INVESTIGATIONS INTO THE GLUTAMIC ACID METABOLISM OF BACTERIUM TULARENSE

by

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INTRODUCTION

Glutamic acid holds a key position in the metabolism of the cell, because this amino acid is involved in many cellular reactions. Two of these are of unique significance to the organism, for together they help transform an inorganic, non-physiological substance-ammonia- into the most essential cellular building blocs-amino acids. These changes are brought about in the cell by two specific enzyme systems, glutamic acid dehydrogenase and the transaminases. The dehydrogenase performs the function of an ammonia "pick up service", that is, it reduces the imino acid, formed by the coupling of ammonia with alpha ketoglutaric acid, to produce glutamic acid; the transaminases, on the other hand, function as transfer agents. Transferring the amino group from glutamic acid to other keto acids, they bring about the synthesis of the corresponding amino acids. The limiting factors of these reactions in animal tissues have been discussed by Adler (1938). He pointed out that alpha ketoglutarate should, as normal intermediate in carbon catabolism, always be present in the cell; the dehydrogenase has been found to be present in all tissues studied, including the brain, where it is the only amino-acid-synthesizing enzyme. Glutamic acid synthesis will therefore proceed in all tissues at all times. Synthesis of other amino acids will be dependent upon the presence of their corresponding keto acids. A shortage of these keto acids would lead to a shortage of the amino acids and to a limitation of the

reaction. Such amino acids would then have to be supplied exogenously, as is the case with all "essential" amino acids.

Glutamic acid dissimilation, though first observed in animal tissues, (Knoop, 1910) has since been found to occur in microorganisms (See historical section for review). Extensive studies in E. coli and yeast have shown the presence of a specific glutamic acid dehydrogenase (Euler, 1937; Adler, 1938). These pioneer studies led to the investigation of many other bacterial species for the presence of this enzyme system. In the present study the dissimilation of glutamic acid by B. tularense, a pathogenic microorganism, will be elucidated.

HISTORICAL

The oxidation of glutamic acid by bacteria was no accidental discovery but the outcome of a carefully planned search for one of the enzymes catalyzing this reaction. Adler et al (1938) investigating E. coli and yeast for the presence of glutamic acid dehydrogenase found the apo-enzyme present in both microorganisms. The apo-enzyme had previously been studied in animal tissues (Euler et al, 1938; Dewan, 1938) where, combined with coenzyme I or II the holoenzyme catalyzed the oxidation of glutamic acid to alpha ketoglutarate and ammonia; or, reversibly, synthesized glutamic acid from these substances.

The apodehydrogenase from E. coli was obtained by grinding previously thawed and frozen cells with quartz, and extracting the cell debris with phosphate buffer. In the presence of coenzyme II and L-glutamic acid, this preparation reduced methylene blue anaerobically.

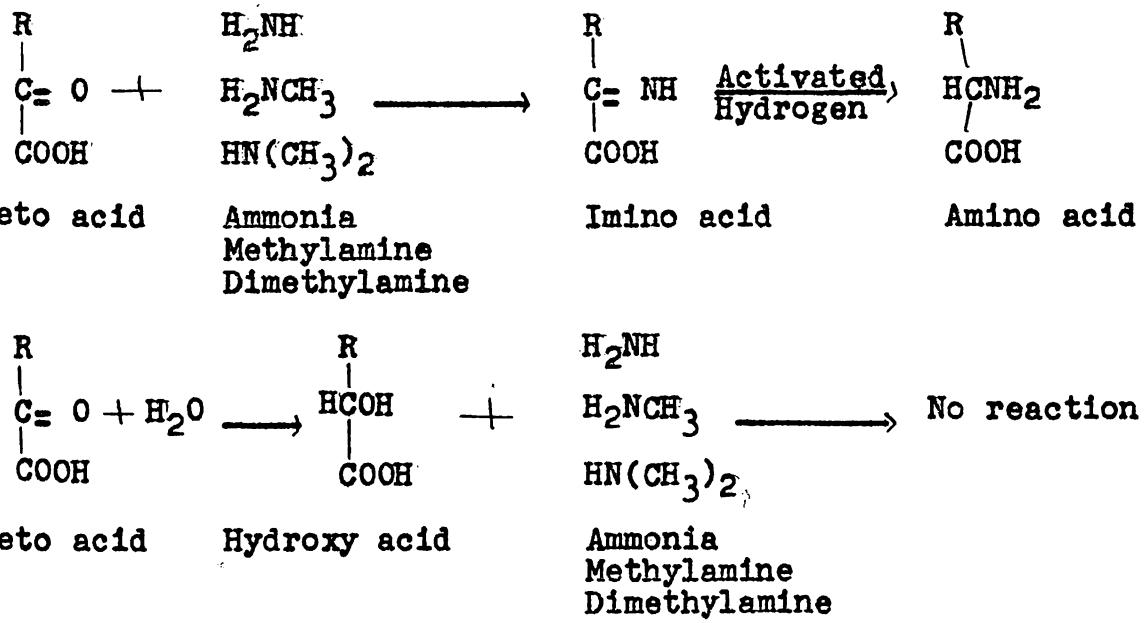
The reaction could also be followed spectophotometrically by measuring increase or decrease of optical density at 340 mu, caused by the reduction or oxidation of coenzyme II. Reduction of the cofactor took place during the dissimilation of glutamic acid, while oxidation of the reduced coenzyme occurred during glutamic acid synthesis. By addition of one of the end products (alpha ketoglutarate or ammonia) to the initial enzyme substrate mixture, reduction of coenzyme and disappearance of glutamic acid were inhibited, indicating the reversibility of the reaction.

The apodehydrogenase derived from E. coli and animal tissues proved to be similar in all respects, except for their cofactor requirement; the bacterial apoenzyme requires coenzyme II, whereas the apoenzyme derived from all animal tissues, except liver, requires coenzyme I (Dewan, 1938; Copenhaver et al., 1950). Liver apodehydrogenase requires either coenzyme I or II. Euler et al. (1938) considered the possibility that liver contains two different glutamic dehydrogenases.

In E. coli, as in animal tissue, the reversible action of glutamic acid dehydrogenase is of greatest significance to the cell, for it leads to the synthesis of three metabolic intermediates: glutamic acid, functioning as amino donor in the synthesis of other amino acids; alpha ketoglutarate, a component of the tricarboxylic acid cycle, a metabolic path leading to the disintegration of the carbon skeleton and yielding large amounts of energy; and ammonia, functioning both in amino acid and amide synthesis.

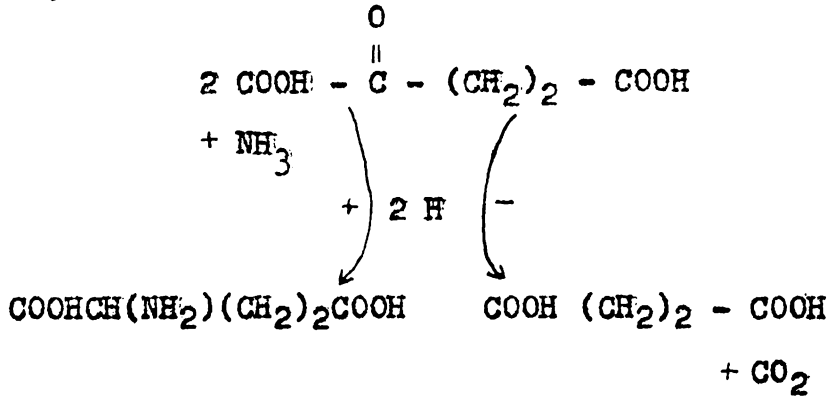
The work of Adler with artificially reconstituted enzyme systems helped clarify a further point which, with less purified enzyme preparations, is often obscured by other reactions occurring simultaneously: that of the mechanism of the reaction. Two possibilities existed for the formation of amino acids from keto acids and ammonia: the synthesis could proceed either through the imino or the hydroxy acid. On the basis of chemical models, described below, Knoop showed the imino acid to be the intermediate.

Knoop and Oesterlin (1925) found that, in the presence of activated hydrogen, a mixture of ammonia and keto acid resulted in the formation of the corresponding amino acid. If both methyl- and dimethylamine could substitute for ammonia in this reaction, then the path through the hydroxy acid could not be excluded; however, if ammonia and methylamine were the only bases which could take part in the condensation, then the imino acid would have been the only possible intermediate. This turned out to be true. The graphic representation of these reactions is given below.



Adler's work verified that of Knoop on the biological level. In this case reduced coenzyme II served as hydrogen donor to the iminoglutaric acid, and L-glutamic dehydrogenase catalyzed the synthesis of L-glutamic acid. In the reverse reaction, iminoglutaric acid disintegrated spontaneously to alphaketoglutaric acid and ammonia.

It was expected that in whole cells, be they of animal or bacterial origin, glutamic acid oxidation would be accompanied by reduction, and glutamic acid synthesis by oxidation of other cellular systems. Krebs and Cohen (1939) studying amination in surviving tissue slices, found that the reductive amination of alpha ketoglutarate by ammonia resulted not only in glutamic acid formation, but also in accumulation of succinic acid and production of carbon dioxide. To account for these findings they postulated a two step reaction: formation of iminoglutaric acid from one mole of alpha ketoglutarate and ammonia, followed by reduction of the imino acid to glutamic acid by a second mole of alpha ketoglutarate, which itself underwent oxidative decarboxylation to succinic acid and carbon dioxide. In the process, alpha ketoglutarate acted as hydrogen donor to the imino acid. No other hydrogen donor could be substituted in this system. These are the reactions:

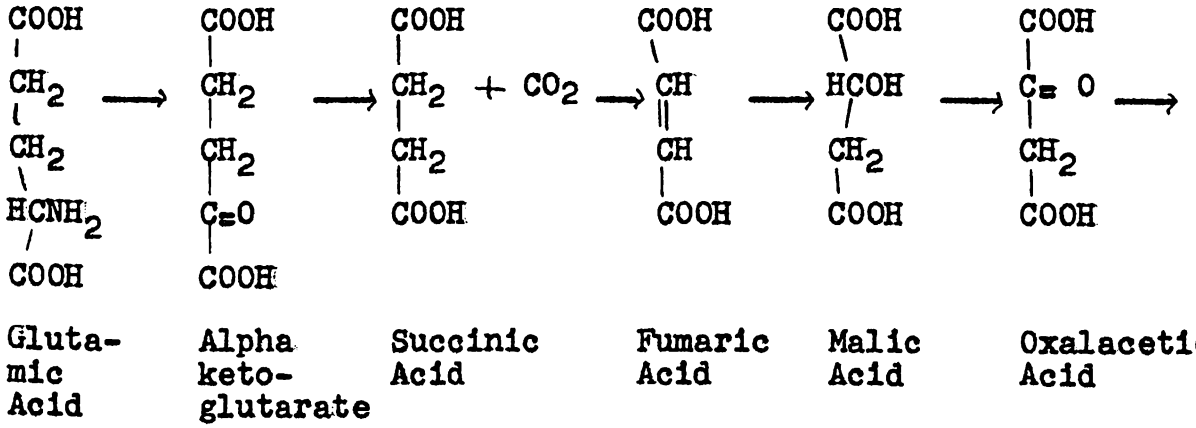


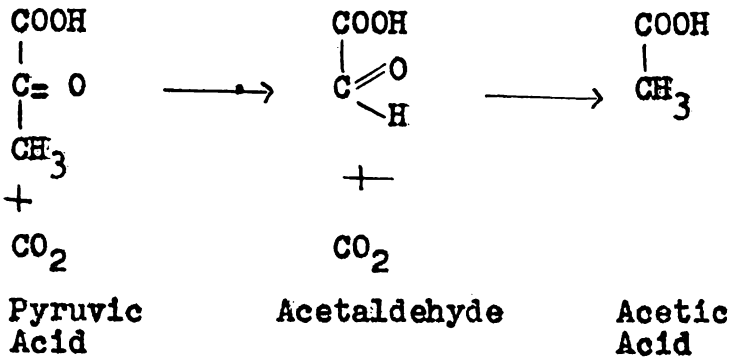
Other dehydrogenase systems which can supply H for the synthesis of glutamic acid in animal tissues are the following: oxidation of beta hydroxy-butyric acid to butyric acid (Dewan, 1938); oxidation of isocitric acid to alpha ketoglutarate (Adler et al., 1939); oxidation of L-malate to oxaloacetate

(Krebs et al., 1948). The reverse reaction, that is oxidation of glutamic acid leading to reduction of other cellular systems, has also been studied. Dewan (1939) using a pig heart preparation was able to link glutamic acid oxidation to the reduction of both pyruvate and oxalacetate. This same oxidoreduction system is present in N. gonorrhoeae (Tonhazy and Pelczar, 1953).

Bacterial cells oxidize glutamic acid in a variety of ways. As stated by Stephenson (1949): "Bacterial deamination proceeds by several methods which differ according to the enzyme make-up of the organism in question and to the condition prevailing in the medium."

Using resting cell suspensions of H. parainfluenzae, Klein (1940) showed that glutamic acid was deaminated and oxidized beyond alpha ketoglutarate, by quantitative measurements of ammonia, carbon dioxide and oxygen. The ratio moles of oxygen used to moles of substrate employed is a measure of the reaction. The ratio indicated that acetic acid might be the end product. This acid was indeed found and identified. The path of the reaction was believed to be the following:





Pyruvic Acid — Acetaldehyde — Acetic Acid

Each proposed intermediate was oxidized by the cell suspension to acetic acid. Additional evidence for this path through succinic acid was found in the fact that malonic acid, which inhibits the oxidation of succinic, also inhibited the oxidation of glutamic acid. The oxidation of glutamic, malic, and acetaldehyde was dependent on the presence of coenzyme I or II.

Resting cell suspensions of N. gonorrhoeae appear to dissimilate glutamic acid along the same metabolic path as H. parainfluenzae (Tonhazy and Pelczar, 1953). Some of the methods used to support this belief were similar to those used by Klein: quantitative measurements of ammonia, carbon dioxide and oxygen; isolation of acetic acid as the end product of the overall reaction; and oxidation by the cell suspensions of the proposed intermediates to acetic acid. Malonate and arsenite inhibited the oxidation of L and D-glutamic acid. Additions of the individual components of the tricarboxylic acid cycle to L-glutamic acid and washed cells increased the Q_{O_2} (N) (microliters oxygen/mgmN/hour) at maximum rate from 73 (glutamic acid alone) to 218 (glutamic acid and intermediate).

This presents good evidence that the cycle is involved in these reactions. The oxidation of D-glutamic acid was similarly stimulated. A conclusion as to the mechanism by which D-glutamic acid was converted to alpha ketoglutarate could not be reached. It could occur either by conversion to the L-form due to the presence of racemase, or by the action of D-amino oxidase.

Synthesis of glutamic acid by N. gonorrhoeae took place through a strong glutamic-aspartic transaminase system. In whole cells both the transaminase and the oxidative system were active simultaneously.

Of 22 amino acids tested only D- and L- glutamic acid were oxidized at an appreciable rate.

Resting cells of B. abortus dissimilate glutamic acid at an appreciable rate (Gerhardt et al., 1950) by oxidative deamination. Although quantitative measurements of oxygen uptake and ammonia and carbon dioxide production were made, no elucidation of the metabolic path was attempted. The data presented indicate that complete oxidation of glutamic acid did not take place. The theoretical oxygen uptake, calculated on the basis that 4.5 moles of oxygen are used per mole of glutamic acid disappearing, was not achieved. No search for intermediates or end products was made.

A path for glutamic acid utilization by this genus-Brucella- was found two years later by Cameron and his co-workers (1952). Utilizing glutamic acid as a substrate,

resting cell suspensions synthesized ornithine, arginine, and citrulline. The process was followed and intermediates were identified by chromatogramming the cell-free supernatant after various periods of incubation. When arginine was used as the substrate, ornithine and alanine were formed. The two species which could carry out these reactions were B. abortus and B. melitensis. These organisms seem to possess the enzyme systems associated with the ornithine cycle. Similarly, this cycle was found to operate in growing cultures of B. subtilis (Wiame, 1951; Wiame, Storck and Bourgeois, 1953). Two members of the cycle, arginine and ornithine, as well as proline and glutamic and aspartic acids served as N source for growth, when added singly to a defined medium.

Using the method of simultaneous adaptation, it was found that all the amino acids were intermediates in the synthesis of glutamic acid; they could not be used directly, without being transformed. The syntheses of glutamic acid were irreversible. The path from arginine to glutamic acid led through proline and was, of course, the reverse of that demonstrated in resting cell suspensions of B. abortus, as described above. A vigorous aspartic-glutamic transaminase system could be demonstrated, in which aspartic acid functioned as the sole amino donor. The carbon skeleton could be supplied by either alpha ketoglutarate or any other member of the citric acid cycle. The suggestion was made that the primary function of this cycle in B. subtilis is to supply intermediates for amino acid metabolism rather than to serve as an energy

trapping mechanism. Under the conditions of the experiment, the de-aminase system was not active in the synthesis of glutamic acid, since B. subtilis could not utilize ammonium ions as nitrogen source.

An extensive study of amino acid metabolism, glutamic acid in particular, was carried out by Jebb and Tomlinson on H. pertussis (1951). Resting cell suspensions do not ferment or oxidize carbohydrates. It was of interest, therefore, to investigate if, and what amino acids could be oxidized to meet the energy requirement of the cells. Among all the amino acids tested, glutamic acid was the most rapidly metabolized, as measured both by oxygen uptake and ammonia production. The ratio of oxygen to ammonia, mole by mole, was larger than 1:2, which indicated that glutamic acid was oxidized beyond alpha ketoglutarate; complete oxidation to carbon dioxide and water did not take place in the absence of inhibitors, since only 80 per cent of the theoretical uptake of oxygen occurred. In the presence of 2,4 dinotrophenol, a known inhibitor of assimilation, oxygen uptake increased to yield the amount consistent with complete oxidation.

With arsenite as inhibitor, oxidation was stopped at the level of alpha ketoglutarate. This intermediate was isolated and identified as the phenylhydrazone. Since alpha ketoglutarate is a known component of the citric acid cycle, it was of importance to find out if other members of the cycle could also be oxidized by the cells. If the rates of oxidation of these

metabolites and glutamic acid were similar, then they might well be intermediates in the oxidation of the amino acid. Unfortunately, alpha ketoglutarate, pyruvate and oxalacetate were oxidized slowly under the experimental conditions used. Most probably the lack of activity could be attributed to slow diffusion of the substrates into the cell due to impermeability of the cell wall. No attempt was made to clarify this point, however, leaving the question of the metabolic route for glutamic acid open. To test if growing cultures of H. pertussis could attack glutamic acid as vigorously as resting cell suspensions, cells were grown in a chemically defined medium containing glutamic acid as the only carbon and nitrogen source. Growth in this medium proved to be almost as dense as that in the control, which contained casamino acids. This experiment proved clearly that glutamic acid could serve as the principal source of energy for these organisms.

Glutamic acid synthesis of another pathogen, B. anthracis, has been studied extensively by Housewright et al. (1950). This organism produces an active transaminase, which catalyzes the exchange of amino groups between aspartic and alpha ketoglutaric acids to form L-glutamic acid.

Resting cell suspensions of B. tularense dissimilate glutamic acid more rapidly than any other amino acid tested. These observations, made by Berger (1950), initiated studies of the glutamic acid metabolism of this organism, which form the basis of this dissertation. Both resting cell suspensions

and cell-free extracts oxidize glutamic acid to alpha ketoglutarate; aerobically this acid undergoes further oxidation, probably through succinic and citric acid; anaerobically the reaction occurs only in the presence of an electron acceptor and does not seem to proceed beyond alpha ketoglutarate.

These conclusions are based on inhibition experiments as well as on the isolation and identification of alpha ketoglutarate from a suspension oxidizing glutamic acid. The experiments are described in the following sections.

MATERIALS AND METHODS

I. Organisms.

A. B. tularense.

The organism used throughout this study was a fully virulent strain of B. tularense SMRB 9, originally received from Dr. Cora Downs, Virus Laboratory, University of Kansas. Stock cultures were maintained on cysteine-blood-agar slants and were transferred monthly from slant to slant.

Broth cultures were prepared by washing the growth off a stock slant with 10 ml of casein-decamin medium. Daily broth to broth transfers were made, using a 5 per cent inoculum. These cultures were incubated at 37 C on a reciprocating platform shaker, having a 4.5 inch stroke and shaking at a speed of 96 excursions per minute. The tubes were held in racks at an angle of 25° from the horizontal. This facilitated aeration, and therefore growth, of the cultures.

A culture newly transferred from blood-cysteine-agar slant to broth showed a definite lag in growth. Four to 9 consecutive subcultures in broth were usually needed to eliminate this lag and to produce optimum growth. Cells derived from optimally growing cultures only were used in all experiments.

A record was kept of the number of serial transfers in broth each culture had undergone before it was used experimentally. No difference in enzymatic activity due to the

difference in the number of transfers could be demonstrated, provided the cells were derived from optimally growing cultures.

B. E. coli.

A culture of E. coli, strain 4157, was obtained from the American Type Culture Collection. This culture had been found to possess vigorous glutamic decarboxylase activity. The culture was maintained on Difco stock agar and was transferred once every 6 weeks.

II. Media.

A. Casein-Decamin broth.

A subculture medium for B. tularense, described by Mills et al. (1949), was used for growth of all broth cultures. This broth contained the following components:

Sodium chloride	10 gm
Glucose	10
Casein hydrolysate	200 ml (representing 20 gm original casein)
Decamin*	3
L-cysteine HCl	2
Vitamin B_1	0.2 mgm
Magnesium sulfate	1×10^{-4}M(final)
Calcium chloride	2×10^{-4}M(")
Ferrous sulfate	1×10^{-7}M(")
Manganese sulfate	1×10^{-7}M(")
Phosphate buffer	2.25×10^{-2}M(final, mixture of 1 M K_2HPO_4 and KH_2PO_4, pH 6.5)
Distilled water to 1 l	

Components were adjusted to pH 6.5 with KOH before they were made up to final volume.

The broth was autoclaved for 13 minutes at 120 lbs pressure.

* Product of Vi Co., 415 Scott Str., Chicago, Ill.

B. Medium for maintenance and growth of E. coli.

Stock culture medium:

Bacto-Beef Extract	3 gm
Peptone	5
Agar	15
Sodium chloride	5
Distilled water to 1.0	1

This medium was autoclaved for 20 minutes at 120 lbs pressure. Liquid cultures of E. coli were grown in medium shown to produce maximum decarboxylase activity, as recommended by Umbreit and Gunsalus (1945).

Peptic ase	1%
Yeast Extract	0.1%
K_2HPO_4	0.25%
Glucose	1%

Adjusted to pH 7.0 with NaOH.

750 ml of broth were dispensed into 1 l Erlenmeyer flasks. These were plugged with cotton and autoclaved for 30 minutes at 120 lbs pressure.

III. Growth experiments with B. tularense.

A. Equipment.

Growth was recorded as a measure of increase in optical density, using the Evelyn Photoelectric Colorimeter to record the change. All tubes used for growth experiments had been previously calibrated for uniform transmission at 650 mu, the wavelength used for reading optical density. Each tube contained 10 ml of sterile casein-decamin broth.

B. Preparation of cultures.

Tubes containing 10 ml of sterile broth were inoculated with 0.5 ml of a 12-hour culture. The optical density of these tubes was measured immediately after inoculation, using an uninoculated broth tube as a blank. All tubes were then incubated and treated as described under section IA.

IV. Manometric techniques.

All manometric glass ware was calibrated and constants were determined and calculated according to the method of Umbreit et al. (1949).

A. Equipment.

Barcroft-Warburg type of respirometers were used for all experiments. Most experiments were performed with vessels having two side arms, but occasionally vessels having one or three side arms were used. The flasks with three side arms have one arm modified to form a double sac.

For oxygen determinations KOH was used in the center well; for carbon dioxide determinations the alkali was replaced by water. A fluted filter paper, one inch square, was placed into the center well of each vessel. The total volumes of the vessels varied and will be recorded for each experiment. At the beginning of the study, the components were added to the vessels with 1 ml pipettes, whose tips had been drawn out into a capillary. Later on, 1 ml Tuberculin and 2 ml syringes, fitted with 16 - 18 gauge needles, were used exclusively.

A temperature equilibration period preceded each test run; also all vessels were checked for leaks before the first reading was made. In all experiments the first reading was taken 10 minutes after the substrate or enzyme solution was tipped. Reading of the manometers was usually continued for two to three hours, until the oxygen uptake ceased. In some experiments the manometers were read for short intervals only. The flasks were shaken at the rate of 120 oscillations per minute.

During the early experiments all manometers contained Brodie's solution (Sodium chloride- 23 gm; Sodium choleate- 5 gm; Evan's Blue- 100 mgm; made up to 500 ml with distilled water); subsequently this was replaced with Krebs' fluid (Potassium bromide- 44 gm; Tritone X-100* 0.3 gm; Evan's Blue- 0.3 gm; made up to 1 l with distilled water). The density of both solutions was measured with a pycnometer.

All Warburg vessels were put through the following washing procedure: a twelve hour soak in Purina Disinfectant was followed by immersion in gasoline to remove all grease, a thorough rinse in tap water, a subsequent soak in Chromic acid cleaning solution for 6 hours, followed by 6 washings in tap and 6 in distilled water. The manometer joints which fit into the Warburg cups were disinfected by being wrapped into cotton, soaked with disinfectant.

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* Non-ionic detergent produced by Rohm and Haas, Philadelphia, Penn.

B. Non-proliferating cell suspensions.

Mass cultures were started by making a 5 per cent inoculum of an optimally growing culture into one or more sterile broth tubes, and allowing these to grow at 37 C on the reciprocating shaker for 5 hours. At that time the cultures are in the log phase. These cultures were then used as inoculum for 100 to 500 ml of casein-decamin medium. To insure adequate aeration, the 100 ml lots were usually grown in 1 l Erlenmeyers and the 500 ml lots in 5 l Fernbach flasks. The flasks were incubated at 37 C and shaken on the same shaker as the tubes.

After a specified time of incubation, ranging from 4 to 24 hours and stated for each experiment, the cultures were centrifuged in covered cellulose-nitrate tubes in a Servall Angle Centrifuge at 5000 RPM. The cells were washed with .45 per cent saline, recentrifuged, resuspended in 0.1 M phosphate buffer (mixture of KH_2PO_4 and K_2HPO_4) pH 5.8, and aerated by shaking at 37 C for half an hour. In several experiments the cells were aerated at 4 C instead of at 37 C, and in some experiments the period of incubation was increased. Finally the cells were recentrifuged, washed once more in .45 per cent saline and were resuspended in saline. They were used immediately.

The dry weight of each suspension was determined by pipetting an aliquot into a previously tared 10 ml Erlenmeyer flask, drying it at 100 to 110 C for 24 hours, and after cooling in a dessicator, reweighing the flask. The weight of a saline blank was always determined and subtracted from

the dry weight of the test suspension.

Each suspension was checked for purity of the bacterial culture by gram staining.

C. Non-proliferating cell suspensions of E. coli.

To obtain cell suspensions with maximum decarboxylase activity, the methods developed by Umbreit and Gunsalus (1945) were followed.

From a stock agar slant a transfer was made into broth, and the culture incubated for 12 hours at 37 C. One drop of this culture was then transferred to another sterile broth tube and incubated without aeration for 8 hours at 26 C. From this tube a 4 per cent inoculum was made into one or two 1 l Erlenmeyer flasks containing 750 ml of medium. The cells were allowed to incubate at 26 C for 39 hours. This length of incubation is necessary to destroy lysine decarboxylase, which is as vigorous as glutamic decarboxylase in young cultures. After this incubation, the cells were centrifuged down in an International Refrigerated Centrifuge, washed once with 0.9 per cent saline, and resuspended in 0.2 M acetate buffer, pH 3.8. This suspension was kept in the refrigerator until used. Cells stored in this way kept their activity for many weeks.

In preliminary experiments an attempt was made to obtain active dried cell preparations by dehydrating the cells in vacuo over calcium chloride or concentrated sulfuric acid.

In another trial the cells were shell frozen in dry ice and ethyl alcohol and then lyophilized. None of these preparations showed decarboxylase activity.

V. Preparation of cell-free extracts of B. tularense.*

A. Sonic disintegration.

Cell suspensions were disintegrated in a Raytheon sonic oscillator at 9 Kilocycles sec^{-1} and 160 to 175 plate volts for 45 minutes. The suspension was held in a closed, cellulose nitrate tube which fitted snugly into the cup of the oscillator. To facilitate contact between the oscillating diaphragm of the cup and the tube, a small amount of ice water was used to fill the space between the cup and the tube. The water was most effective in transmitting the oscillations, as well as keeping the cells cool. The cup itself was cooled by constant circulation of ice water through its cooling system. The temperature of the cell suspension was kept below 20 C.

Cells were grown as described under IV B. Cells were washed twice with .45 per cent saline and were then resuspended in 0.1 M phosphate buffer, pH 7.0 (K_2HPO_4 and KH_2PO_4). Usually cells derived from 500 ml of culture fluid were resuspended and disintegrated in 30 ml of buffer, concentrating them 17 times.

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* Many thanks are due to the staff of the Virus Laboratory for making the Raytheon oscillator available to us.

After disintegration, the extract was centrifuged for 20 minutes at 5000 RPM in the Servall Angle Centrifuge, to sediment the cellular debris and the remaining whole cells. The supernatant was then stored in the freezing compartment of the refrigerator and thawed just before use.

Some extracts were dialyzed. The conditions of the dialysis will be described in the Experimental section.

B. Disintegration by freezing and thawing.

During the course of the investigation the production of cell-free preparations by freezing and thawing was undertaken. A heavy cell suspension was pipetted into the bottom of a small celluloid tube and this was immersed alternately in a mixture of dry ice-acetone and in cold water. In this way the cells were frozen and thawed in succession. It was soon apparent that this process was more tedious and less efficient than disintegration of the cells by sonic oscillations and it was therefore discontinued.

VI. Chromatography.

Whatman paper #10 was employed. The materials to be tested were deposited in discreet spots in 0.01 and 0.02 ml amounts, 4 to 5 cm apart, along the width of the paper, 2.5 to 3.0 cm from the bottom edge. The sheet was carefully rolled until the edges of the shorter sides met. These edges were then clipped together at the bottom and top, transforming the sheet into a cylinder. Ascending chromatography was used. The

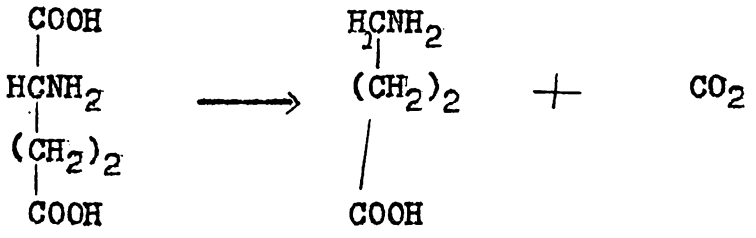
chromatograms were developed by spraying with the appropriate solvent.

To effect concentration of materials to be put on the chromatogram, it was usually necessary to decrease the volume of the solution in which these materials were dissolved. This was accomplished by distilling off water in vacuo, and chromatographing the residue.

VII. Chemical analyses.

A. Determination of glutamic acid.

Glutamic acid was determined manometrically by the method of Umbreit and Gunsalus (1945). L-glutamic acid decarboxylase present in E. coli catalizes the following reactions:



Each mole of glutamic acid gives rise to a mole of carbon dioxide, the volume of which can be measured in the Warburg apparatus. The volume of carbon dioxide liberated is proportional to the amount of glutamic acid present. 22.4 microliters of carbon dioxide are equivalent to 1 micro mole of carbon dioxide. This method has a maximum error of 10 per cent. The concentration of the cell suspensions were always such, that 10 micro moles of glutamic acid were decarboxylated within 30 minutes.

B. Determination of volatile acids.

These determinations were performed in accordance with the method of Neish (1952). The following changes were made: the material to be extracted was acidified with 10 N H_2SO_4, since HCl was found to be ether extractable. The extraction was carried out in a Kutscher-Steudel type extractor. After extraction, usually for 48 to 60 hours, 10 ml of distilled water was added to the ether solution and the ether was evaporated at room temperature with the help of a fan. The water residue was divided into 2 parts: one was titrated with standard alkali; the other was made alkaline with excess NH_4OH and chromatogrammed for fatty acids.

C. Nitrogen determination.

The method of Willits and Ogg (1950) was used, except that an acid-base indicator of the following composition was substituted for the one recommended by the authors: 2 volumes Methyl Red (0.1 per cent solution in 50 per cent alcohol) mixed with 1 volume Brom Cresol Green (0.1 per cent aqueous solution, to which is added 0.143 mM NaOH). 4 drops of indicator are used. Solution turns colorless and is titrated to faint pink with acid (Paretsky, 1953).

As an overall control, a water blank was digested along with each series of nitrogen determinations. Standard ammonium sulfate solution was always distilled before the test solutions, to check the solutions used in the determinations.

D. Ammonia determination.

Johnson's method (1941, a) was used.

E. Keto acid determination.

The method of Friedemann and Haugen (1943) was employed. Keto acids were converted to phenylhydrazones; these were extracted with ethyl acetate and sodium bicarbonate, dissolved in sodium hydroxide, and quantitatively determined by estimation of the color in a Coleman Junior Spectrophotometer at 520 mu. Optimum conditions, as outlined by the authors, were maintained for the formation of the phenylhydrazones of alpha ketoglutaric acid. These conditions consisted of a 25 minute period of incubation at 25 C for the formation of phenylhydrazones.

VIII. Reagents.*

All reagents used were of C.P. grade, unless otherwise indicated. Only special substrates and cofactors will be mentioned in this section.

A. L-glutamic acid.

The glutamic acid used as substrate was tested for purity by chromatography and was found to be free from other amino acids.

B. Alpha ketoglutarate.

Samples were obtained from the Nutritional Biochemical

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* Appreciation is expressed to Dr. T.H. Jukes, Lederle Laboratories, Pearl River, N.Y. for a generous gift of DL-6-thioctic acid; to Robert Hill for preparation of oxalacetate; and to Jack Fellman for preparation of sodium pyruvate.

Corp., Cleveland, Ohio. These samples differed in purity, the least pure melting at 124 C and the best at 114-115 C. The impure sample was recrystallized.

C. Coenzyme A.

Two preparations were used, both obtained from Armour, Chicago, Ill. One, called Liver Concentrate-Armour, Lot 309-111B, contained 10 Lipmann units mgm^{-1}; the other called Liver Coenzyme, Lot R. 341-146, contained 13 Lipmann units mgm^{-1}.

D. Diphospho- and triphosphopyridine nucleotide.

DPN "90" and TPN "80" were obtained from the Sigma Chemical Co., St. Louis, Missouri.

E. Cocarboxylase.

This was obtained from the Nutritional Biochemical Corp.

F. Adenosine triphosphate.

The disodium salt, obtained from the Nutritional Biochemical Corp. was used. It contained 95 per cent ATP, was chromatographically pure (tested by the company) and contained 0.04 micro grams or less of inorganic phosphate mgm^{-1}.

EXPERIMENTAL AND RESULTS

Properties of resting cell suspensions of B. tulrense oxidizing L-glutamic acid.

Oxidation of glutamic acid by resting cell suspensions was found dependent on certain exogenous factors. Investigation of these factors was deemed essential so that optimal reaction conditions could be established.

In a preliminary study of the physiological behavior of B. tularense a growth curve was constructed. This curve related incubation time to growth phase, so that the length of incubation could be expressed in terms of physiological age of the culture.

Cultures for the growth experiment were prepared and incubated as described in section III B, and optical density was measured as described in section III A of Materials and Methods.

One set of cultures was inoculated and allowed to incubate for 12 hours. After this time their optical density was measured hourly or so until the cultures had been incubated a total of 24 hours. On the basis of the data obtained by these readings the period from 12 to 24 hours of the growth curve was plotted.

The second set of cultures was inoculated 12 hours after the first set from the same parent culture. They were incubated and treated exactly as the first set, except that their optical

density was measured hourly, from 1 to 12 hours. Data so obtained were plotted and formed the first part of the growth curve from 1 to 12 hours.

The "parent" culture used as inoculum had previously been grown for 12 hours at 37 C, and was thereafter maintained at room temperature. A culture treated in this manner showed the least change in optical density over a period of 24 hours. This homogeneity was essential, so that the inocula into different tubes over the test period were as much alike as possible.

Fig. 1 represents a growth curve based on data from 2 separate experiments. As may be seen, optimally growing cultures had a very short lag period, a logarithmic (log) period lasting for about 6 hours, and a stationary phase which began after 10 to 12 hours growth. In this phase multiplication and disintegration take place at equal rates. Cultures of 7 hours or less shall be referred to as "young" cultures. They were in the log phase, reproducing at maximum rate. "Old" cultures on the other hand are those, which were approaching the stationary phase; their rate of multiplication decreases, while the rate of disintegration remains the same, so that finally both rates are equal. Data shall be presented which indicate that cell permeability of old cultures differs from that of young ones. Cells of all ages carried out glutamic acid oxidation to the same extent; the ratio, substrate: oxygen: carbon dioxide, was always the same and independent of the age of the culture from which the resting

Inoculum: Culture grown for 12 hours at 37 C; maintained thereafter at room temperature.

Size of inoculum: 5 per cent.

Optical density: determined every hour as described previously.

Duplicate tubes inoculated at 12 hour intervals from same parent culture.

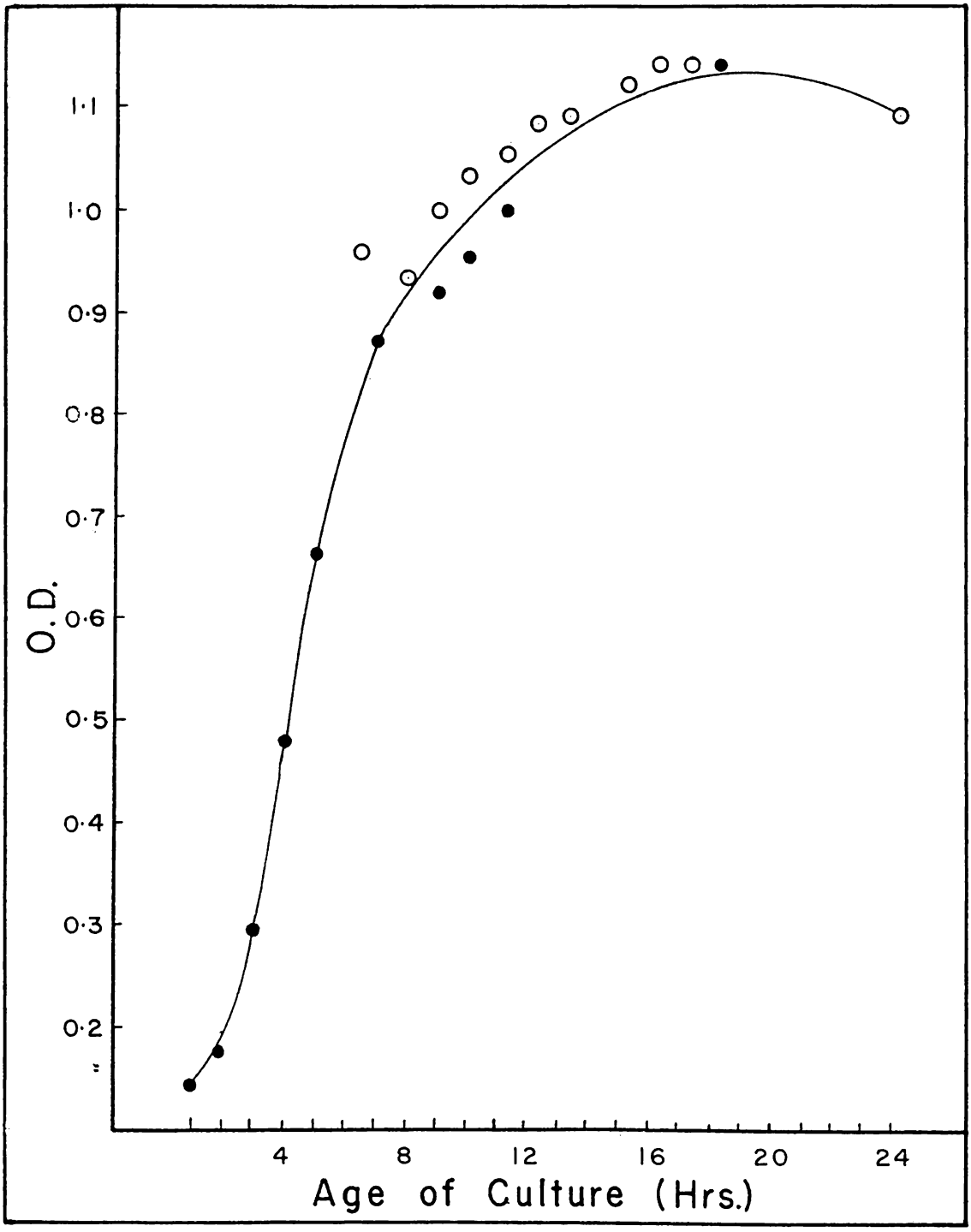
Composite of two experiments as indicated by

○—○ First Experiment

●—● Second Experiment

Fig. 1

GROWTH OF B. TULARENSE IN CASEIN-DECAMIN MEDIUM



cell suspension was derived. The rate of oxidation of glutamic acid by young cultures was greater, however, than that achieved by old cultures; but so was the endogenous respiration, that is, respiration in the absence of added substrate. Equalization of the rates occurred as soon as they were corrected for endogenous oxygen uptake.

The rate of glutamic acid oxidation was greatly affected by the concentration of phosphate. To demonstrate this point, it was necessary to relieve the phosphate of its buffering action by substituting another buffer, so that the phosphate effect could be studied independent of a pH effect. Using Tris (hydroxy methyl) aminomethane (TMAM) as buffer, oxygen uptake of a resting cell suspension on L-glutamic acid was measured in the presence of different concentrations of phosphate. After 30 minutes the experiment was discontinued, and the hydrogen ion concentrations of the solutions were determined immediately with a Beckman pH meter. The results are presented in Fig. 2. It may be seen that the buffering capacity of TMAM at the pH of the experiment was poor, since in the absence of phosphate, or with a phosphate concentration of 10^{-4} M, there was an increase of 0.4 pH units in 30 minutes. That a decrease in the rate of oxidation is nonetheless due to a decrease in phosphate concentration, can be seen clearly by examining the rate of oxygen uptake during the first 10 minute interval. It is most unlikely that a variation in pH existed during this period, and yet a definite lag in oxygen uptake was present in all systems whose phosphate concentration

Cells: Culture grown 14 hours; treated as described; aeration in phosphate buffer extended to 50 minutes; 1.0 ml

Substrate: 10 μM L-glutamic acid, as neutral solution of K salt

Buffer: 0.01 M (final) Tris(hydroxymethyl)-amino methane, pH 6.0

Phosphate: 10^{-1} M to 10^{-4} M (final) as indicated; appropriate dilutions made from stock phosphate buffer, pH 6.0

Center well: 0.2 ml 20 per cent KOH

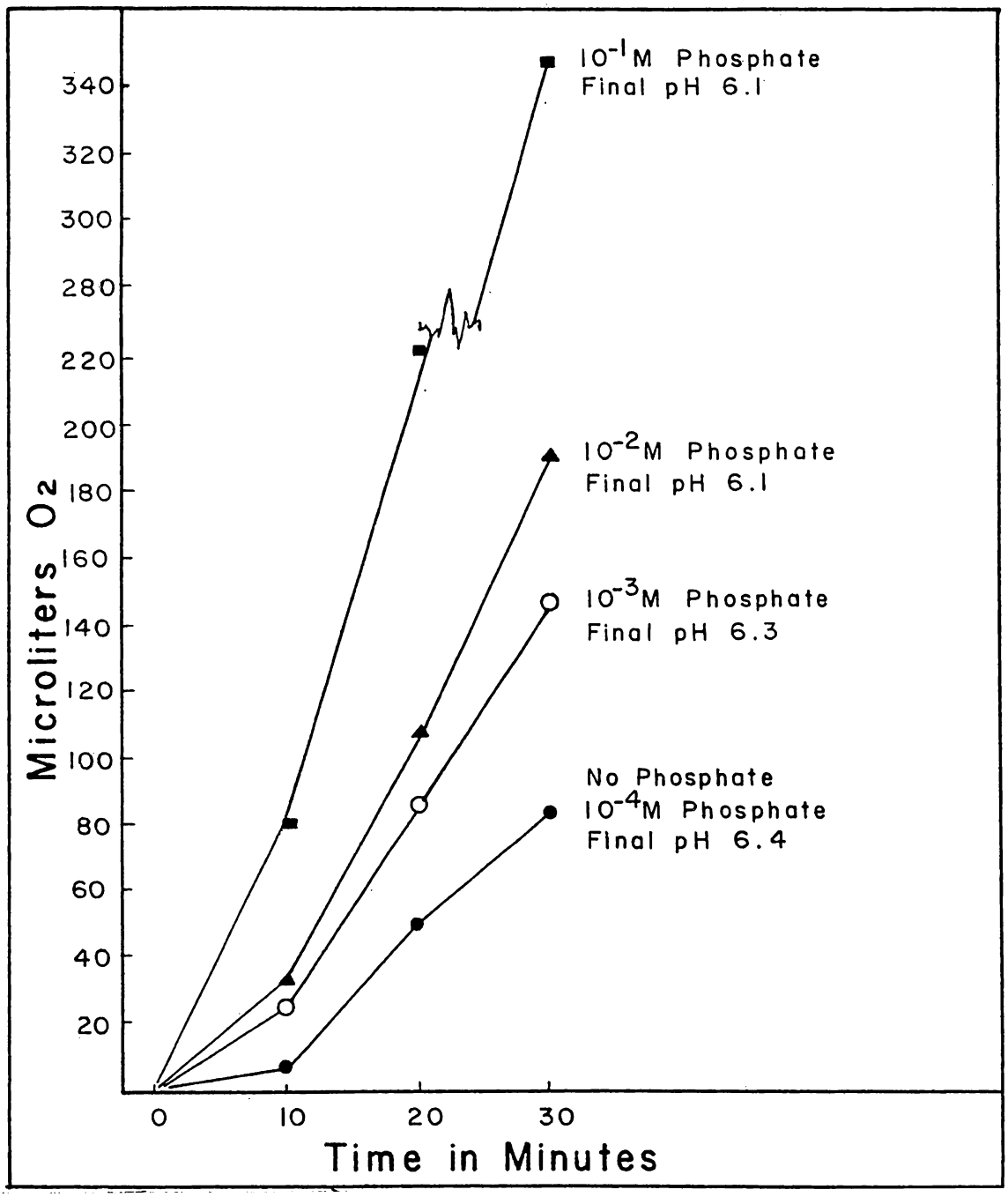
Total volume: 2.2 ml; Temp. 37 C; Gas phase: air

Substrate and phosphate tipped simultaneously 10 minutes after closure of stopcocks at 0 time.

Each graph is corrected for endogenous oxygen uptake, which did not exceed 38 μl in 30 minutes.

Fig. 2

EFFECT OF VARIOUS CONCENTRATIONS OF PHOSPHATE ON OXIDATION OF L-GLUTAMIC ACID BY RESTING CELL SUSPENSIONS OF B. TULARENSE



was below 10^{-1} M. Oxygen uptake in the absence of phosphate and in the presence of 10^{-4} M phosphate was the same, which indicated that this concentration was insufficient to affect the reaction.

Fig. 3, curve B illustrates clearly that under suitable conditions glutamic acid is not oxidized in the absence of phosphate. The amount of oxygen uptake in the presence of phosphate was ten times as great as in its absence. As soon as phosphate was added to an otherwise complete system, the rate of oxidation increased, which made the slopes of curve A and B, Fig. 3, similar. After the reaction had subsided, additional substrate brought about renewed oxygen uptake, whereas additional phosphate had no such effect. Two points were clarified by this observation: phosphate stimulation was entirely dependent upon the presence of sufficient substrate; and, the initial concentration of phosphate was adequate to sustain the reaction.

As stated previously, phosphate performed both an intrinsic and an extrinsic function in glutamic acid oxidation. The extrinsic function, of course, was the maintenance of the proper pH during the course of the reaction.

The study of the pH effect presented some unusual problems. To correlate hydrogen ion concentration with oxidative activity of resting cell suspensions, oxygen uptake was measured at different pH values and the rates were compared. The pH values of the systems were measured before and after the experiment with a Beckman pH meter.

A All components present at 0 time

B All components except phosphate present at 0 time

E All components except substrate present at 0 time

Additions as indicated:

A ↓ Substrate
B ↓ Phosphate

Cells: Culture grown for 15 hours, concentrated from 30 ml of medium; washed three times in 0.45 per cent saline; resuspended in saline 1.0 ml

Substrate: 10 μM L-glutamic acid as neutral solution of K salt

Buffer: 0.1 M (final) phosphate, mixture of K_2HPO_4 and KH_2PO_4, pH 5.3

Minerals: Final concentrations:

Magnesium sulfate	1×10^{-4} M
Calcium chloride	2×10^{-4} M
Ferrous sulfate	1×10^{-7} M
Manganese sulfate	1×10^{-7} M

Center well: 0.2 ml of 20 per cent KOH

Total volume: A before 250 min-3.2 ml; after 250 min-3.3 ml
B before 60 min-3.2 ml; after 60 min-3.5 ml
before 350 min-3.5 ml; after 350 min-3.5 ml
E 3.2 ml

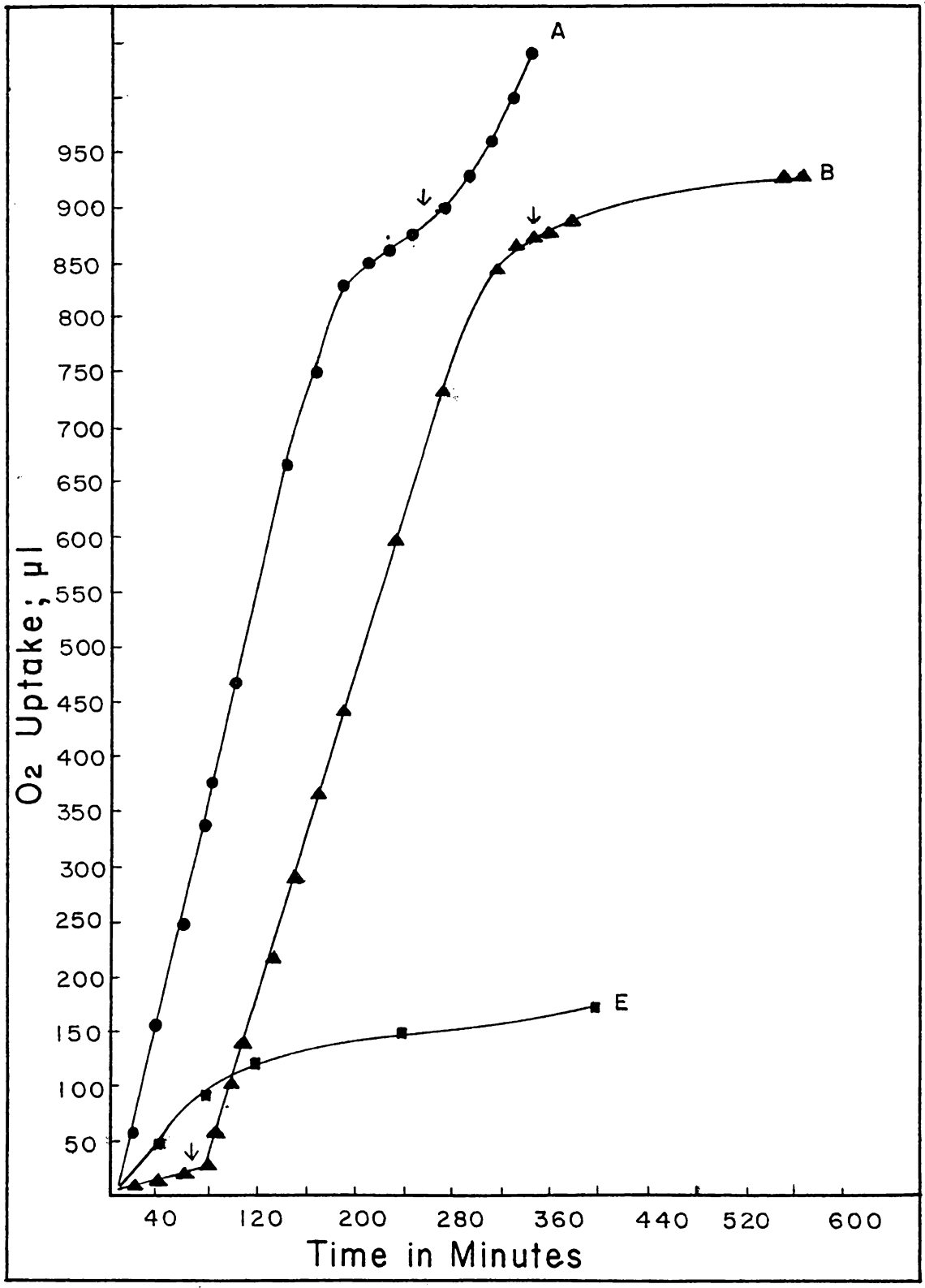
All flask constants corrected for change in volumes

Temp. 37 C; Gas phase: air;

Time: 9 hours and 20 minutes.

Fig. 3

EFFECTS OF ADDITION OF 0.1 M PHOSPHATE BUFFER ON GLUTAMIC ACID OXIDATION AT DIFFERENT STAGES OF THE EXPERIMENT



It was soon apparent that marked changes in pH took place in the course of a 30 minute test run, particularly if the initial pH was 5.0 or below. In several experiments the initial pH values 4.6 and 5.0 rose to 5.3 and 5.5 respectively, whereas a pH value of 5.5 and above remained unchanged. This change in pH was undoubtedly due to ammonia production by resting cell suspensions, both endogenously and in the presence of glutamic acid, and also to the inadequacy of phosphate buffer at a pH below 5.4. An attempt was made to compensate for the low buffering capacity of phosphate at those pH values by using a mixture of citric acid and dipotassium phosphate which maintains its full buffering capacity to pH 2.0, but a rise in initially low pH also occurred with it. Consequently it was not feasible to test values below pH 5.0 for their effect on the rate of glutamic acid oxidation. In early experiments it was also noted that the final pH values of endogenous and experimental systems were not always the same. It was therefore decided to neglect the correction of experimental values for endogenous respiration in these experiments. Table I gives the results of a representative study, which was conducted over a period of 130 minutes. Q_{O_2} was calculated on oxygen uptake from 10 to 40 minutes; both initial and final pH values are listed.

It may be noted that the optimum pH values for the oxidation reaction fall within a broad range, but that there is a sharp depression of rate at pH 7.0. This coincides well with the findings of Berger (1950). Hydrogen ion concentration was maintained best when the initial pH was 6.0. This

TABLE I

EFFECT OF pH ON RATE OF OXIDATION OF L-GLUTAMIC ACID BY RESTING CELL SUSPENSIONS OF B.TULARENSE

pH Initial	pH Final (after 130 min)	O_2 Uptake at interval from 10-40 min	$Q^*_{O_2}$
		µl/30 min	
5.2	5.8	338	113
6.0	6.1	350	117
6.5	6.5	370	123
7.0	6.9	89	30
7.5	7.4	108	36

$$^*Q_{O_2} = \frac{\mu l\ O_2 \text{ uptake}}{\text{mgm dry wt. cells}} \times hr^{-1}$$

Cells: Culture grown for 5 hrs; treated as described; 6 mgm dry wt./ml 1.6 ml

Minerals: Final concentration same as in Fig. 3

Substrate: 10 µM L-glutamic acid, as neutral soln. of K salt

Buffer: 0.1 M (final) K phosphate, pH as indicated

Center well: 0.2 ml 20 per cent KOH

Total volume: 2.2 ml; Temp. 37 C; Gas phase: air

Time: 130 minutes, reading every 10 minutes for 90 minutes, then every 20 minutes

Cells tipped at 0 time, 10 minutes after closure of stopcocks

value was adopted for most of the following experiments.

Properties of L-glutamic acid-oxidizing system of cell-free extracts of B. tularense.

Knowledge of the properties of the glutamic acid-oxidizing system of resting cell suspensions led to a search for the reactions through which these oxidations were taking place. In the course of this investigation it soon became evident that resting cell suspensions presented obstacles which might be avoided, if the oxidizing system could be obtained outside the cell. These obstacles, all properties of whole cells only, were: high endogenous respiration, which was difficult to reduce; inability to oxidize compounds, believed to be intermediates in glutamic acid oxidation, at the same rate as glutamic acid; disappearance of glutamic acid by metabolic paths other than respiration.

An attempt was made to produce cell-free extracts of B. tularense which would oxidize glutamic acid. Table II summarizes the oxidative activity of three independently made cell extracts, derived from three different mass cultures as described under Methods and Materials. It may be seen that oxygen uptake on glutamic acid alone was not much greater than the endogenous respiration. It appeared as if the extracts were either inactivated during preparation, or the extracellular oxidizing system had certain coenzyme requirements which needed to be met for activity to ensue. Since it had been shown previously by Adler (1938) and Klein (1940) that coenzyme I or II is necessary for the oxidation

TABLE II

OXIDATION OF GLUTAMIC ACID BY CELL-FREE EXTRACTS OF B. TULARENSE

Cell-free extract no.	O_2 Uptake			
	None	Glutamic acid	Cofactor solution	Glutamic acid and cofactor solution
			µl / 60 min	
210	28	36	69	149
218-1	109	143	321	585
218-2	11	49	46	268

Extracts: Prepared as described 1.0 ml

Substrate: 40 µM L-glutamic acid, as neutral solution of K salt

Cofactor solution: Amounts / 10 ml 1.0

Adenosine triphosphate (ATP)	5mgm
Coenzyme A (CoA)	50 units
Yeast extract	10 mgm
Cocarboxylase	0.5 mgm
Triphosphopyridine nucleotide (TPN)	1.5 mgm
Diphosphopyridine nucleotide (DPN)	5.0 mgm

Center cup: 0.2 ml of 20 per cent KOH

Total volume: 3.2 ml

Temp. 37 C; Gas phase: air

Tipped substrate 10 minutes after stopcocks were closed

of glutamic acid by certain bacterial extracts and resting cell suspensions, a solution was prepared which contained both of these coenzymes along with ATP, cocarboxylase, coenzyme A and yeast extract. Table II shows the increase in oxygen uptake on glutamic acid when this cofactor solution is added. In every case the increase in microliters of oxygen was more than the sum of the increase in microliters of oxygen when either component was added separately.

As the data show, the cofactor solution stimulated oxygen uptake considerably when added to the extracts in the absense of additional glutamic acid. This effect was due either to oxidizable material present in the solution itself, or to endogenous substrates contained in the extracts, which needed an exogenous supply of coenzyme for their oxidation. The last assumption was verified by the fact that dialysis of the extracts removed the effect.

It was of considerable interest to find out which component or sum of components of the cofactor mixture was stimulating oxygen uptake. This point was explored in two different ways. One experiment was designed to measure increase, the other to measure decrease in oxygen uptake by addition or deletion, respectively, of cofactors from the solution. The first experiment of Table III demonstrates the "attack by addition". In system number I DPN was added to the cell extract in the presence and absence of glutamic acid; system number II contained DPN and CoA; system number

TABLE III

EFFECT OF ADDITION OF COFACTORS ON GLUTAMIC ACID OXIDATION BY CELL-FREE EXTRACTS OF B. TULARENSE.

System no.	Cofactors added	O_2 Uptake at intervals from 30 to 60 min. Substrate µl / 30 min	No substrate µl / 30 min
A. Single addition of cofactors			
I	DPN		18
II	As I + CoA	187	82
III	As II + ATP	192	
IV	As III + Cocarboxylase	182	99
V	As IV + TPN	214	98
VI	As V + Yeast extract	204	94
B. Withdrawal of factors singly from cofactor mixture			
I	All Components	273	111
II	As I without DPN	125	
III	As I without CoA	182	
IV	As I without ATP	192	
V	As I without Cocarboxylase	203	
VI	As I without TPN	215	
VII	As I without Yeast extract	236	
VIII	No components	47	40

Extract: Prepared as described 1.0 ml

Substrate: 40 µM L-glutamic acid, as neutral K salt

Cofactors: Added individually, same concentration as described in Table II

Center well: 0.2 ml of 20 per cent KOH

Total volume: 3.2 ml; Temp. 37 C; Gas phase: air;

Time: 60 minutes, reading every 10 minutes

Substrate tipped 10 minutes after stopcocks were closed.

III, DPN, CoA, and ATP; in system number IV and V two more cofactors were added and finally system VI contained all cofactors present in the original solution. Oxygen uptake was measured in each system under identical conditions, using the same solution of substrate and cell extract. Examination of the data showsthat oxygen uptake on DPN and CoA is not appreciably increased by the additions of ATP, and cocarboxylase, but a boost is obtained by the addition of TPN.

The second experiment of Table III demonstrates the "depression by deletion" design. System number I contains all components; each system below it contains all components except the one indicated. Oxygen uptake was measured in each system under identical conditions, using the same substrate and cell extract. This extract was the same as that used for the first experiment, but since this experiment was the first one of the series, the extract was more active. The coenzyme solutions were prepared new each day but from the same starting materials.

The data reveal that deletion of DPN produced the greatest depression of oxygen uptake; exclusion of CoA, ATP, and cocarboxylase depressed oxygen uptake less, but more than exclusion of TPN or yeast extract. Comparison of the two experiments indicates that DPN is the most essential component of the cofactor mixture; all other components are of lesser importance and are similar in their stimulatory power

except yeast extract. This component, in the concentrations used, contributed no stimulatory factors.

It also may be seen in these experiments that oxygen uptake was stimulated considerably by the addition of co-factors to the extract in absence of glutamic acid. It was therefore decided to repeat the experiment with an extract with lower endogenous respiration, that is, with a dialyzed extract. The results obtained were similar to those presented in Table III, except that the addition and deletion of TPN produced a much greater effect than could be demonstrated in undialyzed extracts. Upon addition of TPN to DPN and CoA, oxygen uptake increased from 140 µl (DPN and CoA alone) to 200 µl; and exclusion of TPN from the total cofactor mixture caused a greater depression in oxygen uptake than was produced by exclusion of yeast extract, cocarboxylase, ATP or CoA.

In the course of the cofactor studies with dialyzed extracts it was found that glutamic acid oxidation would not take place in the presence of cofactor solution alone. In an attempt to stimulate oxidation, Mg^{++} and Mn^{++} were added to the system in form of the sulfate salts. Both ions have been found essential for oxidation of Krebs cycle intermediates by mitochondrial preparations from cats' hearts and rabbit kidney cortex (Ochoa, 1944; Hartman and Kalnitsky, 1950). The results of one experiment are given below:

Metal	O_2 Uptake µl / 70 min
None	0
0.01 M Mg^{++}	352
0.01 M Mn^{++}	60
0.01 M Mg^{++} / 0.01 M Mn^{++}	150

All systems contained 1 ml extract, 40 µM L-glutamic acid, and cofactor solution in the concentration indicated in Table II.

0.01 M Mn^{++} was clearly inhibiting, while 0.01 M Mg^{++} was stimulating. Mg^{++} had been added to all extracts before the previously described experiment on the effect of cofactors was carried out with the dialyzed extract.

It appeared that considerable purification of the extracts was attainable by dialysis, but at the same time this treatment induced instability with respect to oxidation of glutamic acid. Dialyzed extracts which showed vigorous oxidative activity on glutamic acid one day, as demonstrated by oxygen uptake, were inactive when tested the next time. The extracts were stored in the freezer between the test intervals. Attempts to restore the activity by increasing the amount of Mg^{++}, doubling the concentration of cofactors, or adding DL-6-thioctic acid (5.9 µg / ml) to the extract before testing, met with failure. Extracts which could not attack glutamic acid in the presence of oxygen could attack alpha ketoglutarate, succinate and citrate under identical conditions. However, under nitrogen and in the presence of triphenyl tetrazolium chloride (TTC), an electron acceptor, glutamic acid was attacked and a keto acid accumulated. TTC was reduced in this process.

TABLE IV

ACTIVITIES OF THREE DIALYZED CELL-FREE EXTRACTS

Cell-free extract	Days since dialysis	Activities of dialyzed extracts: Glutamic acid	Alpha keto glutarate	Citrate	Succinate	Keto acid accumulation*		
						Incubation, min	Glutamic acid µM	Keto acid µM
		O_2 Uptake: µl/30 min						
218-1,2	2	263						
	8	0	47	42	305	130	80	1.2
	Undialyzed					130	80	1.2
221	1	105						
	4	0						
	5		106	80	509	130	80	3.1
225	Undialyzed	179						
	0							
	Dialyzed for 90 min	0						
	Dialyzed for 12 hrs	0				120	80	0.74

* Reaction carried out anaerobically using N_2 as gas phase. Triphenyl tetrazolium chloride used as electron acceptor.

Substrates: Alpha ketoglutarate: 40 µM
Succinate : 80 µM
Citrate : 40 µM
L-glutamic acid : 80 µM

Mode of dialysis: Extract 218-1,2: 5 hour dialysis with constant stirring at -5 C against M/50 phosphate buffer pH 7.0; thin layer of ice found on surface of buffer at end of dialysis.

Extract 221: 6 hour dialysis with and 6 hours without stirring at 4 C against M/50 phosphate buffer pH 7.0.

Extract 225: Dialyzed 2 samples: one for 90 minutes and one for 12 hours with constant stirring at 4 C against M/50 phosphate buffer pH 7.0.

It appears that the enzyme system catalyzing the dehydrogenation of glutamic acid did undergo reduction, but that it could not undergo reoxidation in the presence of oxygen; when TTC was the electron acceptor, the reaction proceeded. The reaction between enzyme and substrate was unimpaired, but the reaction between enzyme and molecular oxygen was blocked. As shown in Table IV, column 9, dialyzed extracts accumulated as much keto acid as undialyzed ones, when the reaction was carried out anaerobically in the presence of TTC as electron acceptor, though the dialyzed extracts could not carry out this reaction aerobically.

It may also be seen from this Table that conditions which inactivate the electron carrier system linked with glutamic dehydrogenase, did not affect the electron carrier systems linked with the oxidative systems of the Krebs cycle components. This observation, though a most provocative one, was not further investigated.

Since the activity of dialyzed extracts on glutamic acid had been found unpredictable, their use in all further experiments was discontinued, and undialyzed extracts only were tested.

Oxidation of metabolic intermediates by resting cell suspensions and cell-free extracts of B. tularense.

When the properties of resting cell suspensions and cell-free extracts had been investigated and optimum conditions for glutamic acid oxidation had been established, both systems could be employed to study the path of this oxidation. Studies with tissues and other microorganisms have shown that one of the most common modes of attack on glutamic acid is by dehydrogenation and conversion to alpha ketoglutaric acid. This substance is a member of the tricarboxylic acid cycle; consequently, glutamic acid might be oxidized through this cycle.

A common procedure for testing possible intermediates in a postulated path is a comparison of the rate of oxygen uptake of the test substance with that of the metabolites believed to be intermediates in the reaction. If the rates are the same, the metabolites may indeed be intermediates. The rate of oxidation of glutamic acid by resting cell suspensions was compared with that of four metabolic intermediates. It was soon found that the rate of oxidation of these intermediates was dependent upon the age of the culture from which the resting cell suspension was derived. Alpha ketoglutarate, sodium succinate, and sodium acetate were oxidized more rapidly by "Old" cultures, that is cultures that had reached the stationary phase of growth. Sodium pyruvate, however, was oxidized at the same rate by either old or

"young" cultures, that is cultures that were in the log phase of growth.

However, oxidation of these intermediates, when tested under optimum conditions for glutamic acid oxidation, occurred at about one tenth the rate of that of glutamic acid, when "old" cultures were used. These results then could be interpreted in two ways: either glutamic acid was not oxidized through these intermediates, or these substances failed to penetrate the cell wall, or penetrated it at a slow rate, so that the lack of oxygen uptake was due to lack of enzyme-substrate contact rather than to the lack of enzyme systems. Studies of cell wall permeability of bacteria have shown that unionized materials will penetrate the cell whereas the same materials in their ionized state will not (Gerhardt et al., 1953). It was decided to apply this observation to the problem at hand, that is, to test these acidic intermediates at low pH values, so that their non-ionic molecular state would increase and their ionic state would be depressed. Increased penetration of substrate at low pH values would lead to greater oxygen uptake than at high pH values.

A resting cell suspension derived from "old" mass cultures was incubated with three metabolic intermediates at pH 5.0, 6.0 and 7.0, and oxygen uptake was measured. Data presented in Table V show indeed that oxygen uptake decreased with increase in pH. With all three substrates tested the activity fell off at pH 7.0, though this is more marked

TABLE V

EFFECT OF pH ON OXIDATION OF THREE METABOLIC INTERMEDIATES BY RESTING CELL SUSPENSIONS OF B. TULARENSE

Substrate	O_2 Uptake			Q_{O_2}*		
	Initial pH	5.0	6.0	7.0	Final pH		
	Final pH	5.5	6.3	7.2	5.5	6.3	7.2
	µl / 30 min						
Alpha ketoglutaric acid		122	126	70	3.4	2.7	.84
Succinic acid		193	94	80	6.4	1.3	.51
Sodium pyruvate		126	164	117	3.6	4.3	2.1
None		41	62	68			

* $Q_{O_2} = \frac{\mu l\ O_2 \text{ uptake}}{\text{mgm dry wt. cells}} \times hr^{-1}$ corrected for endogenous respiration

Cells: "Old" culture; treated as described; final resuspension of cells in buffered saline, pH as indicated; 47.5 mgm dry weight/ml 1.0 ml

Substrates: 3 µM alpha ketoglutaric acid and succinic acid as neutral solution of Na salt 3 µM sodium pyruvate

Buffer: 0.1 M (final) phosphate, mixture of K_2HPO_4 and KH_2PO_4 pH as indicated

Buffered saline: 0.45 per cent saline brought to proper pH by addition of a few drops of 0.02 M phosphate, pH as indicated

Diluent: 0.002 M (final) phosphate, made from same stock solution as buffer, pH as indicated

Center well: 0.2 ml of 20 per cent KOH

Total volume: 2.2 ml; Temp. 37 C; Gas phase: air

Time: 105 minutes, first reading after 15 minutes, then every 10 minutes to 65 minutes, thereafter every 20 minutes. Values reported obtained at 25 to 55 minute interval

Cells shaken in Warburg cups for 137 minutes before addition of substrate.

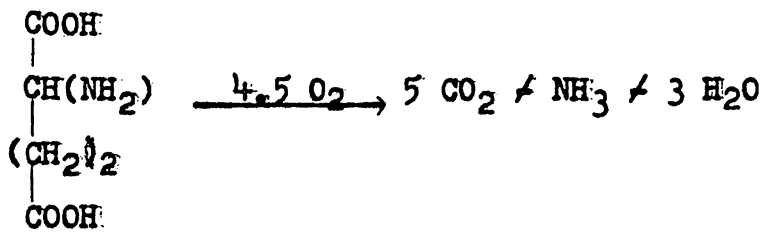
with alpha ketoglutaric acid and succinic acid than with sodium pyruvate. Succinic acid was most rapidly oxidized at the lowest pH, 5.0, while peak activity on alpha ketoglutarate and pyruvate occurred at pH 6.0. It is of interest to note that the activity of the cell suspension alone showed a reverse trend, that is, it increased with increasing pH, from 41 µl oxygen uptake at pH 5.0 to 68 µl at pH 7.0. This may indicate a general depression in activity at low pH, an effect which is masked by the presence of substrate. Under optimum conditions the oxidation of these intermediates still takes place at a lower rate than the oxidation of glutamic acid, when resting cell suspensions are used. Cell-free extracts, on the other hand, present the reverse picture; the optimum rate of glutamic acid oxidation was always less than the rates of oxidation of alpha ketoglutarate, succinate, fumarate, and citrate. However, with cell-free extracts the differences in rates were less pronounced than with whole cells.

On the basis of these experiments it is apparent that cultures of B. tularense, grown in casein-decamin medium, do possess enzyme systems which oxidize both glutamic acid and Krebs cycle intermediates; but whether or not their oxidative paths are the same was not yet clarified.

To gain better insight into this problem, three modes of attack were planned:

1. Quantitative measurements of glutamic acid disappearance and corresponding oxygen uptake and carbon dioxide appearance; such measurements, expressed as ratio of mole of substrate per mole of oxygen

or carbon dioxide, indicate the extent of the reaction. Complete oxidation of 1 mole of glutamic acid should cause the uptake of 4.5 moles of oxygen and the appearance of 5 moles of carbon dioxide. This is the overall reaction:



If it were found that the actual ratios differed from the theoretical ones, it might indicate that complete oxidation did not occur but that the reaction stopped at some intermediate product; since this product was not oxidizable by the cells, it would accumulate in the medium.

2. Demonstration that the reaction proceeds along certain metabolic paths by the use of specific inhibitors known to interfere with these paths.

3. Accumulation and identification of intermediates. This mode of attack is dependent upon the other two.

Experiments to investigate the extent of glutamic acid oxidation.

A. Ratio of glutamic acid disappearance to oxygen uptake and carbon dioxide production by resting cell suspensions.

The quantitative estimation of glutamic acid was essential for these experiments. It was decided to assay for glutamic acid enzymatically, by making use of a L-glutamic acid decarboxylase found in E. coli #4157. The decarboxylase present in the E. coli suspensions, prepared as described under Materials and Methods, attacked both L-glutamic acid and glutamine, but no other amino acid or amide. When tested on resting cell suspensions of B. tularense, in the absence of exogenous glutamic acid, no carbon dioxide was produced; in the presence of known amounts of glutamic acid, mixed with resting cell suspensions, 90 to 99 per cent of the added amino acid was accounted for in terms of carbon dioxide, even when the initial mixture was incubated together for three hours previous to the assay. (Oxidation of glutamic acid by B. tularense suspension was prevented during that time by lack of phosphate.) In the course of these experiments it was important to know if all glutamic acid had disappeared from the system when oxygen uptake and carbon dioxide production had stopped, that is, when the respiratory rate of the test system had dropped to that of the endogenous system. The following experiment was devised to test this point: 10 μM of glutamic acid were allowed to be oxidized by resting cells in a

Warburg vessel. After all activity had ceased, the flask was taken off the manometer. The contents of the center well (KOH) and the filter paper were carefully washed out and replaced with water and a new paper; the vessel was re-equilibrated at 30 C and E. coli suspension tipped from the side arm. No carbon dioxide was produced, although active production took place in a control vessel into which glutamic acid and E. coli were tipped simultaneously; the endogenous vessel, like the test vessel, showed no carbon dioxide production. In two such experiments no glutamic acid was found after the respiratory rate dropped to the endogenous level. These experiments gave adequate assurance that oxygen uptake of resting cell suspensions ceased only after all glutamic acid had disappeared. This was also valid for carbon dioxide production. Knowing the amount of glutamic acid added initially, and converting both oxygen uptake and carbon dioxide production from microliters to micromoles, the ratio, substrate: O_2:CO_2 could be calculated. These ratios, determined for several independent experiments, are listed in Table VI.

Examination of this Table shows, that complete oxidation of the substrate did not take place. The greatest amount of oxygen uptake was 84 per cent of the theoretical (Exp. 130) and the lowest 71 per cent (Exp. 212), whereas the carbon dioxide output was approximately the same for all experiments, 80 per cent. At the concentrations employed a substrate concentration effect could not be demonstrated; that is, the

substrate: O_2:CO_2 ratio could not be increased by increasing the amount of substrate from 5 to 20 μM. The value of the endogenous respiration warrants some consideration. Measured as oxygen uptake, it varied from 72 μl (Exp. 130) to 106 μl (Exp. 212). An attempt was made to keep these values at this low level (compared to 300 or 400 μl in many other experiments of this type) by using either dilute suspensions (Exps. 119 and 130), or by additional aeration of the cell suspension before tipping in the substrate (Exp. 212).

Twelve experiments of this type were performed; in no instance did the amount of oxygen uptake or carbon dioxide output correspond to the amount which would be expected if complete oxidation of the substrate had taken place. These results indicate that glutamic acid either disappears along a non-respiratory path or B. tularense does not contain the enzyme systems necessary to bring about dissimilation of glutamic acid to carbon dioxide and water.

In these experiments it was not possible to measure glutamic acid disappearance in terms of ammonia production, since the bacteria produced large amounts of ammonia endogenously. For heavy suspensions this amount varied under similar conditions from 65 to 100 μg per 1 ml of suspension.

B. Experiments to inhibit assimilation.

One of the paths along which glutamic acid could be disappearing non-oxidatively was by assimilation. If this process were taking place under the conditions prevailing for glutamic

TABLE VI

INCOMPLETE OXIDATION OF GLUTAMIC ACID BY RESTING CELL SUSPENSIONS OF B. TULARENSE

Exp. No.	Substrate μM	O_2 Uptake* μM	CO_2 Output* μM	Substrate: O_2 : CO_2 Observed	O_2 : CO_2 Theoretical (complete oxidation)
119	5	18.5	19.7	1: 3.69: 3.95	1: 4.5: 5.0
130	10	37.7	42.2	1: 3.77: 4.22	
212	10	32.1	39.0	1: 3.21: 3.90	

* All values corrected for endogenous respiration

Cells: Exp. 119: Culture grown for 15 hours; washed 3x in .45 per cent saline and resuspended in .45 per cent saline 1.0 ml

Exp. 130: Culture grown for 4 hours; treated as in Exp. 119 1.0

Exp. 212: Culture grown for 15 hours; treated as described 1.0

Substrate: Glutamic acid as neutral solution of K salt; quantity as indicated

Buffer: 0.1M (final) phosphate, mixture of K_2HPO_4 and KH_2PO_4 pH 5.9

Center well: 0.2 ml of 20 per cent KOH for O_2 uptake data; 0.2 ml of H_2O for CO_2 output data

Total volume: Exps. 119 and 130 - 3.2 ml; Exp. 212 - 2.15 ml;

Temp. 37 C; Gas phase: air

Time: Exp. 119: Total time of experiment: 3 hours
Exp. 130: Total time of experiment: 6 hours 30 minutes
Exp. 212: Total time of experiment: 2 hours 50 minutes

Exp. 119: Tipped cells 10 minutes after taps were closed
Exp. 130: Tipped cells 10 minutes after taps were closed
Exp. 212: Tipped substrate 30 minutes after taps were closed.

acid oxidation, then more glutamic acid would be disappearing than could be accounted for in terms of oxygen uptake and carbon dioxide production. Since a direct measurement of disappearance of glutamic acid by assimilation was not possible, an indirect procedure was employed: an attempt was made to depress the reaction by the use of 2,4 dinitrophenol (DNP), a drug known to have this specific property (Clifton, 1946).

Six separate experiments were performed. These consisted of testing the effect of 7.5×10^{-5} and 7.5×10^{-6} M (final) concentrations of the drug on the oxidation of glutamic acid by normal cell suspensions and by suspensions which had previously been incubated with 7.5×10^{-5} and 7.5×10^{-6} M DNP. The most effective treatment was found to be pre-incubation of the cells with 7.5×10^{-5} M DNP and subsequent testing of these suspensions in the absence of additional inhibitor. Concentrations of the drug outside of those mentioned were either inhibitory or without effect. Total oxygen uptake on 10 uM of glutamic acid was measured in all cases, and it was hoped that in the presence of DNP, oxygen consumption would increase to the amount concomitant with complete oxidation of the substrate, 4.5 moles per mole of glutamic acid. Unfortunately this expectation was not fulfilled. The greatest increase obtained in all experiments was a boost in oxygen uptake from 3.33 to 3.85 moles per mole of substrate.

When sodium azide was used as inhibitor, an increase in oxygen uptake could not be demonstrated with the dilutions tested. In a series of five fold dilutions, from 5×10^{-3} M

(final) to $1x10^{-4}$ M (final), the concentrations were either too high, so that oxidation was completely inhibited, or too low, so that no effect was demonstrable.

The results of these experiments are difficult to interpret. They neither affirm nor deny the existence of assimilation. Therefore the problem posed by the disappearance of glutamic acid along a non-oxidative pathway is essentially still unsolved.

C. Search for acidic and Ninhydrin-reacting end products.

It was stated previously that the oxygen uptake and carbon dioxide output data could be interpreted in another way: that complete dissimilation of glutamic acid does not take place but that the process stops at some metabolic intermediate which accumulates in the medium. The accumulation of such end products is most common in bacteria. H. parainfluenzae and N. gonorrhoeae (Klein, 1940; Tonhazy and Pelczar, 1953) both oxidize glutamic acid to acetic acid. Consequently a search for acidic end products was made.

In a typical experiment cells were harvested sterilly from 500 ml of culture fluid and treated as described for Warburg experiments. They were then diluted to 50 ml and divided into two lots. One lot was added to phosphate and water, the other to phosphate /0.1 M (final), pH 5.9/, and glutamic acid (2 mM) in a 250 ml Erlenmeyer, and both flasks, containing 50 ml each, were incubated at 37 C with shaking for 9 hours. In the beginning, in the middle, and at the end of the

incubation period 2 ml samples were taken out of each flask and assayed for glutamic acid. It was found that 1.43 mM were used up during this period. After 9 hours the contents of the flasks were made acid with 10N H_2SO_4 and made up to 50 ml with distilled water. The material was then centrifuged and the supernatant extracted with ether in a Kutscher-Steudel type extraction apparatus. All further manipulations have been described under Methods and Materials. In 4 such experiments no acidic end products were detected; the amount of alkali needed to titrate the control was similar to the amount needed for the neutralization of the experimental extract. As was stated previously, 10 ml of distilled water were added to the ether before the solvent was evaporated, and half of the aqueous solution was always saved for chromatography. The method of Kennedy and Barker (1951) was used. In all chromatograms aqueous solutions derived from the experimental and control flasks were used; since it was found that fatty acids alone always travelled faster on the chromatogram than when they were mixed with the test solutions, "internal controls" were always set up. These consisted of mixtures of known fatty acids and the control solution. It is believed that the retardation of movement of the acids by the solutions was due to salts present in these solutions.

The results obtained by chromatography were also entirely negative; no fatty acids or Krebs cycle components were detected in the test solutions by this method. These experiments excluded the possibility of an acidic end product of glutamic

acid dissimilation under these conditions.

There was however, another, yet unexplored pathway for the disappearance of glutamic acid; this was disappearance by transamination of the exogenous glutamic acid with the keto acids of the cell.

To investigate this possibility the ether extracted, aqueous extract which remained in the Steudel-Kutscher apparatus was concentrated in vacuo to about one tenth of its volume and chromatogrammed for Ninhydrin-reacting substances, according to the method of Feldman and Gunsalus, 1950. The spots obtained with the experimental and control solution were the same, except, of course, for the glutamic acid spot, which was present only in the sample from the experimental flask.

It is difficult to reconcile complete glutamic acid oxidation with the substrate: O_2 : CO_2 ratios obtained. Yet no resolution of this problem can be offered at present on the basis of these experiments.

Studies with specific inhibitors.

The experiment described and discussed in the three previous sections did not contribute in a positive way towards the central question under investigation: "Are glutamic acid and Krebs cycle intermediates oxidized along the same pathway?" This consideration initiated the second mode of attack on this problem: the use of specific inhibitors.

Experiments were designed to test the effects of inhibitors known to interfere with the oxidation of the following Krebs cycle components: alpha ketoglutarate; succinate; citrate. Semicarbazide and hydroxylamine have been successfully employed for blocking the oxidation of keto acids; therefore these substances were used in an attempt to block glutamic acid oxidation at thestage of alpha ketoglutarate. This attempt was unsuccessful with these inhibitors, since oxidation was eithernot at all inhibited or completely depressed. Sodium arsenite was more satisfactory. A concentration of 10^{-5} M (final) produced 46 per cent inhibition of oxygen uptake, when 10 µM of glutamic acid were oxidized in a Warburg apparatus by a cell suspension containing 2.6 mgm dry weight of cells. A concentration of 10^{-4} M (final) was completely inhibitory to this system. That alpha ketoglutarate accumulated under these conditions could be demonstrated and will be discussed in the next section.

As it is well known that malonic acid is a specific inhibitor for succinic acid (Quastel and Whetham, 1924), this

substance was tested on both succinic and glutamic acids. In experiments with whole cells malonate would not inhibit, though the experiments were performed under optimum conditions for succinic acid oxidation (pH 5.0). When cell-free extracts were employed, however, inhibition was achieved. The results of one experiment are represented in Fig. 4. Using another extract and 400 μM of malonate, 75 per cent inhibition was obtained on 20 μM of glutamic acid, and 79 per cent inhibition on 40 μM glutamic acid. These results are shown in Fig. 5.

The final reaction to be tested by the use of an inhibitor was the condensation reaction of acetate and oxalacetate to form citrate. The blocking agent used was sodium fluoroacetate. This experiment is summarized in Table VII. In the absence of the inhibitor, oxygen uptake on glutamic acid and acetate was the same over the period measured, but in the presence of fluoroacetate, inhibition of acetate oxidation was greater than the inhibition of glutamic acid oxidation. This effect might be caused by a dual role played by glutamic acid: it may be a precursor for both acetate and oxalacetate. The following series of reactions would make this possible: glutamic acid could be oxidized to oxalacetic acid, which might undergo two different reactions simultaneously: one would be the condensation reaction with acetate; the other reaction would lead through pyruvate to acetate, which would condense with more oxalacetate. Since oxalacetate reverses the inhibition of fluoroacetate in

Extract: Prepared as described 1.0 ml

Substrates: 4 μM sodium acetate; 4 μM sodium succinate; 4 μM alpha ketoglutarate as neutral Na salt; 20 μM oxalacetate as neutral Na salt; 40 μM L-glutamic acid as neutral K salt

Inhibitor: 250 μM of malonic acid as neutral Na salt

Cofactor solution: Same as described in Table II 1.0

Center well: 0.2 ml of 20 per cent KOH

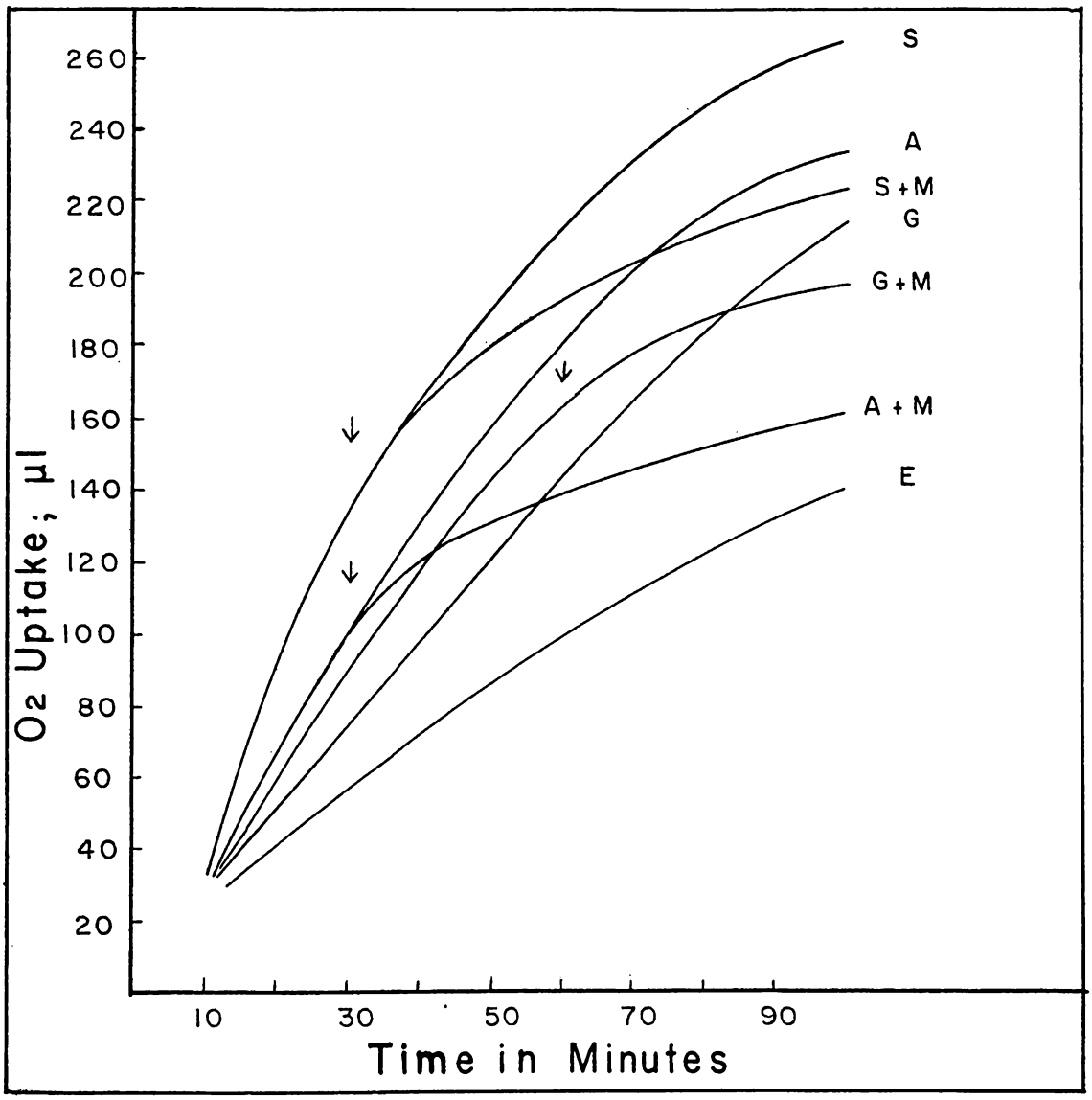
Total volume: 3.5 ml; Temp. 37 C; Gas phase: air

Substrate and cofactor solution tipped simultaneously at 0 time, 10 minutes after stopcocks were closed

Addition of inhibitor as indicated

Fig. 4

OXIDATION OF KREBS CYCLE INTERMEDIATES BY A CELL-FREE EXTRACT OF B. TULARENSE AND INHIBITION BY MALONATE



S-Succinate
S/M- " / malonate

A-Alpha ketoglutarate
A/M- " " and malonate

G-Glutamic acid
G/M- " " and malonate

E-Endogenous, Oxalacetate, Acetate

↓-Addition of Malonate

Extract: Prepared as described 1.0 ml

Substrate: 20 and 40 μM of L-glutamic acid as neutral K salt

Inhibitor: 400 μM of malonic acid as neutral Na salt

Cofactor solution: TPN, DPN and CoA same concentration as described in Table II

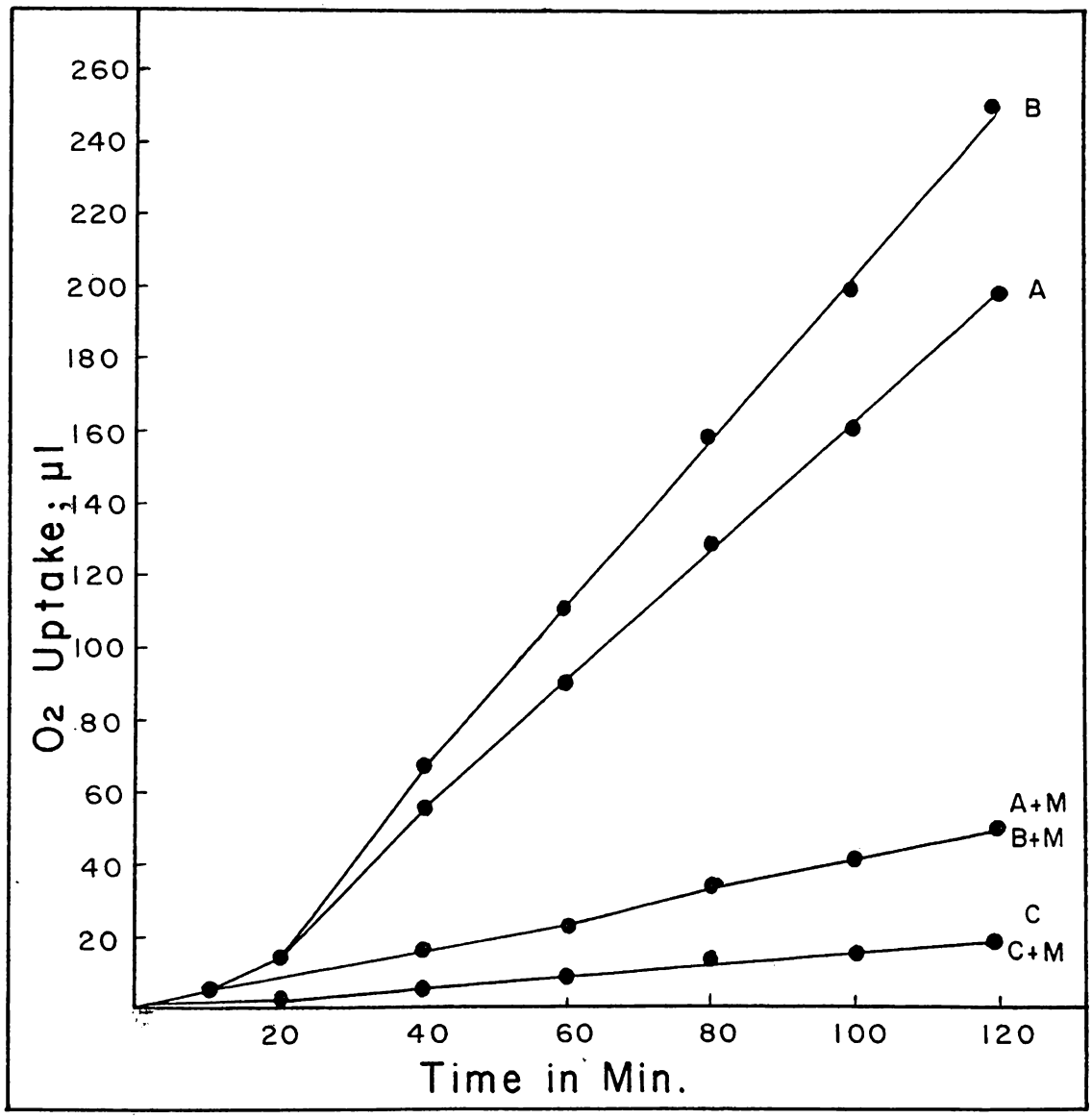
Center well: 0.2 ml of 20 per cent KOH

Total volume: 3.2 ml; Temp. 31 C; Gas phase: air

Substrate tipped at 0 time.

Fig. 5

OXIDATION OF L-GLUTAMIC ACID BY A CELL-FREE EXTRACT OF *B. TULARENSE* AND INHIBITION BY MALONATE



A 20 µM of L-glutamic acid
A + M 20 µM of " " and malonate.
B 40 µM of " "
B + M 40 µM of " " " "
C No substrate
C + M " " and malonate.

TABLE VII

OXIDATION OF SODIUM ACETATE AND L-GLUTAMIC ACID BY RESTING CELL SUSPENSIONS OF B.TULARENSE AND INHIBITION BY SODIUM FLUOROACETATE

Substrate	Sod. fluoroacetate *	O_2 Uptake	Inhibition **
μM	μM	μl / 100 min	%
none	none	196	
none	5	95	
none	20	75	
L-glutamic acid			
100	none	934	
100	5	455	51
100	20	256	76
Sodium acetate			
100	none	950	
100	5	92	100
100	20	61	100

** Corrected for endogenous respiration

Cells: Culture grown for 14½ hours; treated as described; incubated in phosphate in ice box for 1 hour 1.0 ml

Substrate: 100 μM of sodium acetate
100 μM of L-glutamic acid as neutral solution of K salt

Buffer: 0.1 M (final) K phosphate, pH 6.1

Inhibitor: Sod. fluoroacetate, dissolved in M/50 phosphate, pH 6.0, concentrations as indicated

Center well: 0.2 ml of 20 per cent KOH

Total volume: 3.3 ml and 3.6 ml; flask constants corrected for different volumes

Temp. 37 C; Gas phase: air

Pre-incubated inhibitor with cell suspension for 15 minutes

* Sodium fluoroacetate 90%-Technical-(Compound 1080) Monsanto Chem. Co.
Inert material 10%

rabbit kidney cortex (Elliott and Kalnitsky, 1950) this mechanism might also be operating in this system.

This experiment clarifies another point which, although not under direct investigation, is worth mentioning: oxidation of acetate was completely inhibited by fluoroacetate, which is a strong indication that acetate is oxidized through citrate.

The results of these experiments show that the oxidation of glutamic acid is blocked under the same conditions and by the same specific inhibitors as alpha ketoglutarate, succinate, and citrate, all components of the tricarboxylic acid cycle. This constitutes good "circumstantial" evidence that these components and glutamic acid are oxidized along the same metabolic paths. To gather "specific" evidence on this point it was necessary to shift to the third mode of attack: accumulation and identification of an intermediate of glutamic acid metabolism.

Accumulation and identification of alpha ketoglutarate.

The accumulation of keto acid was measured anaerobically in the presence and absence of triphenyl tetrazolium chloride (TTC). The experiment was carried out by allowing a resting cell suspension of B. tularense to attack glutamic acid in a closed system in which air had been replaced by nitrogen, using TTC as electron acceptor. After 15 hours incubation at 37 C, the cells were inactivated by syphoning into the system 1.7 N acetic acid before they were exposed to air. Cells and supernatant were then separated by centrifugation, the cells were washed once and then made up to a specific volume. To determine the keto acid in the internal environment, the cells were disintegrated in the Raytheon oscillator. The extract was centrifuged and assayed by the method of Friedemann and Haugen. Specific details of this experiment and results are given in Table VIII.

TTC upon reduction changes from a colorless, water soluble dye into a water insoluble, red formazan. Since the colorimetric determination of keto acids depends upon the formation of a red color (reaction of NaOH and phenylhydrazone) it was necessary to determine if the reduced TTC, present in the test solution, would interfere with the keto acid determination. It was found not to interfere, because the phenylhydrazone was soluble in $NaHCO_3$ and reduced TTC was not, so that separation was brought about by extracting the solution with $NaHCO_3$, before the color was developed.

It is quite evident from the data presented in Table VIII that no accumulation of keto acid took place in the absence of an electron acceptor, indicating the participation of an electron carrier system in this reaction. Aerobically, of course, oxygen fulfills this role. Under aerobic conditions accumulation of keto acid took place only in the presence of arsenite. Table IX summarizes the data on which the preceding assertion is based. The experiments were carried out essentially as the one just described, except that incubation of the cells took place aerobically by shaking the flasks containing the cells, glutamic acid, and inhibitor. Keto acid was again determined in the internal environment by breaking the cells open and assaying the extract.

The effect of arsenite, so evident in the internal environment, could hardly be demonstrated in the external environment, where a small amount of keto acid was demonstrable both in the absence and presence of the inhibitor (maximum accumulation 12.5 μM from 2000 μM glutamic acid in 15 hours, under the experimental conditions described in Table IX). It is possible that the conditions for accumulation of keto acid in the external environment were less than optimal, and that greater accumulation could have been produced, if a study of optimal conditions had been made.

TABLE VIII

ANAEROBIC ACCUMULATION OF KETO ACID UNDER VARIOUS CONDITIONS INSIDE WHOLE CELLS OF B. TULARENSE

Contents of tubes (Variables only)	Keto acid *	
	µM / 3 ml	Total µM
L-glutamic acid	0.28	0.45
L-glutamic acid and TTC	2.66	4.44
L-glutamic acid, TTC, and sod. arsenite	2.03	3.38

* Corrected for endogenous accumulation of keto acid, which was determined on all three systems; maximum accumulation was 0.11 uM/3 ml

Cells: Culture grown for 8 hours 45 minutes; treated as described, 5ml/tube

Buffer: 0.1 M (final) K phosphate, pH 5.9

Substrate: 2 mM L-glutamic acid, as neutral solution of K salt

Triphenyl tetrazolium chloride (TTC): 2% (final)

Sodium arsenite: 5×10^{-5} M (final)

Total volume: 10 ml; Temp. 37 C

Gas phase: Nitrogen ; Time of incubation: 15 hours

Cells seperated by centrifugation, and, after washing, disintegrated in the Raytheon oscillator for 45 minutes at 150 - 160 plate volts.

Alpha ketoglutaric acid was used for preparation of standard curve.

TABLE IX

AEROBIC ACCUMULATION OF KETO ACIDS INSIDE WHOLE CELL OF *B. TULARENSE* IN PRESENCE AND ABSENCE OF SODIUM ARSENITE

Exp. no.	Age of culture	Incubation time	Glutamic acid	Treatment	Total keto acid accumulated *
	hours	hours	μM		μM
195	14	19	2,000	none	0.35
			2,000	5×10^{-5}M arsenite	2.3
215	9	15	2,000	none	0.28
			2,000	5×10^{-5}M arsenite	3.1

* Corrected for endogenous accumulation with and without arsenite; values approximately one tenth of experimental ones.

Exp. 195: Cells: Culture grown for 14 hours as previously described 10 ml/flask

Buffer: 0.1 M (final) K phosphate, pH 5.9

Substrate: as indicated

Sodium arsenite: Concentration as indicated (final)

Total volume: 25 ml; Gas phase: air; Temp. 37 C

Exp. 215: All reagents and conditions as described for Exp. 195, or as indicated in Table; except:

Cells: Culture grown for 9 hours as previously described 5 ml/flask

Total volume: 10 ml

A comparison of the effect of arsenite on an anaerobic system and on the aerobic ones indicates that the inhibitor blocked further oxidation of the keto acid specifically; in the absence of oxygen this reaction was blocked automatically and the inhibitor was superfluous. The small reduction in accumulation of keto acid in its presence is believed to be due to excess inhibitor.

The method of Friedemann and Haugen (1943), used in these experiments for the estimation of keto acids, is a general one, though optimal conditions for the assay of alpha ketoglutarate were employed. Nonetheless it was felt that more precise identification of this acid was necessary. For this purpose, 2,4 dinitrophenylhydrazones (DNPH) of oxalacetate, pyruvate, and alpha ketoglutarate were prepared. These, and the unknown accumulated in various experiments, were treated and chromatographed according to the method of Cavallini et al. (1949 a,b). Preliminary experiments showed that the unknown was not the DNPH of pyruvate, since the unknown moved much more slowly on the chromatogram than the pyruvate derivative; in fact the unknown moved at approximately the same rate as the DNPH of alpha ketoglutarate and oxalacetate. Separation of these derivatives and the unknown was difficult, because their RF values were close. (The RF values reported by Cavallini for the DNPH of oxalacetate and alpha ketoglutarate were 0.28 and 0.26 respectively). The identification of the unknown therefore could not be based on a comparison of the RF values of the single compounds alone; instead, mixtures of the unknown and known DNPH were chromatogrammed together. If,

in this mixture, the unknown and the known compounds travelled at the same rate, so that only one round spot appeared on the chromatogram, then the unknown and known were the same compound; if an elongated spot or two spots appeared, then the unknown and known were different compounds. The results of such a chromatogram are given below:

2,4 Dinitrophenylhydrazone of	RF*	Remarks
Oxalacetate	0.22	round spot
Alpha ketoglutarate	0.24	round spot
Oxalacetate + alpha ketoglutarate	0.24	elongated spot, twice the size of single spots
Oxalacetate + unknown	0.19 0.15	2 spots
Alpha ketoglutarate + unknown	0.20	1 spot

Solvent: Butanol 50; Ethanol 10; water 40 (v/v)

* $\frac{\text{Distance travelled by 2,4 dinitrophenylhydrazones}}{\text{" " " solvent}}$

From these data it is evident that the unknown is the phenylhydrazone of alpha ketoglutaric acid.

The foregoing experiments indicate that dehydrogenation of glutamic acid does occur in B. tularense, and that the end product of this reaction is alpha ketoglutarate. Furthermore, the reaction proceeds only in the presence of a suitable electron acceptor. The greatest conversion of glutamic acid to alpha ketoglutarate was carried out by cell-free extracts at pH 7.0. One tenth of the initial substrate was converted in 2¼ hours.

DISCUSSION

Oxidation of glutamic acid by resting cell suspensions of B tularense is dependent upon the presence of phosphate in the environment. This dependence is best demonstrable with dilute bacterial suspensions. The data presented in Fig. 3, graph B, indicate that in the absence of phosphate, oxidation of glutamic acid does not proceed. In another experiment, 10 uM of glutamic acid and a dilute suspension of B. tularense were incubated together for 3 hours in the absence of phosphate. 96 per cent of the initially added glutamic acid was recovered at the end of the incubation period. The phosphate dependency is less evident in the data presented in Fig. 2, since a considerable amount (84 µl) of oxygen was taken up in the absence of phosphate, although the boost obtained by additional phosphate is undeniable. This respiration in the absence of phosphate increased with increasing cell concentration. The cell suspension used in the experiment presented in Fig. 2 was derived from 125 ml of culture, whereas the cells used in the experiment represented in Fig. 3 were derived from a 30 ml culture. It may well be that intracellular phosphate supports oxidation in the absence of an exogenous source.

The stimulation of acetate oxidation of Corynebacterium creatinovorans by phosphate is similar to the phosphate stimulation of glutamic acid oxidation of B. tularense.

However, in the acetate system it was not possible to stop the oxidation by mere withdrawal of the phosphate (Guzman Barron et al., 1950). The function of the phosphate, in the acetate as well as in the glutamic acid oxidizing system is not clear. If, in the acetate system, phosphorylation preceded oxidation, then replacement of acetyl phosphate for acetate should have increased the rate of oxidation or shortened the induction period. These effects were not observed.

In studies of glutamic acid oxidation by other microorganisms (H. parainfluenzae, H. pertussis, B. abortus, etc.) a "phosphate" effect has not been reported, nor was it found that a coupling of this oxidation with an exergonic reaction was necessary, before glutamic acid disappearance could be demonstrated. However, in studies of amino acid assimilation by Strep. faecalis and Staph. aureus Gale found that assimilation of glutamic acid would take place only in the presence of another, energy yielding, reaction (Gale, 1947; Gale and Taylor, 1947). This energy was usually supplied by an exergonic metabolic reaction such as glycolysis. It is possible that in B. tularense a coupling of glutamic acid dissimilation to an exergonic reaction is essential, and that phosphate is needed for the exergonic reaction to take place. This hypothesis needs to be verified experimentally by following the disappearance of glutamic acid in the presence of an energy yielding substrate such as glucose, in the absence of phosphate. It is of interest to note that in Gale's experiments phosphate

could neither replace glucose, nor was it essential for assimilation of glutamic acid in the presence of glucose.

A most interesting phosphate effect was demonstrable with rat liver mitochondria (Lardy and Wellmann, 1952). Using alpha ketoglutarate as substrate, the rate of oxygen uptake decreased to zero when the concentration of inorganic phosphate fell to 2×10^{-4} M. Addition of more phosphate (30 µM) brought about renewed oxidation immediately. Included in this system were hexokinase and glucose as an acceptor of high energy phosphate bonds. In the presence of sufficient inorganic phosphate, but in the absence of the acceptor system, the rate of oxidation of the substrate was low. An 8 to 15 fold increase in the oxidation rate was obtained by addition of the phosphate acceptor system to the reaction mixture. These experiments show clearly that inorganic phosphates and phosphate acceptors play a large role in the regulation of rate of metabolic processes. The role of phosphate is well illustrated by the Pasteur effect. Johnson (1941, b) believes that this effect (i.e., a decrease in the rate of utilization of carbohydrate upon admission of oxygen to the system) can be explained on the basis of low concentrations of inorganic phosphates or phosphate acceptors, brought about by phosphorylations occurring in the presence of oxygen.

Like resting cell suspensions, undialyzed cell-free extracts of B. tularense dissimilate glutamic acid, using molecular oxygen as the overall electron acceptor. Dialyzed

extracts, on the other hand, are unstable with respect to molecular oxygen. They can carry out oxidation of the substrate, but the enzyme system so reduced cannot be reoxidized by molecular oxygen. This same observation was made by Adler et al. (1938) using cell-free extracts of E. coli. By measuring oxidation of reduced CoII they found that dehydrogenation could not take place in air, and concluded that the system was lacking in flavin enzyme. When the reaction was carried out anaerobically in the presence of Methylene Blue, reduction took place. Stumpf and Green (1944) induced a similar phenomenon while investigating L-amino acid oxidase of Proteus vulgaris. Using HCN, they inhibited the enzymatic activity of their material 88 per cent, when tested aerobically, while no inhibition could be demonstrated when tested anaerobically. This showed that the inhibitor blocked oxidation of the reduced enzyme by molecular oxygen, whereas the reduction of the enzyme by the substrate was not affected.

Unfortunately the observations of the blocked oxidative pathway of cell-free extracts of B. tularense were made towards the end of this investigation, so that the characterizations of the enzymes and coenzymes involved in the block remain as a problem for further study. The study might be carried out in two ways: attempts to relieve the block by addition of substances known to function in the electron carrier systems of other microorganisms and animal tissues, i.e., flavin adennine nucleotide, and analyzing for the presence of reduced CoI and CoII by measuring optical density of blocked

and unblocked systems in the presence of glutamic acid and oxygen at 340 mu. It may also be worthwhile to try to induce a similar block in undialyzed extracts by the use of specific inhibitors, such as CN^-, known to block the cytochrom systems.

Studies on the coenzyme requirements of the cell-free extracts were indecisive in the sense that several coenzymes (ATP, CoA, cocarboxylase) produced the same amount of stimulation. It is believed that with a more highly purified extract the stimulatory actions of these coenzymes would not be the same. Of course such a purified extract would have to be assayed anaerobically, since its oxidative pathway might be blocked.

The influence of the cell wall on the metabolic reactions of B. tularense was shown clearly by the following facts: compounds, such as alpha ketoglutarate and succinate, that may be predicted as intermediates in the dissimilation of glutamic acid, are oxidized at markedly lower rates than glutamic acid by whole cells; citrate was not oxidized by whole cells at pH 5.9; and malonate failed to inhibit the oxidation of succinate and glutamate at pH 4.5 and 5.0. Cell-free extracts, on the other hand, oxidized all these compounds at rates higher than glutamic acid, and inhibition of succinate and glutamic acid by malonate could be demonstrated at pH 6.5. Influence of cell wall permeability was also strikingly demonstrated by measuring the rate of

oxidation of alpha ketoglutarate, succinate, and pyruvate at different pH values. A definite decrease in rate with increase in pH could be demonstrated. These same observations were made by Gerhardt et al. (1953) on resting cell suspensions of Brucellae, and by Guzman Barron et al. (1950) on resting cell suspensions of O. creatinovorans.

These data indicate again that the permeability of the cell wall must be taken into consideration when evaluating experiments with whole cells. The lack of activity of whole cells on a specific substrate does not necessarily indicate a lack of the concomitant enzyme system in the internal environment of the cell.

The first step towards a study of the overall mechanism of glutamic acid oxidation was the establishment of the ratio, substrate: oxygen: carbon dioxide. These values proved to be less than the expected ones, if complete oxidation of glutamic acid had taken place. It is believed that complete oxidation did take place, but that it could not be demonstrated because the ratio was calculated on the basis of overall disappearance of glutamic acid, rather than on the amount which was actually respired. This problem could be attacked more successfully by the use of radioactive glutamic acid. By measuring the radioactivity of the initially added acid, and the amount assimilated by the cells, the actual quantity respired could be obtained and on this basis the substrate: oxygen: carbon dioxide ratio calculated.

The first step in glutamic acid oxidation by B. tularense, under the conditions of these experiments, was its dehydrogenation and subsequent deamination to alpha ketoglutarate. In all other microorganisms previously investigated for this reaction, this step was found to be linked to an electron carrier system of which either CoI or CoII is a member. That such a system is also operating in B. tularense is shown by the fact that the accumulation of alpha ketoglutarate is inhibited if the reaction is carried out anaerobically in the absence of an exogenous electron acceptor. Aerobically, oxygen acts as acceptor. The isolation of alpha ketoglutarate, from a suspension metabolizing glutamic acid, was a good indication that the amino acid is oxidized through this substance. Since this keto acid is a member of the tricarboxylic acid cycle, it was of interest to find out if fumarate, succinate, and citrate would also be oxidized. These components were tried as substrates for cell-free extracts and were found to be oxidized by them. Another indication that this cycle is involved in glutamic acid oxidation is the fact that this oxidation was blocked by inhibitors which block the respiration of alpha ketoglutarate, succinate, and citrate. Final proof of the operation of the Krebs cycle in B. tularense must, however, await the isolation of both succinate and citrate from suspensions oxidizing glutamic acid.

SUMMARY

1. Resting cell suspensions of B. tularense oxidize glutamic acid vigorously in the presence of 0.1 M phosphate buffer. The pH optimum for this reaction is broad, from pH 5.5 to 6.6. A definite drop in the rate of oxidation occurs at pH 7.0.

2. Cell-free extracts of B. tularense require Mg^{++} and a coenzyme solution, containing DPN, TPN, CoA, ATP, and cocarboxylase, for an optimum rate of oxidation of glutamic acid. DPN is the only essential member of this solution. Oxidation of glutamic will not take place in its absence, though it will occur when the other components are missing.

3. Dialyzed extracts are unstable with respect to aerobic oxidation; that is, they are inactive in the presence of molecular oxygen. When another electron acceptor, such as triphenyl tetrazolium chloride, is substituted for oxygen, these extracts oxidize glutamic acid to alpha ketoglutarate.

4. Resting cell suspensions oxidize glutamic acid to alpha ketoglutarate. In the presence of oxygen, this acid is further oxidized, but in the presence of sodium arsenite the reaction can be reduced and the keto acid accumulates. The action of arsenite causes accumulation of keto acid inside of the cells.

5. Resting cell suspensions oxidize succinate and alpha ketoglutarate at a slower rate than glutamic acid,

though the oxidative rate of these substances and pyruvate is increased with decreasing pH. Citrate is not oxidized by whole cells at pH 5.9, nor does malonate inhibit the oxidation of either succinate or glutamic acid at pH 4.5 and 5.0.

6. Cell-free extracts oxidize fumarate, succinate, alpha ketoglutarate, and citrate at a higher rate than glutamic acid. The oxidation of succinate and glutamate by these extracts is inhibited by malonate.

7. Alpha ketoglutarate was isolated from resting cell suspensions and cell-free extracts oxidizing glutamic acid, and was identified chromatographically.

8. Complete oxidation of glutamic acid is believed to take place, though the substrate: oxygen: carbon dioxide ratio was less than it would be, if complete oxidation had taken place. This is believed to be due to the disappearance of glutamic acid along a non-oxidative path, probably by assimilation.

9. Glutamic acid oxidation is inhibited by sodium arsenite, sodium malonate, and fluoroacetate, compounds which inhibit the oxidation of alpha keto glutarate, succinate and citrate.

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