 percent at 0.0124M formaldehyde.

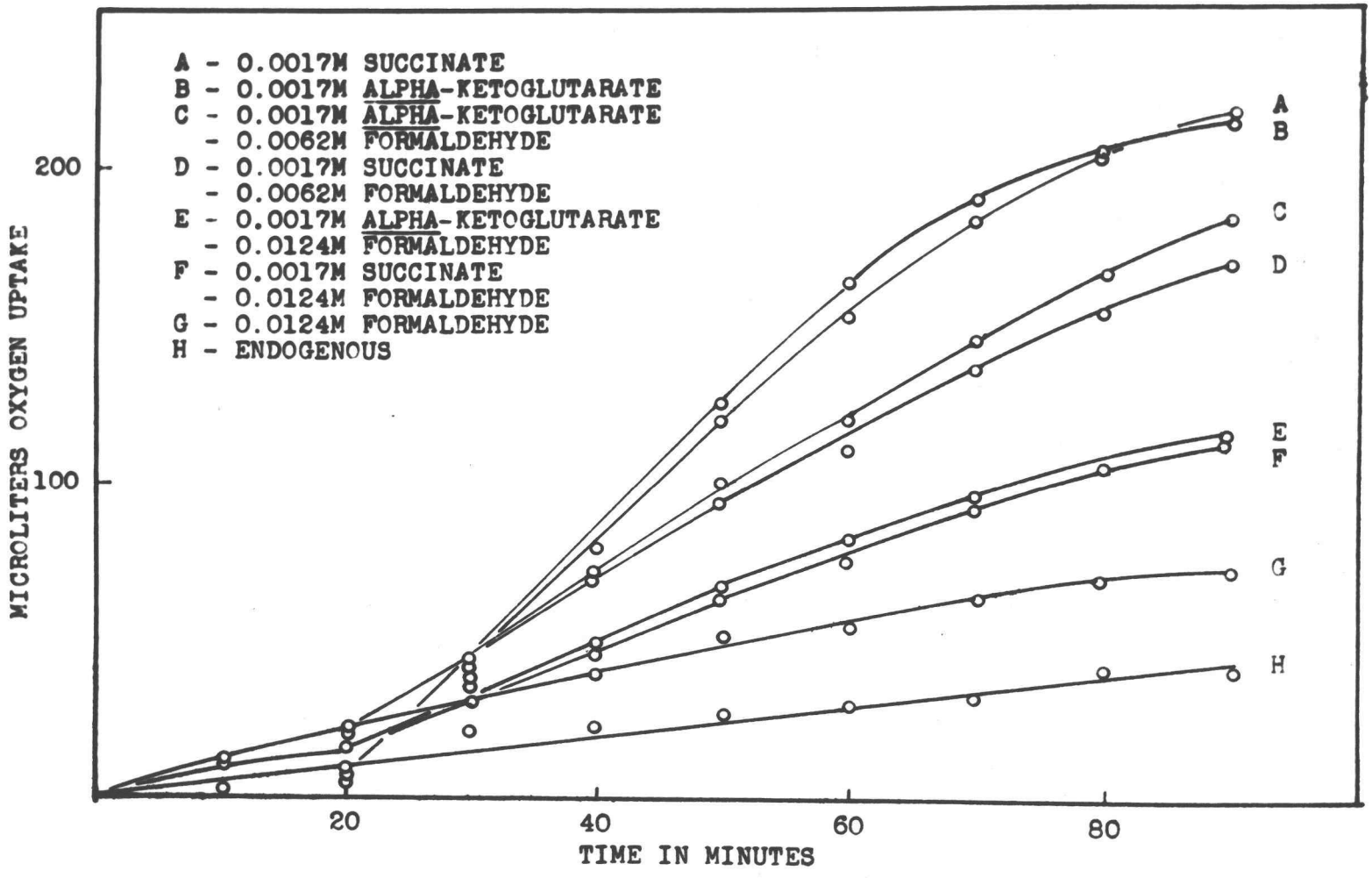

Figure 9. Inhibition of oxidation of alpha-ketoglutarate and succinate by formaldehyde.

Trans-1,2-Cyclopentanedicarboxylic acid: Seaman and Houlihan (34, pp. 437-440) observed that a concentration of 0.04M CPC[1] increased the rate of oxygen uptake of whole cells of Tetrahymena geleii a flagellated protozoan, in the presence of 0.02M acetate, pyruvate and succinate. This increase was shown to be due to an increase in cell permeability. CPC exerted a competitive inhibition with succinate when cell homogenate were used. Since permeability is a factor in working with brucellae, the effect of CPC on the rate of oxidation of various substrates has been tested. The results are shown in Table 8.

The only stimulation of oxygen uptake occured when L-asparagine was used as the substrate. Aspartic acid was not oxidized, with or without CPC. Glutamate and alpha-ketoglutarate oxidation was inhibited 18.4 percent and 56.4 percent respectively by 0.00556M CPC. A continuation of this study in this laboratory (7) has produced some rather interesting results which, in order to present a more complete picture, are shown in Table 9. At pH 6.8 use of 0.04M CPC resulted in a 12 percent inhibition of succinate, five percent inhibition of glutamate, 2.5 percent stimulation of L-asparagine oxidation. Aspartate oxidation and the endogenous respiration were

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[1] Trans-1,2-Cyclopentanedicarboxylic acid

unaffected. At pH 5.5, 0.04M CPC inhibited oxidation of succinate 56 percent, glutamate 68 percent, alpha-keto-glutarate 45 percent and asparagine 100 percent. Aspartate showed some oxygen uptake at pH 5.5 which was inhibited almost completely by 0.04M CPC. The use of 0.08M CPC gave the same general picture, although, as would be expected, results were not so pronounced. Marked inhibition of the endogenous respiration by 0.04M CPC at pH 5.5 indicated partial inactivation of vital cell processes by CPC.

Table 8

The effect of CPC on the rate of oxidation of various compounds at pH 6.8

Molarity CPC	Oxidation rate			
	L-asparagine	L-aspartic	L-glutamate	alpha-keto glutarate
0.0222	258(77)	77(77)		
0.0056	250(77)	77(77)	520(77)	102(68)
0.0011	234(77)	77(77)	594(77)	
0.0000	203(77)	77(77)	637(77)	234(66)

Table 9

Effect of trans-1,2-cyclopentanedicarboxylic acid (CPA) on oxidation of various compounds at pH 6.8 and 5.5*

SUBSTRATE	pH 6.8		pH 5.5	
	$Q_{O2}(N)$	% Inhibition#	$Q_{O2}(N)$	% Inhibition#
0.0033M Succinate	175(48)		428(77)	
0.0033M Succinate plus 0.04M CPA	154(48)	16.5	178(77)	71.2
0.0033M Succinate plus 0.008M CPA	163(48)	9.5	358(77)	20.0
0.04M CPA	73(48)	-52.0	33(77)	57.1
0.008M CPA	48(48)	0	79(77)	-2.6
0.0033M Glutamate	348(57)		300(48)	
0.0033M Glutamate plus 0.04M CPA	330(57)	6.2	95(48)	81.4
0.0033M Glutamate plus 0.008M CPA	330(57)	6.2	218(48)	32.5
0.0033M Alpha-ketoglutarate	163(57)		425(48)	
0.0033M Alpha-ketoglutarate plus 0.04M CPA	159(57)	3.8	235(48)	50.4
0.0033M Alpha-ketoglutarate plus 0.008M CPA	159(57)	3.8	366(48)	15.6
0.04M CPA	57(57)	0	19(48)	60.4
0.008M CPA	57(57)	0	32(48)	33.3

Table 9-continued

SUBSTRATE	pH 6.8		pH 5.5	
	Q_{O2}(N)	% Inhibition#	Q_{O2}(N)	% Inhibition#
0.0033M Aspartate	65(55)		107(48)	
0.0033M Aspartate plus 0.04M CPA	65(55)	0	19(48)	100
0.0033M Aspartate plus 0.008M CPA	65(55)	0	28(48)	100
0.0033M Asparagine	180(55)		55(48)	
0.0033M Asparagine plus 0.04M CPA	214(55)	-27.2	7(48)	100
0.0033M Asparagine plus 0.008M CPA	183(55)	-2.4	24(48)	100
0.04M CPA	53(55)	3.6	7(48)	85.4
0.008M CPA	72(55)	-30.9	19(48)	60.4

* These data were compiled by Mr. Arthur Anderson
# Calculations corrected for endogenous respiration.
Rate of oxidation suppressed below the endogenous rate.

2,4-Dinitrophenol: 2,4-dinitrophenol has been used to inhibit bacterial respiration and also to decrease substrate assimilation. However if used in the proper concentration it may increase the rate of oxygen uptake in the presence of substrate. This action of DNP[2] is associated with its action in breaking high energy phosphate bonds and thus making the energy unavailable for anabolic processes in the cell. Barron et al (9,p.139) found that inhibition of acetate oxidation by 0.00005M DNP occurred at pH 5.5 but not at pH 7.0. In this work the promary purpose of using DNP was to decrease substrate assimilation and thus obtain the maximum oxygen consumption per mole of substrate metabolized. By comparing this oxygen uptake with theoretical values for various stages of oxidation, an indication of the products of oxidation may sometimes be obtained. A study of the date in Table 10 shows the results obtained for substrates tested. Figures 10 and 11 illustrate the actual curves obtained for acetate and succinate and also give the points used as a basis for calculation of moles of oxygen taken up per mole of substrate. The results obtained with alpha-ketoglutarate and glutamate were below the theoretical for complete oxidation. Possibly, in glutamate oxidation the amino group tends to increase

[2] 2,4-dinitrophenol

assimilation by providing a readily available nitrogen source. The value of 1.85 moles oxygen uptake per mole of glutamate without DNP checks closely with that of Gerhardt's data (19, p. 80). The alpha-ketoglutarate used was from a commercial source and no determination of its purity was made. The theoretical oxygen uptake value of one mole of succinic acid is 3.5 moles. The results of two experiments using different lots of cells with 0.000033M DNP at pH 5.5 gave values of 3.56 and 3.18 moles of oxygen, respectively. A carbon dioxide evolution determination which was run at the same time as the second oxygen uptake determination showed that 3.56 moles had been evolved out of the theoretical four. These values are sufficiently close to the theoretical that complete oxidation may be assumed. This observation becomes particularly true when the R.Q. of 1.12 is considered (theoretical R.Q. is 1.14). The oxygen uptake and carbon dioxide evolution values obtained for acetate were 93 and 81 percent respectively, of the theoretical for complete oxidation. In this instance complete oxidation of acetate is indicated. Since formic acid has a theoretical oxygen mole ratio of 1.5 the excessive oxygen uptake infers complete oxidation if only one end product is assumed.

Table 10

Stoichiometric ratios for the dissimilation of various compounds

SUBSTRATE[x]	CONCENTRATION OF 2,4-DINITROPHENOL	MOLES O_2 PER MOLES SUBSTRATE		
		Actual	Theory	% Theory
0.00167M Glutamate	0	1.85	4.5	41
0.00167M Glutamate	0.000125M	2.57	4.5	57
0.00167M Glutamate	0.0000625M	1.53	4.5	34
0.000833M Alpha-ketoglutarate	0.000033M	3.12	4.5	69
0.000833M Succinate	0.000033M	3.56	3.5	102
0.000833M Succinate	0.000033M	3.18 (3.56)#	3.5 (4.0)#	91 (89)#
0.000833M Acetate	0.000033M	1.86 (1.70)#	2.0 (2.0)#	93 (85)#

[x] Reaction at pH 6.8.
Values in parentheses are for CO_2.

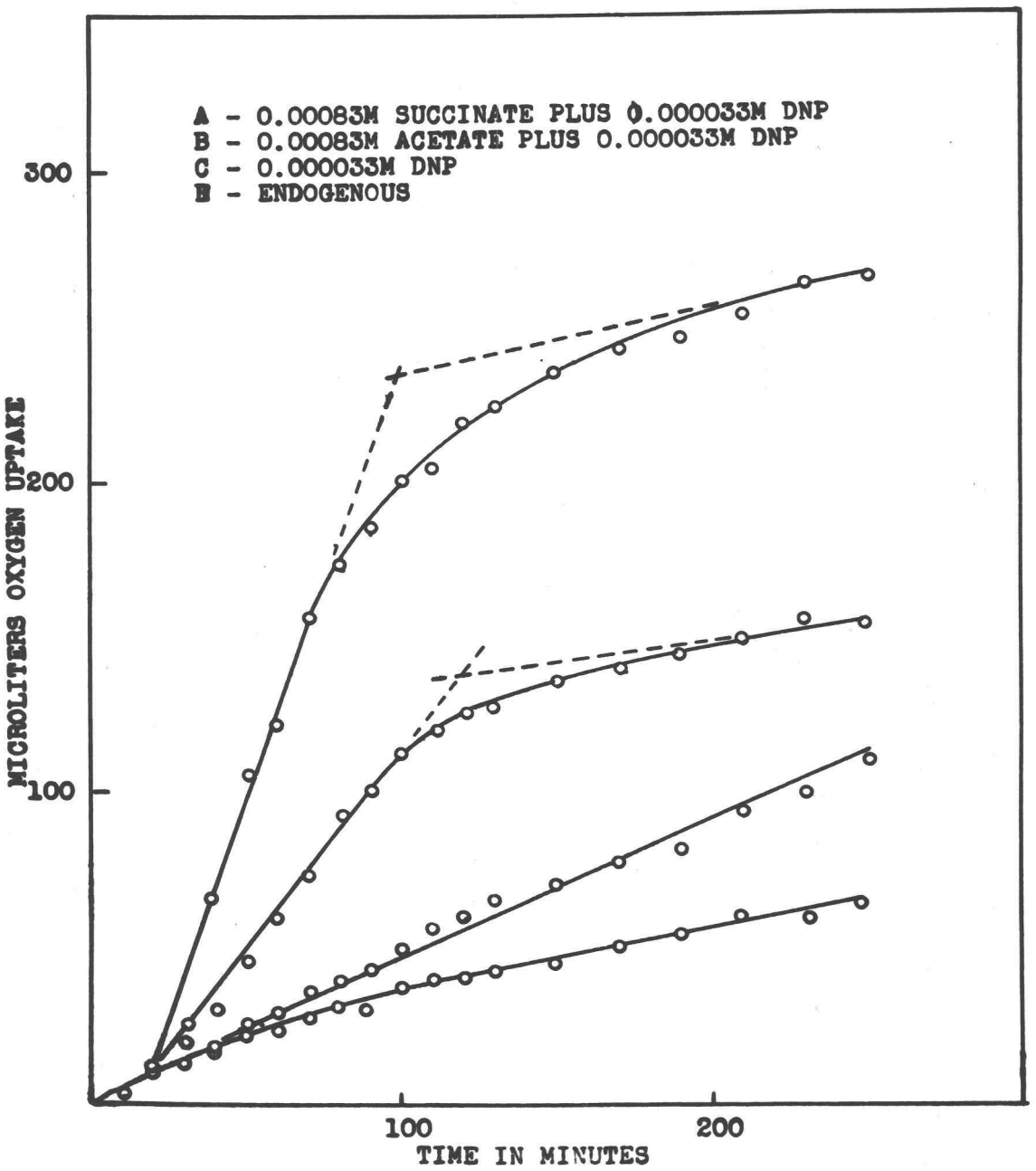


Figure 10. Oxidation of succinate and acetate at pH 5.5 in the presence of DNP.

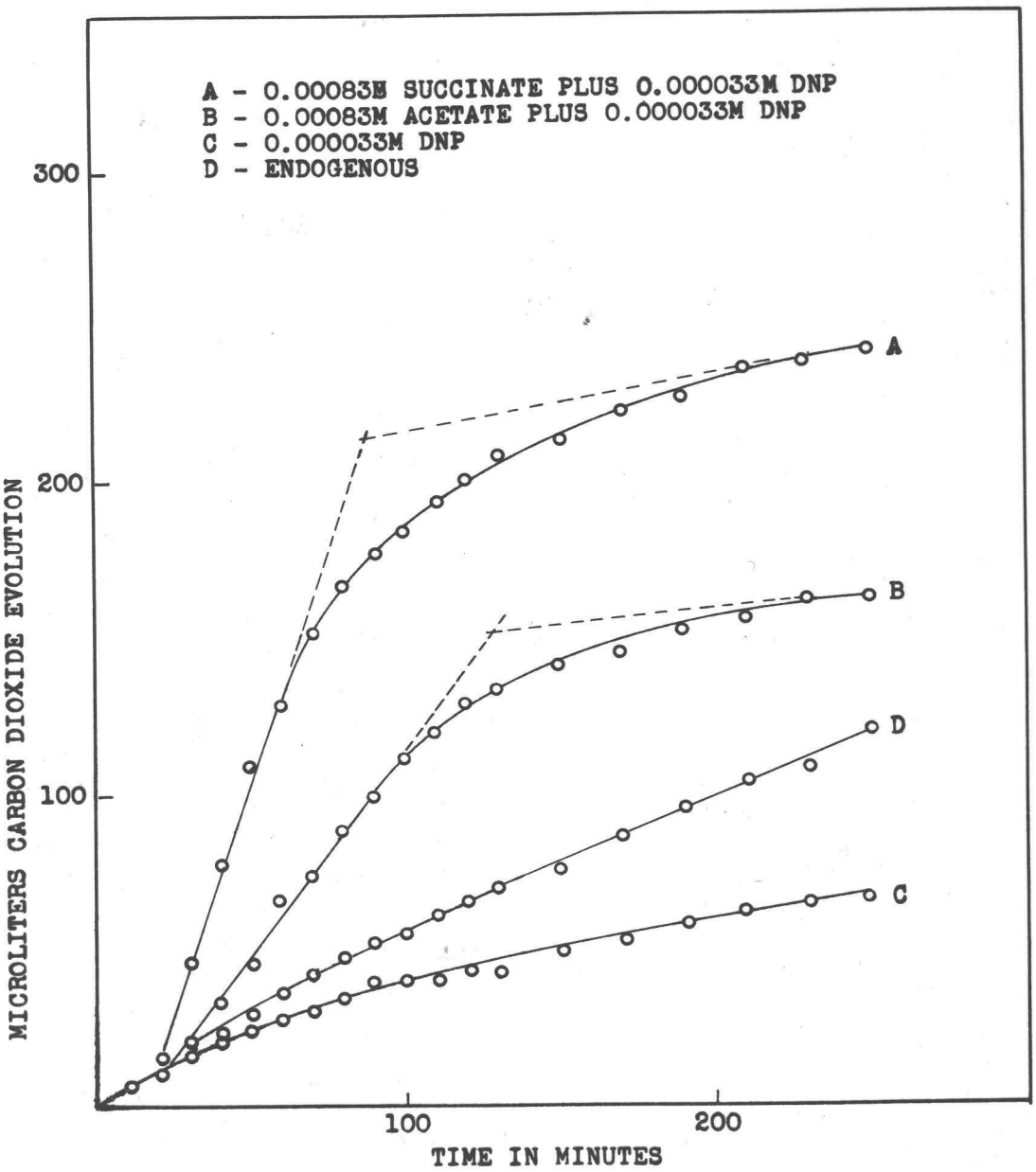


Figure 11. Carbon dioxide evolution from succinate and Acetate at pH 5.5 in the presence of DNP.

Freezing and Thawing of Cell Suspensions: Cells were frozen and thawed in an attempt to reduce the effect of cell permeability. The methods used have been described earlier in this thesis. As might be expected the overall enzymatic activity of the cells was reduced. The oxidation rates of succinate and alpha-ketoglutarate did not reach the values previously obtained by lowering the pH or increasing the substrate concentration. The oxidative activity of the cells toward glutamate was greatly reduced. Preliminary experiments showed that the optimum oxidation rate of alpha-ketoglutarate and succinate was obtained by freezing ten times. The effect of freezing and thawing on the permeability of the cells toward malonate has already been described. The $Q_{O2}(N)$ values of different lots of cells varied greatly as is shown in Table 11. The results in Table 11 indicate that all tested intermediates of the tricarboxylic acid cycle were oxidized at a significant rate except oxalacetate and citrate. Acetate was oxidized more rapidly than oxalacetate or citrate but more slowly than the other compounds tested.

Table 11

Effect of freezing and thawing* of cells on oxidation of various compounds

SUBSTRATE#	Q_{O2}(N)		
Glutamate	134(91)	170(54)	52(19)
Alpha-ketoglutarate		207(54)	70(19)
Succinate	386(91)	209(54)	93(19)
Fumarate		181(54)	50(19)
Malate			52(19)
Oxalacetate			28(19)
Acetate			41(19)
Citrate			22(19)

* Cell suspensions rapidly frozen and thawed 10 times.
# Reaction at pH 6.8, substrates 0.0033M concentration.

Cell Free Preparations: In a continuation of attempts to remove permeability effects several experiments were preformed with cell free preparations, made by grinding with alumina as described in the section on Experimental Methods. In four experiments two preparations showed no activity. One of the preparations which showed oxygen uptake with succinate was inactive with glutamate as substrate. The other active preparation was tested on succinate, alpha-ketoglutarate and citrate, the oxygen uptake curves for this preparation are shown in Figure 12. Succinate oxidation was rapid at first then declined. Alpha-ketoglutarate and citrate showed negative uptakes for the first few readings. However, when these negative values were plotted both subtrates gave a straight line plot. $Q_{O2}(N)$ values of the cell free material were: succinate 114 (19) (calculated between five and twenty minutes), alpha-ketoglutarate 46 (19) and citrate 28 (19). The activity of the preparation on all three TCA intermediates appeared to be significant and seems to indicate that enzymes capable of oxidizing citrate are present in brucellae.

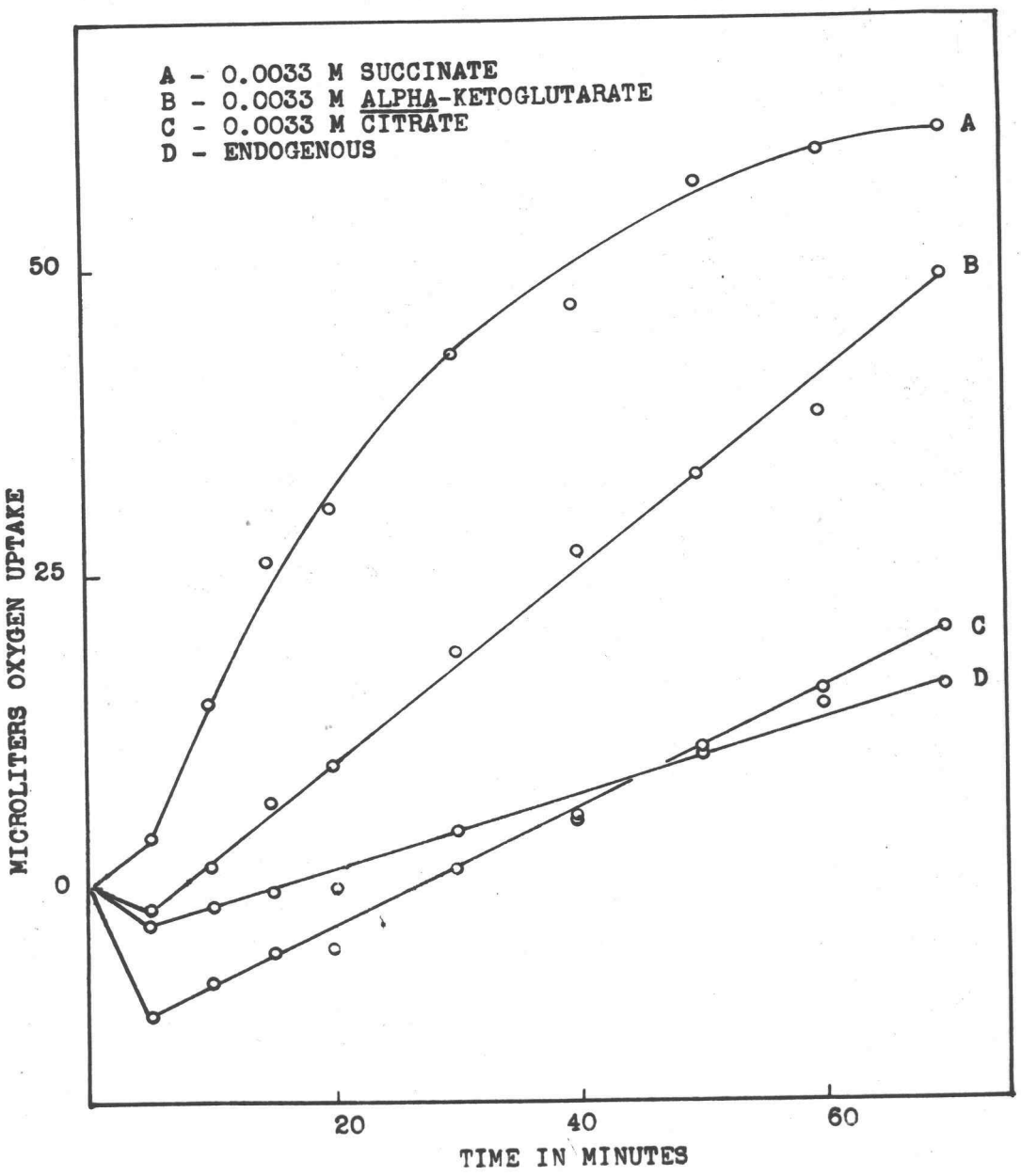


Figure 12. Oxidation of succinate, alpha-ketoglutarate, and citrate by cell-free preparation.

Citrate formation: Since the results obtained in other experiments do not definitely establish the existence or non-existence of a tricarboxylic acid cycle in brucellae, an attempt was made to determine whether the organism synthesized citrate. It has been previously mentioned that formation of citrate is not a criterion of the presence of a Kreb's cycle. However since both isocitrate and alpha-ketoglutarate are oxidized by resting cells the demonstration of citrate formation should tend to lend support to the existence of a tricarboxylic acid cycle. Oxalacetate, pyruvate and glutamate were used as substrates. Incubation of heat killed cells with oxalacetate gave a higher value than control cells, indicating either that the oxalacetate was contaminated with citrate or that oxalacetate interferes with the test under the conditions used. The results of three experiments are tabulated in Table 12. Citrate formation was calculated by subtracting the citrate value of the control cells (minus substrate) from the cells to which substrate had been added. Since oxalacetate alone gave a high value for citrate, the value for oxalacetate with heated cells was subtracted from the value for oxalacetate with unheated cells. The error, if any, in the values shown should tend to make them low since, if oxalacetate interferes with the determination, its concentration

should be lower with the untreated cells due to oxidation. This lower concentration should give less interference and a larger portion of the value should be due to citrate formation. The experiments show that very little, if any citrate accumulates in a resting cell suspension with oxalacetate or oxalacetate and pyruvate substrates. If citrate is formed, the lack of accumulation would indicate the oxidation of this acid. Monofluoracetate has been used as an inhibitor of citrate oxidation. Unfortunately none was available during this study.

Table 12

Recovery of citrate from dissimilation of glutamate, oxalacetate, and pyruvate

SUBSTRATE*	CELLS#	MOLES CITRATE RECOVERED PER ML	MOLES CITRATE FORMED
None	Heated	0	
Oxalacetate	Heated	0.133	
None	Normal	0	
Oxalacetate	Normal	0.146	0.013
Oxalacetate	Heated	0.138	
Pyruvate	Heated	0.003	
None	Normal	0.003	
Oxalacetate plus pyruvate	Normal	0.072	0.002
Glutamate	Normal	0.003	0
Glutamate plus pyruvate	Normal	0.003	0

* All substrates 0.02M concentration.

Approximately 1.0 mg. nitrogen per ml.

DISCUSSION

Adaptive Enzymes: The simultaneous adaption technique is based on the fact that exposure of an organism to an adaptively oxidizable substrate also induces the formation of enzymes capable of oxidizing the intermediates of this substrate. That is, if a compound proceeds through the reaction chain A-B-C-D-etc. exposure of the organism to compound A, which is oxidized by an adaptive enzyme, will simultaneously cause adaption to compounds B-C-D; if the organism is exposed to compound B it will be adapted to C and D but not A. Similarly cells which are not adapted to compound A should be adapted to compounds B, C and D.

In this work although possible adaptive curves were shown for glutamate and asparagine, oxidation of the postulated intermediates, alpha-ketoglutarate and succinate was not of an adaptive nature regardless of the media on which the cells were grown. These results indicate the possibility of a tri-or dicarboxylic acid cycle. If a TCA cycle is present alpha-ketoglutarate and succinate oxidation would not be under adaptive control since these compounds would lie on the main pathway of metabolism and enzymes for their oxidation would be continuously present. Oxidation of glutamate would require only oxidative deaminati on to alpha-ketoglutarate, similarly deamidation

and transamination of asparagine would yield oxalacetate. Other workers have demonstrated that brucellae possess enzymes to carry out these processes (6, pp. 98-102).

If a dicarboxylic acid cycle is present in brucellae essentially the same results would be obtained with the exception that cells which were grown on lactate as a carbon source should not be adapted to alpha-ketoglutarate. However if the reaction alpha-ketoglutarate-succinate were freely reversible the cells might be adapted to alpha-ketoglutarate. Also since glutamic acid is essential for normal growth it must by synthesized, probably with alpha-ketoglutarate as an intermediate. If this is the pathway of glutamate synthesis then cells should be continuously adapted to alpha-ketoglutarate and glutamate. Both asparagine and glutamate contain an available nitrogen source and the apparently adaptive curves obtained with these compounds might possibly be explained a being due to cell growth and multiplication. However, consideration of data obtained from other work in this laboratory (16, pp. 8-11) shows that even in a complete synthetic G-W medium the generation time for Brucella abortus, strain 19 is 9.3 hours. Since the observation of oxygen uptake with asparagine and glutamate cover a period of 4.5 hours and the oxygen uptake is

increased tenfold during this period the results obtained cannot be wholly explained by growth.

Hydrogen Ion and Concentration Effects: Assuming the theory that the cell wall is permeable only to the unionized molecule of certain acidic organic compounds is correct, it is possible to calculate what effect lowering the pH should have on the effective substrate concentration. Succinic acid provides a good material to use as an example. Use of the formula $pH = pK_a + \log\frac{salt}{acid}$ and the value of 4.18 for the pK_a of succinic acid shows the ratio of salt: acid is 416 at pH 6.8 and 21 at pH 5.5. Therefore lowering the pH from 6.8 to 5.5 has the effect of increasing the substrate concentration approximately 20 times. Approximately the same holds true for the other members of the citric acid cycle. Apparently citrate is either not oxidized or the citrate does not penetrate the cell regardless of its condition of ionization.

Allowing the pH to remain at 6.8 and increasing the substrate concentration has the same effect, with regard to the unionized acid concentration, as lowering the pH. Succinate in a concentration of 0.167M (the concentration at which maximum oxidation rate was attained) at pH 6.8 has an unionized acid concentration of approximately 0.0004M and showed $Q_{O2}(N)$ rate of 636(120). Succinic acid in a concentration of 0.0033M and a pH of 5.5 has

an unionized acid concentration of 0.0002M and a $Q_{O_2}(N)$ rate which has shown a maximum of 622(98). These are the expected results if the original assumption is correct.

The oxidation rates attained by these methods are probably not the maximum possible. The lower pH is not the optimum for the enzymes of the TCA cycle while osmotic effects may reduce the rate of oxidation when a high substrate concentration is used. In addition a high substrate concentration may result in an inhibition or stimulation if an impurity is present which is inhibitory or oxidizable. However the results obtained with lowered pH and increased substrate concentration show that glutamate may be oxidized through the lower intermediates in the Kreb's citric acid cycle and illustrate the importance of consideration of the effects due to cell permeability.

Inhibitors:Malonate: Demonstration of malonate inhibition in bacteria has not generally been successful. Barron and co-workers (9, p. 142) found that at pH 7.0 malonate did not cause inhibition of succinate and acetate oxidation using C. creatinovorans. However, at pH 5.3, 0.04M malonate caused complete inhibition. From these results he concluded that permeability to malonate is a function of pH. Pardee and Potter (33, p. 244-249) using rat tissue homogenates found that a concentration

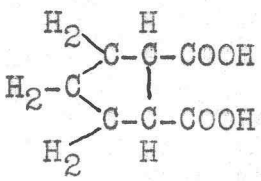
of greater than 0.04M malonate inhibited oxalacetate oxidation in addition to succinate. This oxalacetate inhibition was attributed to the formation of a magnesium malonate complex. It is possible that high concentration of malonate are necessary to demonstrate inhibition with brucellae because the cell wall is only permeable to the unionized molecule. If this is true then at pH 5.5 and a 10.1 total malonate: total succinate ratio, the ratio of the unionized molecule is 1:2.4 due to the greater acidity of the malonate. If the latter figure is the effective inhibitor substrate ratio then the inhibition of 26 percent which was obtained is close to the result which would be expected. The high substrate inhibitor ratio necessary to produce significant inhibition in the frozen and thawed cells may indicate that quite a large percentage of the cells are still impermeable to malonate.

Arsenious oxide: The use of very low concentrations of arsenious oxide causes marked inhibition. It is not possible to deduce from this data whether glutamate is oxidized via alpha-ketoglutarate since succinate is also inhibited markedly.

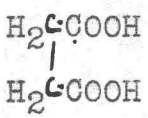
Formaldehyde: Formaldehyde inhibition studies were not carried beyond the preliminary stage, however the results obtained indicated the oxidation of

alpha-ketoglutarate via succinate. Formaldehyde might prove useful in inhibitor studies where permeability effects rule out the use of malonate.

CPC: The results with CPC indicated that the normally rather slow rate of asparagine oxidation may be due to low permeability of the cell wall to this material. The inhibition effects, which were most marked at pH 5.5 confirm the results obtained by Seaman and Houlihan using homogenates of T. geleii (34, p. 439). Examination of the structural formula of CPC shows the arrangement of the carboxyl groups to be very similar to those in succinate, and it might therefore be expected to act as a competitive inhibitor. The use of CPC is



CPC



Succinic acid

open to the same criticism as malonate, namely that the concentration used probably causes inhibition of other reactions in the cell. This is indicated by the marked inhibition of the endogenous respiration of the cells.

DNP: The use of DNP as an assimilation inhibitor showed that the optimum concentration range for this chemical was very narrow; perhaps the concentration required is that which allows just enough high energy

phosphate to remain in the system to keep the cell functioning. Too low a concentration of DNP caused stimulation of oxidation; too high a concentration caused complete inhibition. The maximum inhibition of substrate assimilation seemed to occur at a concentration of DNP which brought about a slight decrease in the oxidation rate. This optimum concentration was a function of the density of the cell suspension, the hydrogen ion concentration and the substrate used. The complete oxidation of succinate and acetate possibly implies a cyclic oxidative mechanism, since the writer has found no reference to complete oxidation occurring in a biological system by any other process.

Freezing and Thawing: The stimulation of oxidation of TCA intermediates by frozen and thawed cells emphasizes the fact that permeability of the cell wall to the substrate being studied is always an important factor. The results also indicate that glutamic acid deaminase is particularly labile. The lowered activity of the frozen cells toward TCA intermediated may be due to release and consequent dilution of necessary co-factors.

Cell Free Preparations: The preparation of and active cell free extract from brucellae has not previously been reported in the literature. The success in the present work showed alumina grinding to be a method for

obtaining active preparations. Addition of necessary co-factors might improve the activity of these preparations; these co-factors might be the same as those used in cyclophorase preparations.

Citrate formation: The failure to show accumulation of citrate in resting cell suspensions does not necessarily indicate the absence of a citric acid cycle since it is possible that citrate is oxidized before it can accumulate in significant amounts. Also the results obtained while not significant individually show that tests for citrate were always either zero of weakly positive, no negative results were obtained.

SUMMARY AND CONCLUSIONS

The use of adaptive enzyme techniques showed that the enzymes for the oxidation of glutamate and asparagine may be adaptive but that those for the oxidation of alpha-ketoglutarate, succinate and acetate are not. These results indicated the presence of a cyclic oxidative mechanism, possibly the Kreb's citric acid cycle. Members of the TCA cycle are oxidized slowly at pH 6.8 and0.0033M concentration by resting cells. However if the pH is lowered to 5.5 alpha-ketoglutarate, succinate and malate are oxidized more rapidly than glutamate. Fumarate, pyruvate, acetate and oxalacetate were oxidized 94, 80, 77, and 51 per cent, respectively, as fast as glutamate. Citrate was not oxidized. Glutamate oxidation was slightly depressed at the lower pH. Preliminary tests at pH 4.5 showed the cells to be without activity.

Stimulation of oxidation rates at lowered pH has been postulated to be due to the increased permeability of the cell and wall to the unionized molecule. If this theory is correct then an increase in substrate concentration should also lead to an increase in oxidation rates. Testing of various substrates at concentrations greater than that normally used (0.00333M) showed that oxidation rates of alpha-ketoglutarate, succinate, aspartate and glutamine were markedly stimulated, while the effect on

glutamate was only moderate. Apparent oxidation of citrate occurred at 0.0555M with a $Q_{O_2}(N)$ of 108(87).

Removal of permeability effects by freezing and thawing or grinding with alumina was attempted. Frozen and thawed cells oxidized all members of the TCA cycle except citrate. The enzymes responsible for the primary oxidation of glutamate are almost totally destroyed by this treatment. Cell free preparations made by grinding with alumina were very unstable. A preparation was made which gave the following $Q_{O_2}(N)$ values; succinate 114(19) alpha-ketoglutarate 46(19) and citrate 28(19). The oxidation of citrate, which is of particular interest, seems to be significant. Analysis of the supernatant liquid of a resting cell suspension exposed to various substrates showed no appreciable accumulation of citric acid. Monofluoracetate, which is an inhibitor of citrate oxidation, would have been useful at this stage but none was available.

The use of 2,4-dinitrophenol as an assimilation inhibitor yielded the following stoichiometric ratios per mole of substrate oxidized; glutamate, oxygen consumed 2.57 moles; alpha-ketoglutarate, oxygen consumed 3.12: succinate, oxygen consumed 3.37, carbon dioxide evolved 3.56; acetate, oxygen consumed 1.86, carbon dioxide evolved 1.70. These values in the case of succinate and

acetate indicate complete oxidation to carbon dioxide and water. The value for alpha-ketoglutarate is 78 per cent of theoretical, and for glutamate it is 57 per cent.

The effects of several inhibitors were studied. A malonate inhibition of succinate of 26 per cent occurred at pH 5.5 with 0.00333M substrate and a 10:1 inhibitor substrate ratio. The same substrate inhibitor ratio using frozen cells resulted in a 59 per cent inhibition of succinate oxidation. Arsenious oxide, an inhibitor of alpha-ketoglutarate acid oxidation, was also tried. Marked inhibition of glutamate, alpha-ketoglutarate and succinate occurred even when a concentration of 0.0000-325M was used. Formaldehyde, although oxidized slowly by brucella, acts as an inhibitor of alpha-ketoglutarate and succinate at 0.0124M. This concentration of inhibitor decreased the rate of oxidation of the above two compounds 53.5 and 48 per cent, respectively.

The effect of trans 1,2-cyclopentanedicarboxylic acid on the permeability of the cell to various substrates was tested. Asparagine was the only compound whose oxidation was stimulated. At pH 5.5, 0.04M acid inhibited the oxidation of glutamate, alpha-ketoglutarate succinate and aspartate.

The results obtained with the various techniques used indicate the possibility of the presence of a citric acid

cycle in brucella. Evidence for the existence of such a cycle are as follows:

(1) Demonstration that alpha-ketoglutarate, succinate and acetate oxidases are constitutive enzymes.

(2) Oxidation of citric acid by cell free preparation.

(3) Complete oxidation of succinate and acetate.

(4) Oxidation of all members of the citric acid cycle tested.

No results which contraindicated the presence of a citric acid cycle were obtained.

Inhibitor studies show that glutamate is probably oxidized via alpha-ketoglutarate and succinate.

BIBLIOGRAPHY

1. Ajl, Samuel F. Acetic acid oxidation by Escherichia coli and Aerobacter aerogenes. Journal of bacteriology 59:499-507. 1950

2. _____and M.D. Kamen. Studies on the mechanism of acetate oxidation by bacteria. Federation proceedings 9:143-144. 1950.

3. _____and D.T.C. Wong. Studies on the mechanism of acetate oxidation by bacteria. IV. Acetate oxidation by citrate-grown Aerobacter aerogenes studied with radioactive carbon. Journal of bacteriology 61:379-387. 1950

4. _____and M.D. Kamen. Studies on the mechanism of acetate oxidation by Escherichia coli. Journal of biological chemistry 189:845-857. 1951.

5. _____et al. Studies on the mechanism of acetate oxidation by Micrococcus lysodeikticus. Journal of biological chemistry 189:859-867. 1951

6. Altenbern, Robert A. and Riley D. Housewright. Alanine synthesis and carbohydrate oxidation by smooth Brucella abortus. Journal of bacteriology 62:97-105. 1951

7. Anderson, Arthur W. Unpublished data. 1951.

8. Attimonelli, R. The oxidizing action of species of Brucella on the fatty acids. Boll. ist. sieroterap. milan 21:238-240. 1946. (in Chemical abstracts 40:3153. 1946)

9. Barron, E.S. Guzman, M.I. Ardao and M. Hearon. The mechanism of acetate oxidation by Corynebacterium creatinovorans. Archives of biochemistry 29:130-153. 1950.

10. Bernheim, Frederick. Effect of formaldehyde on certain oxidative enzymes. Proceedings of the society of experimental biology and medicine 76:133-134. 1951.

11. Bonner, James and S. C. Wildman. Enzymatic mechanisms in the respiration of spinach leaves. Archives of biochemistry 10:497-518. 1946.

12. Campbell, Jack J.R. and Flora N. Stokes, Tricarboxylic acid cycle in Pseudomonas aeruginosa. Journal of biological chemistry 190:853-858. 1951.

13. Caselli, P. Sull'attivita desaminative delle brucelle. Riv. ist. sieroterap., Ital. (Naples), Sez. I, 22:251-256. 1947.

14. D'Ambrosio, L. La utilizzazione dei monosaccaridi die parte della Brucella abortus. Boll. ist. sieroterap. milan. 22:246-248. 1943.

15. Dickman, S.R. and A.A. Cloutier. Factors affecting the activity of aconitase. Journal of biological chemistry 188:379-388. 1951.

16. Evenson, Margery A. The nutrition of brucellae: Utilization of trace minerals for growth. M.S. thesis. Oregon State College. 1951.

17. Gerhardt, Philipp. Studies on the physiology of Brucella. M.S. thesis. University of Wisconsin. 1947.

18. _____and J.B. Wilson. The nutrition of brucellae: Growth in simple chemically defined media. Journal of bacteriology 56:17-24. 1948.

19. _____The metabolism of amino acids and related compounds by brucellae. PhD thesis. University of Wisconsin. 1949.

20. _____L.A. Tucker and J.B. Wilson. The nutrition of brucellae: Utilization of single amino acids for growth. Journal of bacteriology 59:777-782. 1950.

21. _____, H.B. Levine and J.B. Wilson. The oxidative disimilation of amino acids and related compounds by Brucella abortus. Journal of bacteriology 60:459-467. 1950.

22. Johnson, M.J. Isolation and properties of a pure yeast polypeptidase. Journal of biological chemistry 137:575-586.1941.

23. Karlsson, J.L. and H.A. Barkerl. Evidence against the occurence of a tricarboxylic acid cycle in Azotobacter agilis. Journal of biological chemistry 175:913-921. 1948.

24. Kien-Hun, B. and W. Kan. Uber die methlenblaureduktion durch Bact. abort. infectiosi Bang und Bact. melitensis. Z. Hyg. Infekt. 117:399-402. 1935.

25. Levine, H.B. and E.D. Garber. Detection of rough disociants of Pasteurells pestis with tetrazolium chloride. Journal of bacteriology 60:508. 1950.

26. Lineweaver, Hans and D. Burke. The determination of enzyme disociation constants. Journal of the American chemical society 56:658-666. 1934.

27. Mannozzi-torini, M. and R. Vendramini. Studi sul metabolismo delle brucelle. 1. L'ossidazione degli aminoacidi das parte della Brucella abortus. Boll. ist. sieroterap. milan. 20:4-12. 1941.

28. _____and _____. Studi sul metabolismo delle brucelle. 2. Ulteriori recerche sulls ossidazione degli aminoacidi da parte della Brucella abortus. Boll. ist. sieroterap. milan. 20:13-18. 1941.

29. McAlpine, J.A., W.N. Plastridge and G.D. Brigham. Studies on the metabolism of the abortus-melitensis group. 5. Factors influencing sugar utilization. Journal of infectious diseases 45:485-489. 1929.

30. McCullough, N.B. and L.A. Dick. Growth of Brucella in a simple chemically defined medium. Proceedings of the society of experimental biology and medicine 52:310-311. 1942.

31. McIlwain, H. Preparation of cell-free bacterial extracts with powdered alumina. Journal of general microbiology 2:288-291. 1948.

32. Novelli, G. David and F. lipman. The catalytic function of coenzyme A in citric acid synthesis. Journal of biological chemistry 182: 213-228. 1950.

33. Pardee, Arthur B. and V.R. Potter. Malonate inhibition of oxidations in the Kreb's tricarboxylic acid cycle. Journal of biological chemistry 178:241-250. 1949.

34. Seaman, Gerald R. and R.K. Houlihan. trans-1,2-Cyclopentanedicarboxylic acid a succinic acid analog affecting the permeability of the cell membrane. Archives of biochemistry 26:436-441. 1950.

35. Sleeper, B.P. and R.Y. Stanier. Bacterial oxidation of aromatic compounds. I. Adaptive patterns. Journal of bacteriology 59:117-127. 1950.

36. _____, M. Tsuchida and R. Y. Stanier. Bacterial oxidation of aromatic compounds. II. Preparation of enzymatically active dried cells and the influence thereon of prior patterns of adaption. Journal of bacteriology 59:129-133. 1950.

37. Stanier, R.Y. Simultaneous adaption: a new technique for the study of metabolic pathways. Journal of bacteriology 54:339-348. 1947.

38. _____, Personal communication. 1951

39. Steinbach, K.F. Investigations into bacterial respiration with special reference to hydrogen donators. Thesis. University of Auerich. (Printed by J. Hagmann, Substrasse 47, Zuerich). 1940.

40. Stern, Joseph R. and Severo Ochoa. Enzymatic synthesis of citric acid. Federation proceedings 9:234-235. 1950.

41. Umbreit, W.W., R.H. Burris and J.F. Stauffer. Manometric techniques and tissure metabolism. Minneapolis, Minn., Burgess, 1949. 227p.

42. Werkman, C.H. and P.W. Wilson. Bacterial physiology. New York, Academic Press Inc., 1951. 707p.

43. Wilson, Joseph B. Personal communication. 1951.

44. Zobell, C.E. and K.F. Meyer. Metabolism studies on the Brucella group. IX. Physiochemical requirements in synthetic medium. Journal of infectious diseases 51:361-381. 1932.