A Study of the Oxidation of 2-Ketogluconate Using Cell Preparations of *Pseudomonas aeruginosa*

by

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ABSTRACT

Pseudomonas aeruginosa is known to dissimilate glucose by way of a pathway which does not involve phosphorylation at the hexose level. The established intermediates in this pathway are gluconate, 2-ketogluconate, pyruvate and finally the compounds of an unconventional tricarboxylic acid cycle. The major gap in our knowledge concerns the fate of 2-ketogluconate. The enzymes responsible for the degradation of this compound have proven to be very unstable and previous attempts to obtain an active cell preparation or cell free extract have met with little success. There is some evidence that drying cells in an atmosphere of carbon monoxide preserves the enzyme in question while interfering with the complete oxidation of 2-ketogluconate. This should result in the accumulation of an intermediate product and thus would make possible the elucidation of one more step in the sequence of reactions. For this reason the work on monoxide-dried cells was continued in the hope, that they would serve as a source of the 2-ketogluconate enzyme.

This technique produced a preparation with a good ability to oxidize 2-ketogluconate. However, the viscous nature of the preparation made centrifugal separation of the remaining live cells almost impossible.

Glucose and gluconate grown cells when suspended in a 45% solution of sucrose and subjected to sonic vibrations produced a reproducible cell free preparation with a good ability to oxidize 2-ketogluconate. This preparation had an optimum pH of 7.4 and a respiratory quotient of 3. The mechanism of this oxidation remains unexplored. Pyruvic acid was identified as a product of this reaction.

The crude sucrose sonicate was not stimulated by ATP and no phosphorylation could be detected aerobically or anaerobically by measuring acid stable (ester) phosphate. The preparation was not inhibited by 2.5×10^{-2} sodium fluoride. Moreover, a crude sonicate of gluconate grown cells in the presence of ATP and magnesium, showed no phosphorylated compounds, by the chromatographic methods employed. The study for detection of phosphorylated compounds was carried out under strict anaerobic conditions.

No new intermediates were isolated in the pathway of 2-ketogluconate breakdown and this pathway still remains unknown. However, this work has provided several methods of obtaining active cell preparations.

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INTRODUCTION

The universal existence and acceptance of the Embden-Meyerhof scheme as a mechanism for the anaerobic dissimilation of carbohydrates and the Warburg-Dicken's system plus the tricarboxylic acid cycle as mechanisms for the aerobic dissimilation of carbohydrates has caused many workers to view any new metabolic pathway with suspicion. This attitude is particularly true with the pathway described for Pseudomonas aeruginosa ATCC 9027 (29). In contrast to the established pathways this one does not involve phosphorylation at the hexose level and therefore seems to contradict our accepted ideas on energy generation and transfer. It has been shown that this organism oxidizes glucose to carbon dioxide and water by way of gluconic, 2-ketogluconic (29) and pyruvic acids (34). The pyruvic acid in turn is oxidized by way of an unconventional tricarboxylic acid cycle (8).

Studies on oxidation of hexoses are relatively new, the initial work being done by Dickens (13). In a preliminary note in 1936 he outlined a scheme of carbohydrate oxidation which consisted of the esterification of the hexose to hexosemonophosphate, oxidation of this compound to the phosphohexonate, further oxidation to 2-ketophosphohexonate, decarboxylation to a pentose phosphoric acid. These oxidations and decarboxylations were pictured as continuing

until pyruvate was formed.

At that time evidence in favour of this scheme was slight. No proof of the formation of pentose phosphoric acids by oxidation of hexosemonophosphate or if phosphohexonate existed. The first data supporting the formation of 2-ketophosphohexonic acid as an intermediate was advanced by Lipmann (22) on the basis of oxygen uptake experiments. Lipmann found that phosphohexonic acid was oxidized by ground yeast, and that for each mole of phosphohexonate used one atom of oxygen was consumed, and 1 to 1.5 moles of CO_2 appeared. The amount of CO_2 was reduced to about 1 mole in the presence of monobromoacetate. Lipmann then advanced the theory that 2-keto-6-phosphogluconic acid was first formed ($\frac{1}{2}O_2$ consumed), this was then decarboxylated ($1CO_2$ liberated) and the further process consisted of the fermentation of the theoretically expected d-arabinose phosphate. This last stage was thought to be inhibited by bromo acetate. Some evidence tending to show keto-acid formation was provided by experiments (Dickens 1936 (13)) in which HCN appeared to act as a ketone fixative. In other experiments addition of carboxylase to a carboxylase free enzyme preparation caused a further oxidation and decarboxylation of phosphohexonate.

Warburg and Christian (1936 (35)) showed that the "end point" in Lipmann's experiments was only apparent and was in

reality due to a gradual destruction of enzyme activity. Dicken's experiments with HCN and carboxylase (13) apparently were too indirect, and could not be rigidly interpreted.

Later in 1938 Dicken's (12) established two critical points:

1. Pentose phosphoric acids arose from the oxidation and decarboxylation of hexose phosphoric acids.
2. Pentose phosphoric acids were readily oxidized and fermented by yeast extracts.

In the first part of this important paper the oxidation of phosphohexonic acid was described and the occurence of five carbon phosphoric esters giving the pentose reaction (Warburg & Christian 1937) was confirmed. In addition further oxidation of the phosphohexonic acid to a 4 carbon phosphoric ester was shown to occur. In the second part of the same paper it was shown that ribose pentose phosphoric acid was oxidized by yeast extract in the presence of coenzyme two.

In a subsequent paper (Dickens 1938) it was shown that pentose phosphoric acid was readily fermentable by yeast enzymes in the presence of coenzyme one and inorganic phosphate. The important point which arose during this work was that the pentose phosphoric acid most readily attacked both oxidatively and by fermentation was not arabinose - 5 phosphate which would be expected to arise from simple two carbon

oxidation and decarboxylation of glucose but was the stereo-isomer, ribose-5-phosphoric acid. Ribose itself was not fermented either by yeast cells or yeast extract, nor was it oxidized by brain slices or by yeast extract. Dickens advanced the theory that ribose-5-phosphate originated from hexoses by a process of phosphorylation, oxidation and de-carboxylation.

Although Dickens' work gave the field of aerobic metabolism a tremendous impetus it remained for Smyrniotis and Seigmuller to carry this line of thought to a fruitful stage. Horecker and Smyrniotis in 1950 (18) purified from yeast the enzyme which catalyzed the oxidation of 6-phosphogluconate. They found that pentose phosphate was produced quantitatively, according to the equation:

6-phosphogluconate + TPN $\longrightarrow$ pentose phosphate + CO_2 + $TPNH_2$

In addition 85 per cent of this pentose phosphate corresponded to ribose-5-phosphate on a paper chromatogram. Various other procedures such as the enzymatic removal of phosphate and paper chromatography with four solvent systems confirmed the presence of ribose. Their results placed ribose-5-phosphate in the direct pathway of phosphogluconate oxidation.

Finally in 1951 Smyrniotis and Seigmuller (17) employing a purified yeast preparation showed that glucose was oxidized by way of glucose-6-phosphate and 6-phosphogluconate. They then postulated that the next intermediate in the sequence was 3-keto-6-phosphogluconate which was decarboxylated to ribulose-5-phosphate. Ribulose-5-phosphate was identified as the

product of 6-phosphogluconate oxidation but the postulated intermediate still remains hypothetical never having been confirmed by experiment.

It was a year later that Etner and Doudoroff (14) showed a variation to the previously described scheme of Warburg and Dickens as completed by Smyrniotis and Seigmuller. These workers, using saccarophilia, showed that the pathway was identical to that previously described with the exception that 6-phosphogluconic acid was split to yield 3-phosphoglyceraldelhyde and pyruvic acid. The 3-phosphoglyceraldelhyde was then oxidized to pyruvate.

As previously stated the intermediate 3-keto-6-phosphogluconate is still hypothetical. But, recently in Belgium, J. De Ley (10) employing Aerobacter cloacae concluded that 2-keto-d-gluconic acid is phosphorylated by a specific kinase when employing adenosine triphosphate and magnesium. This worker concluded that his preparation contained a mixture of enzymes which decomposed the primary phosphorylated product into unidentified phosphate esters and hence he was unable to illustrate the presence of 2-keto-d-gluconate-6-phosphate. In addition De Ley claims that 2-keto-d-gluconokinase acts only when the bacteria are previously cultivated on 2-ketogluconate or, in other words, that the enzyme is adaptive in nature. Basing his argument on the premise that 2-keto-d-gluconate is formed during the reaction of this kinase, he

he felt that this compound was an intermediate in the direct oxidation of glucose.

Narrod and Wood (27) found that fluorescens A312 contained a magnesium dependent 2-ketogluconate phosphorylating enzyme. They used the formation of acid-stable (ester) phosphate and acid formation as a criteria for phosphorylation. With crude preparations they found that a 2,4-dinitrophenylhydrazine reactive keto acid was formed in the presence of both 2-ketogluconate and ATP but not 2-ketogluconate alone.

More support for a phosphorylated route comes from Claridge and Werkman (9) while working with aeruginosa 9027. These workers presented data to indicate that two glucose dissimilating mechanisms are operative in this organism. Cell free extracts of this organism were reported to phosphorylate glucose and the presence of triose-phosphate dehydrogenase and aldolase was shown. The aldolase had only weak activity and had its point of optimum activity at pH8.3. This would indicate that glycolysis is not important in this organism.

As previously stated the work from our laboratory does not support the phosphorylated mechanisms and favors a scheme which is operative without phosphorylation at the hexose level. The starting point of this work might be

traced back to Lockwood in 1940 (23) who reported that large quantities of gluconic and 2-ketogluconic acids accumulated during the growth of fluorescens on a glucose medium. Barron and Friedmann (2) later showed that the oxidation of glucose by aeruginosa was not affected by sodium fluoride and therefore did not involve phosphorylation. Again in 1946 Lockwood and Stodola (24) showed that fluorescens NRRL B-6 gave alpha ketoglutaric acid as a major product. Also in the same year these workers obtained a preparation which could oxidize pentoses to the corresponding pentonic acids (25). Four years later in 1950 Koepsell (20) found that fluorescens formed alpha ketoglutarate and pyruvate from gluconate during growth. The accumulation of these acids was influenced by the iron content of the medium. The above mentioned were interested only in the commercial production of organic acids and were not interested in pathways of metabolism. Koepsell, however, did mention that 2-ketogluconate undoubtedly was formed by the dephosphorylation of 6-phospho-2-ketogluconate.

Norris and Campbell (6) found detectable amounts of gluconic and 2-ketogluconic acids in sixteen and twenty-four hour cultures of aeruginosa when glucose was used as the growth substrate. Since the oxidation systems for these compounds remained strong over this period of time they concluded that the compounds must be formed and oxidized continuously thus being intermediates in a pathway of

glucose oxidation. The absence of inhibition by sodium fluoride, the lack of activity under anaerobic conditions, the absence of phosphorylated hexoses and the quantitative accumulation of 2-ketogluconate with dried cell preparations led Campbell and Norris (6) to conclude that a conventional glycolytic scheme was not present in aeruginosa.

Warburton, Eagles and Campbell (34) in 1951 demonstrated that at the end of sixteen, twenty-eight and forty hours there was an accumulation of pyruvic acid in glucose grown cultures of aeruginosa. In addition, these cultures had a strong ability for oxidizing pyruvate. In the same year Campbell and Stokes (7) offered data to show that resting cells had no ability to oxidize citrate cis-aconitate, isocitrate, alpha ketoglutarate, succinate or fumarate, whereas dried cells had the ability to oxidize the foregoing compounds and malate, acetate, oxalacetate and pyruvate in addition. These workers concluded that this organism must possess a conventional tricarboxylic acid cycle.

Many arguments have been offered for and against this non-phosphorylated scheme. For example, it has been stated that these compounds are produced as side reactions by members of the Warburg-Dickens' pathway. Wood and Schwerdt (36 and 37) have shown that cell free

extracts of fluorescens have many of the enzymes of the Warburg Dickens pathway but lack hexokinase and therefore are unable to oxidize glucose by way of a conventional Warburg Dickens. The work of Claridge and Werkman (9) would appear to refute the data since they have shown that aeruginosa can phosphorylate glucose.

No evidence to date can refute the data that aeruginosa can oxidize glucose by way of gluconic, 2-ketogluconic and pyruvic acids. However, no data are available on the fate of 2-ketogluconate during oxidation.

It was in 1935 that Bernhauer and Gorlick (3) first demonstrated that 2-ketogluconic acid was produced during bacterial dissimilation. Later Bernhauer and Knoblock (4 and 5) showed that when Acetobacter suboxydans was grown on calcium gluconate or glucose plus calcium carbonate, 2-ketogluconate and 5-ketogluconate accumulated. These data were criticized on the grounds that an impure culture must have been employed since no known organism produces both intermediates.

Twelve years after this work Katznelson and Tanenbaum (19) found that Acetobacter melanogenum oxidized glucose, gluconate and 2-ketogluconate to a common end product. This end product was tentatively identified as 2,5-diketogluconic acid. It was characterized chemically but unfortunately its instability at room temperature and

physiological pH prevented its being employed for manometric studies. McDonald (26) has been able to show that when cells of aeruginosa were dried in vacuo over calcium chloride in the presence of carbon monoxide and the absence of light they retained a limited ability to oxidize 2-ketogluconate and for all purposes behaved as a cell free preparation, being freely permeable to all substrates.

Although this work revealed no new intermediates it was felt that continutation of this method would make possible the identification of intermediates between 2-ketogluconate and pyruvate. It was the purpose of this thesis to continue using gas dried cells and also to discover any other readily available means for securing a preparation which would have a limited ability to oxidize 2-ketogluconate and thus accumulate identifiable intermediates. Such information might serve as a key to establishing the mechanisms whereby 2-ketogluconate is degraded to pyruvic acid.

METHODS

Bacteriological

The organism used throughout this study was *Pseudomonas aeruginosa* ATCC 9027 and was maintained on stock culture media consisting of:

Tryptone	-	1.0%
K_2HPO_4	-	0.5%
Glucose	-	0.1%
Glycerol	-	0.5%
Yeast Extract	-	0.1%
Agar	-	0.5%
Gelatin	-	2.0%

The stock cultures were transferred quarterly and examined microscopically at each transfer. Stock cultures were stored at approximately 6°C.

The medium used for obtaining active cells with a low rate of endogenous respiration was that of Campbell *et al.* (6). The medium was dispensed in 100 ml quantities in Roux flasks. After inoculation these were incubated at 30°C. for approximately 18 hours. The inoculum was prepared from a stock culture by transferring to a glucose agar slant at least three times at 24 hour intervals. When gluconic acid was the carbon source the growth from a glucose agar slant was washed into a Roux flask containing the previously described medium plus gluconate. This flask after incubation for 18 to 24 hours served as the source of the one per cent inoculum for the larger volume of media.

Sonic disruption of cells

Gluconate grown cells for cell free studies were washed once with M/30 pH 7.0 phosphate buffer after harvesting. The resultant cell paste was resuspended in a 45 per cent solution of commercial sucrose at a rate of 200 to 250 milligrams of wet cells per ml of sucrose solution. The cell suspension was subjected to sonic oscillation for fifteen minutes in a Raytheon 10 kilocycle oscillator. The resulting mixture known as the sonicate was placed in previously chilled plastic centrifuge cups and carried in a chipped ice bath to a Serval SS-1 centrifuge maintained at approximately -10°C. The sonicate was then centrifuged for exactly five minutes at 25000 times gravity and used immediately for manometric studies. The unused portion was kept frozen for future use.

The cells used for studies of the effect of fuel gas and varying levels of iron were treated as for desiccation studies except that all glassware employed was chemically cleaned. Chemically clean glassware was not used in the plating procedure.

Crushing of cells at low temperatures:

Glucose grown cells were harvested as previously described for treatment by sonic oscillation. The cylinder of a Carver laboratory press was previously chilled in a mixture of dry ice, and alcohol, dry ice and acetone or

just dry ice. The washed cell paste was pipetted into the chilled cylinder where it immediately froze. This was then subjected to a pressure of 12,000 pounds or until the frozen mass melted with the extreme pressure. The resulting proteinaceous mixture was then centrifuged at 5000 revolutions per minute at approximately 10°C for 15 to 20 minutes. After this treatment it was immediately used for manometric studies.

EXPERIMENTAL METHODS

Previous work with resting cells of Pseudomonas aeruginosa 9027 has shown that this organism has a strong ability to oxidize glucose, gluconate and 2-ketogluconate. In addition cells of this organism dried under vacuum with phosphorous pentoxide as a desiccant, retained their ability to oxidize glucose or gluconate quantitatively to 2-ketogluconate (32). Cell free extracts also will convert glucose or gluconate to 2-ketogluconate. This quantitative conversion is possible because dried cell preparations have no synthetic abilities and therefore do not exhibit oxidative assimilation. In addition they have lost their ability to oxidize 2-ketogluconate. Since dried cells are freely permeable and do not oxidatively assimilate, a block in the catabolic pathway will result in the quantitative accumulation of an intermediate. This in turn will make possible the isolation and identification of this intermediate compound and will aid in plotting the general direction of the pathway.

Hill worked for several years on various modifications of this drying technique but found that his treatments did not preserve the ability of the organism to oxidize 2-ketogluconate. However, McDonald obtained cell preparations with some activity towards 2-ketogluconate.(26). These active preparations were obtained by drying resting

cells *in vacuo* over calcium chloride in an atmosphere of fuel gas or carbon monoxide.

Despite the fact that McDonald obtained a preparation with activity toward 2-ketogluconate, many major problems remained outstanding. Firstly, the method of desiccating cells in an atmosphere of carbon monoxide produced a very high rate of endogenous respiration which made a quantitative interpretation of results impossible. Secondly, desiccation over phosphorous pentoxide in the presence of a partial vacuum produced cells with no activity on 2-ketogluconate suggesting inactivation of this enzyme by oxidation. Finally no available methods of rupturing bacterial cells had yielded a cell free preparation capable of oxidizing 2-ketogluconate.

Monoxide Dried Cells

The previously described mechanism whereby dried cell preparations accumulated intermediates was applicable to McDonald's monoxide drying method. It was felt that continued use of this method would reveal any intermediates between 2-ketogluconate and pyruvate and point to the pathway of oxidation of the former compound. The first problem which had to be approached was the reduction of the high rate of endogenous respiration while maintaining activity against 2-ketogluconate.

(a) Methods employed to reduce endogenous respiration. As

pointed out by Norris (28) the rate of endogenous respiration is of particular importance in a study of aerobic microorganisms. The importance may be attributed to the fact that the endogenous respiration is normally high and may be completely suppressed in the presence of a readily oxidizable substrate and therefore may be disregarded. More often, however, it is only partially inhibited by substrate. On the other hand a stimulation of the endogenous respiration may result in the presence of oxidizable substrate. Norris and Campbell (28) have shown that the endogenous respiration of aeruginosa is unaffected by substrate when this substrate is glucose. However, it was later found that this situation differed with different substrates. It seems probable that if the substrate is dissimilated by a pathway that has nothing in common with the pathway of storage product breakdown, then the rates of the two reactions will be independent. However, if a single set of enzymes is being competed for one might expect the endogenous to be suppressed in the presence of oxidizable substrate.

(b) Methods. The most usual approach to the problem of endogenous respiration is to work with cultures whose endogenous ability has been reduced to a point where it is not considered a serious factor.

However, it is not always possible to achieve this ideal and attempts must be made to reduce storage products that have accumulated. The first technique to accomplish this was that used by Quastel and Whetham on resting cells of E. coli. This is known as the starvation technique (38). Resting cells of aeruginosa were desiccated according to McDonald's technique. The cells were then suspended in one hundred to one hundred and fifty mls of distilled water and vigorously aerated 4 - 6 hours thus forcing the organism to oxidize its stored products. After this treatment the cells were harvested and again treated as previously with monoxide gas. The dried cells were used in manometric studies and although the endogenous activity was lowered the activity on 2-ketogluconate had disappeared. Several weeks of study using varying aeration times produced negative results.

Aging at low temperatures is a second method used to lower endogenous respiration. Monoxide dried cells were placed in a small test tube which was inserted in a 125 ml Buchner flask containing a shallow layer of anhydrous calcium chloride. Nitrogen was bubbled through the flask as well as the test tube containing the dried cells, for five minutes. The flask was then sealed shut with screw clamps and stored at -10°C.

The nitrogen atmosphere was employed in order to prevent inactivation by oxidation. This procedure did result in the production of dried cells with a low rate of endogenous activity but it also inactivated the 2-ketogluconate enzyme. Numerous attempts with variations of from 2 - 24 hours storage always gave the same results.

A third procedure called for storing the cells as a wet pack in the refrigerator at 10°C. Samples were removed at intervals from 4 - 24 hours and dried under monoxide gas. Results show little or no change in endogenous activity and again the cells were inactive against 2-ketogluconate.

The next attempt was to vary the time required for drying the cells. The normal procedure produced dried cells in from 48 - 72 hours. In order to dry cells quickly they were harvested and then spread over a porous plate with a minimum amount of distilled water. The cells were then scraped from the plate and dried in the usual fashion. Again as in the previous methods the results were negative. It was then felt that a high endogenous respiration was essential if 2-ketogluconate activity was to be maintained with monoxide dried cells.

(c) Attempts to obtain reproducible results with carbon monoxide-dried cells. Resting cells have a strong ability to oxidize 2-ketogluconate and there is no accumulation of intermediate

compounds. It is therefore essential that no living cells be introduced with the monoxide dried cells or a preparation capable of oxidizing all intermediates would result. Also the oxygen uptake data would always be positive and would be assumed to be due to the dried cells. Resting cells are impermeable to citrate, and therefore cannot oxidize it, whereas cell extracts or dried cells are able to oxidize citrate. Therefore the ability to oxidize citrate can be taken as a characteristic of a cell preparation. Cell extracts or dried cell preparations have no synthetic abilities and therefore cannot form adaptive enzymes. Malonate is oxidized by adaptive enzymes and so this substrate can be used for determining the presence of normal living cells. In addition plating cells on nutrient agar and counting the colonies could be used as a check on a number of living cells in the dried cell preparation.

Plate counts showed only a few colonies for a 1:100 dilution of 3 mgs. of the cell preparation and (figure I) shows the cell preparation was overwhelmingly a cell free extract. The curves in (figure II) represent data that were obtained at infrequent intervals and which were of little use to this study because of their non-reproducibility. The following methods, which

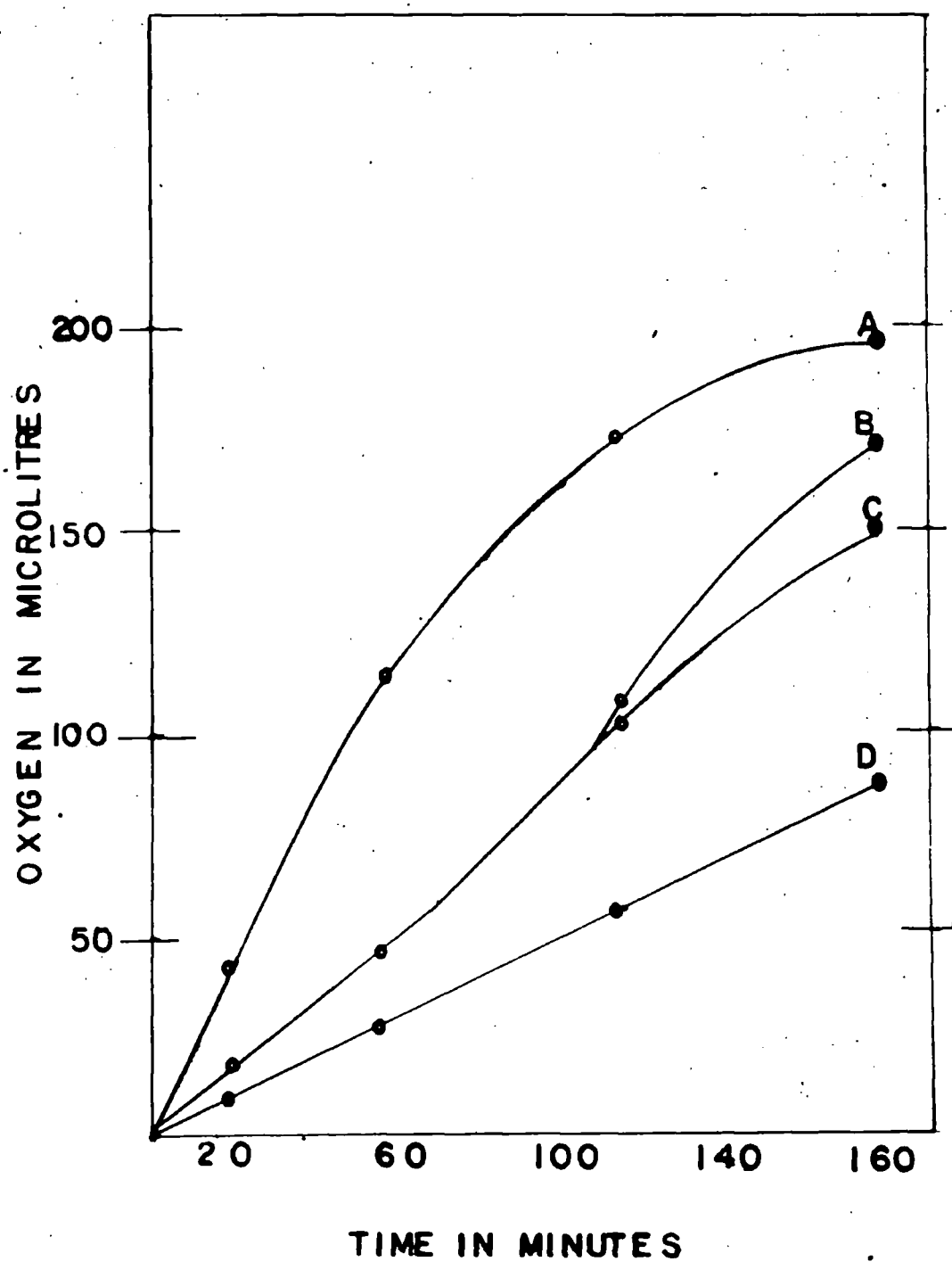


Figure I - Testing carbon monoxide dried cells for the presence of living cells by the Malonate-Citrate technique.

The oxygen uptake is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4. Each Warburg cup contained 20 mgs of dried cells.

A - Glucose
B - Citrate
C - 2-ketogluconate
D - Endogenous and malonate

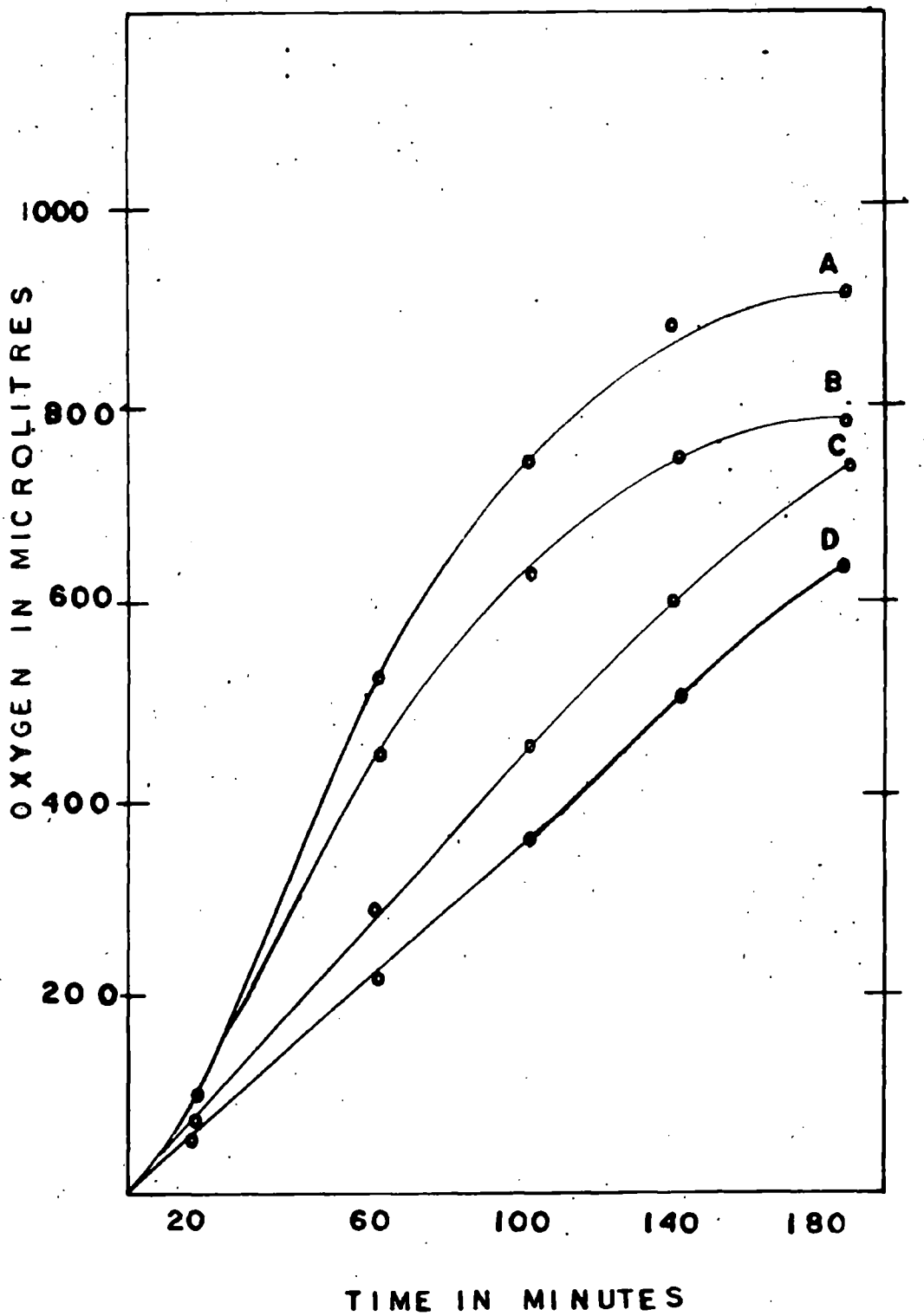


Figure II - Testing the activity of carbon monoxide dried cells on various substrates.

The oxygen uptake is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4. Each Warburg cup contained 20 mgs of dried cells.

A - Glucose
B - Gluconate
C - 2-ketogluconate
D - Endogenous

were used over a period of several months, were employed in an effort to limit the variables encountered during the growth and drying and therefore give a preparation that could be reproduced on any occasion.

By drying cells under a partial vacuum of hydrogen McDonald had produced a cell preparation with a weak ability to oxidize 2-ketogluconate (26). It was felt that a mixture of carbon monoxide and hydrogen gas would be more effective than carbon monoxide alone since hydrogen would exert a reducing effect on any sulfhydryl enzymes. Although the combination of gases which could be used are innumerable it was felt that a mixture of hydrogen and carbon monoxide would best serve the purpose.

Hydrogen was manufactured by dropping 6 N HCl onto mossy zinc and passing the resulting gas through water to absorb all HCl fumes. The carbon monoxide was manufactured as described previously. Both gases were permitted to mix freely in a Buchner flask before entering the desiccator. The results were again discouraging and there was little difference between monoxide dried cells as opposed to monoxide plus hydrogen dried cells.

The next step was to go back to the theory that a partial vacuum allowed oxidation and hence inactivation

of the 2-ketogluconate enzyme. It was decided that a petri dish filled with small pieces of white phosphorous could be placed in the desiccator during the evacuation and thus absorb all the oxygen present. These preparations were also inactive. Many variations of this theme were attempted, such as, carbon monoxide plus hydrogen dried in the presence of white phosphorous, or hydrogen or carbon monoxide alone with white phosphorous in the desiccator. But all of many duplicate or even quintuplicate trials produced negative results.

Most of the positive results were obtained with preparations which had been dried in the absence of light. It was felt that 20-30 minutes required for the gassing procedure might have been a sufficient exposure to light to inactivate the cells. During drying the desiccator was stored in the dark. To check on this possible source of trouble the desiccator was encased in a box completely sealed off from any light, subjected to gassing and left to dry without ever leaving the box. Several attempts at this procedure gave results comparable to the normal drying procedure under monoxide and were abandoned. A combination of carbon monoxide, hydrogen and white phosphorous with cells dried

in complete darkness also proved futile and again produced an inactive preparation. In addition an enzyme is much more stable in the presence of its substrate so the above procedure was applied to cells dried in the presence of 2-ketogluconate. Again, after numerous attempts results were negative or not reproducible. Salts of bile such as glycocholate have been shown to have a solubilizing effect on various enzymes and have been particularly successful in this laboratory when used to solubilize the gluconate oxidizing enzyme. It was felt that these salts might exert a solubilizing action on dried cells and the 2-ketogluconate activity might be preserved in the supernatant fluid. Dried cells at 20 mgs/ml were suspended in 1 gm. of glycocholate at a final concentration of 10% and left at room temperature for varying periods. The cells were then centrifuged and the ability of the supernatant to oxidize 2-ketogluconate tested. The results were negative.

The next course that remained was to see if drying had caused the preparation to lose its ability to synthesize coenzymes thus accounting for the lack of activity. The co-factors were made up as follows and were used at these concentrations for manometric studies:

Coenzyme A	-	20.00 units/.1 ml
DPN	-	0.02 micromoles/.1 ml
Glutathione	-	50.00 micromoles/.1 ml
Cysteine	-	0.30 mls of a 20% solution

Despite various combinations of these co-factors no stimulation was detected. Many weeks were spent trying to stabilize the dried cell preparation so that a reproducible preparation could be employed for further studies. Countless manometric studies produced negative results and the exhaustive study with the McDonald technique of monoxide dried cells would not appear to produce a stable workable preparation.

Fuel Gas Dried Cells

Fuel gas had the following composition:

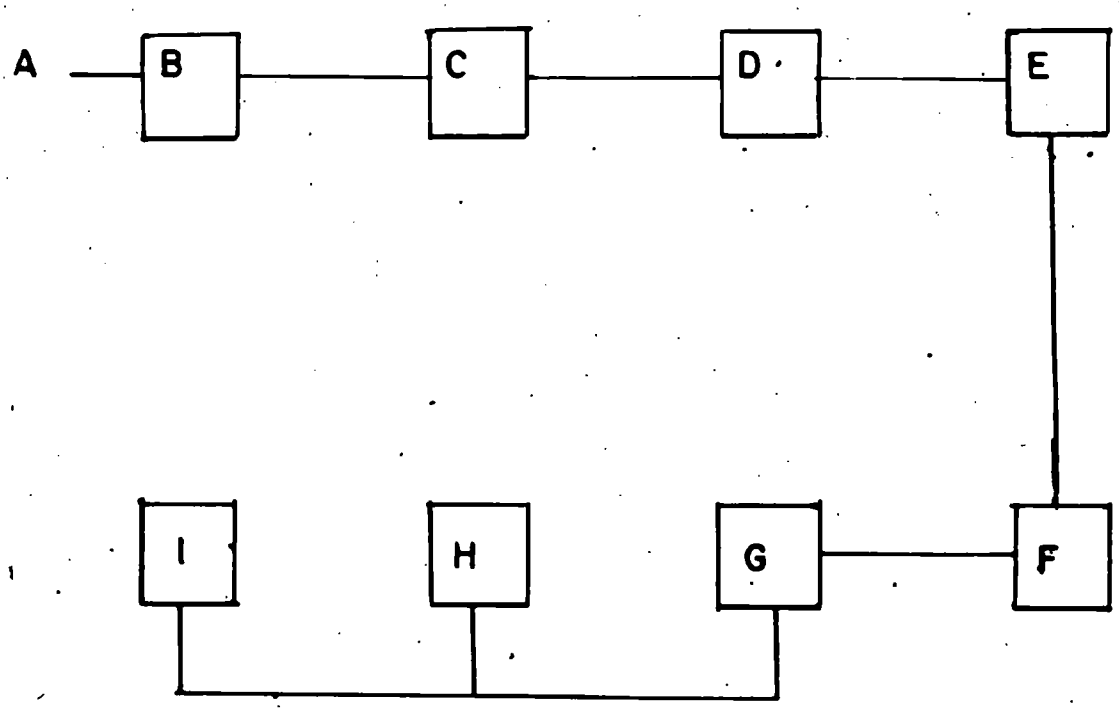
41.7%	hydrogen
27.4%	carbon monoxide
14.4%	methane
7.7%	nitrogen
3.8%	unsaturated gases ethylene, propylene, acetylene
3.6%	carbon monoxide
0.4%	oxygen

Although McDonald dried cells in an atmosphere of fuel gas and obtained no activity with them it was felt that this gas should be a good atmosphere in which to dry the cells provided chemical traps could be introduced to remove any toxic elements. Fuel gas provided an atmosphere containing the high percentage of carbon monoxide and hydrogen, both of which previously proved successful for drying cells, in a convenient and inexpensive state.

The gas was passed through alkaline pyrogallol (45% KOH and 15% pyrogallic) to remove oxygen; water, to catch any water soluble compound in the fuel gas; another pyrogallic trap to remove remaining traces of oxygen, and concentrated sulfuric acid to dry it before finally entering the vacuum desiccator. The desiccator was evacuated by vacuum pump and after refilling three times was left with a partial vacuum in an atmosphere of fuel gas. A schematic drawing shows the essentials of the setup (figure III).

Again, as with monoxide-dried cells, fuel gas-dried cells gave results which were not reproducible. Malonate-citrate and plating techniques previously described showed that a cell preparation had been produced thus warranting further experimentation.

(a)Attempts to obtain reproducible results from fuel gas dried cells. In agreement with Stern and Melnick in 1941 (31) it was felt that fuel gas bound the iron of cytochrome into an iron-monoxide complex. It was felt that any excessive iron might act as a catalyst for the oxidation of sulfhydryl groups during the drying period and so experiments with known limiting amounts of iron were carried out. Chemically clean glassware and iron added as ferrous sulphate septahydrate were employed.



FUEL GAS TRAPS
DIAGRAMMATIC

Figure III - Essentials of setup used in fuel gas drying technique Squares A to F represent a 250 ml Buchner flask containing the following:

A - Source of fuel gas
B - Alkaline pyrogallol (45% KOH and 15% pyrogallic acid
C - Water
D - An additional pyrogallol trap
E - Concentrated H_2SO_4
F - An empty Buchner to receive the dry gas
G - Vacuum pump
H - Vacuum guage
I - Vacuum Desiccator

The mineral medium of Campbell *et al.* (6) with the following variables was dispensed into Roux flasks.

Flask 1	-	No added iron
Flask 2	-	0.1 ppm iron autoclaved in medium
Flask 3	-	0.1 ppm iron added aseptically
Flask 4	-	2.0 ppm iron autoclaved in medium
Flask 5	-	2.0 ppm iron added aseptically
Flask 6	-	5.0 ppm iron autoclaved in medium
Flask 7	-	5.0 ppm iron added aseptically

Cells were harvested and then dried under fuel gas. The dried cells were used in the Warburg reaction vessel at the rate of 20 mgs per cup. Table 1 shows the results of the preliminary manometric trials.

TABLE I

THE EFFECT OF ADDING VARYING LEVELS OF IRON

Substrate	Iron addition ppm	Treatment	Oxygen Uptake
2-ketogluconate	0	-	(Oxidation not completed at 3 hours)- 175
"	0.1	added aseptically	125
"	0.1	autoclaved in	125
"	2.0	added aseptically	0
"	2.0	autoclaved in	0
"	5.0	added aseptically	0
"	5.0	autoclaved in	0

From table I it would appear that activity towards 2-ketogluconate decreased with increasing concentrations of iron in the growth medium. In addition, cells grown with no added iron had an unimpaired ability to oxidize the substrate to completion, showing no evidence of a block in the pathway of oxidation. The results were in accord with the original premise, but further substantiation with chemically clean glassware was necessary in order to insure the removal of all residual iron.

All glassware was left overnight in hot 10% nitric acid. It was then rinsed 10 times with tap water, twice with distilled water, filled with, or soaked in, glass distilled water and autoclaved at 15 pounds for 15 minutes. After autoclaving, the flasks were rinsed twice with glass distilled water. It was also necessary to remove iron from a 30% solution of glucose, which served as the growth substrate in these studies. This was accomplished by passing it through ion - exchange resin IRC - 50. The resin was conditioned by allowing it to remain overnight in 10% hydrochloric acid and then washing free of acid with distilled water. The resin now being in the anionic form was capable of adsorbing all cations from a 30% glucose solution. Since the glucose was neutral it

would pass freely through the resin and leave the iron behind.

The medium previously described (6) was made up in glass distilled water and sterilized at 15 pounds pressure for 15 minutes. Each Roux flask was inoculated with 5 drops of a 1/100 dilution of glucose grown cells and incubated for 18 hours at 30°C. The culture grown in the absence of iron had a very low count and the cells were extremely colorless as contrasted to normal pink cells obtained with 0.5 ppm iron. The cells were then dried under a partial vacuum of fuel gas in chemically clean petri plates.

TABLE II
MANOMETRIC STUDIES ON CELLS GROWN IN CHEMICALLY CLEAN GLASSWARE

Substrate	Iron ppm	Oxygen uptake μl
2-ketogluconate	0.0	88
"	0.4	135
"	0.8	0
"	1.0	0
"	5.0	0

The results from table II show that cells grown with no added iron had a limited ability to oxidize 2-ketogluconate. Although the cells were grown and dried in the absence of added iron, undoubtedly traces were

present and the results confirm the original hypothesis. Cells grown in chemically clean glassware in the presence of 0.4 ppm iron were similar to those grown in normal glassware with 0.5 ppm iron. The marked oxidative stimulation by iron demonstrates the dependence of over-all metabolism on an adequate hydrogen transport system. Further work in this field should prove to be very fruitful.

A series of experiments using 2-ketogluconate as the source of carbon in place of glucose were undertaken. The cells were desiccated as previously and the experiments were identical to those carried out when glucose was the sole source of carbon. From numerous Warburg runs the results were identical to those for glucose grown cells. Again, unreproducible results necessitated a new approach to the problem.

A search was begun for a chemical method to measure the disappearance of the substrate and in this manner substantiate the oxygen uptake data obtained in the presence of a high endogenous respiration. At the same time, it was felt that a quantitative measure of substrate disappearance would aid in identifying any intermediate compounds formed

during the oxidation of 2-ketogluconate. For this purpose the Folin-Malmros reducing sugar method was employed. This method was carried out as outlined by Umbreit et al. (33). Pure compounds such as 2-ketogluconate worked very well but a series of trials with dried cells were very disappointing. Unfortunately, the endogenous or control vessel also gave a blue color (positive test) even in the absence of 2-ketogluconate. All the variables were altered but still the positive test remained for the control vessel and the method was abandoned.

The method of Friedman and Haugen (15) which employs 2,4-dinitrophenylhydrazine as a reagent for alpha keto acids was also tested. The hydrazone is extracted with sodium carbonate and may be measured quantitatively. Despite many trials it would appear that the hydrazone of 2-ketogluconate will not form under the conditions described by these workers. The method of Lanning and Cohen (21) which is simple and rapid was tried. This makes use of the condensation of o-phenylenediamine with the ketohexonic acids to form 2-hydroxyquinoxalines.

The reagent was freshly prepared 2.5% aqueous solution of o-phenylenediaminedihydrochloride or one containing 15 mgs of free amine per ml. of 0.25 N HCl.

To 2 mls of a neutral solution containing 10 to 100 gammas of 2-ketohexonic acid was added 1 ml of reagent. The reaction tube was heated in a boiling water bath for 30 minutes and cooled to room temperature. Optical density when measured at 330 mμ and 560 mμ gave a ratio of 1.5 for 2-ketogluconate.

A standard curve was set up using solutions containing the following amounts of calcium 2-ketogluconate made to volume in a 25 ml volumetric flask. (Figure IV)

(1) 12.1 gms.
(2) 24.2 "
(3) 36.3 "
(4) 48.4 "
(5) 60.5 "
(6) 72.6 "

The reagent blank contained water as a solvent, since it behaved the same as M/15 pH 7.0 phosphate buffer in spectrophotometric studies.

Respirometer studies were carried out with fuel gas dried cells at 20 mgs of dried cells per cup.

PROTOCOL FOR MEASURING
2-KETOGLUCONATE DISAPPEARANCE

pH 7.4 M/15 Phosphate Buffer	Endogenous Cup	Reaction Cup
	1.5 mls	1.5 mls
Water	1.0 mls	0.8 mls
Cells	0.5 mls	0.5 mls
2-ketogluconate	-	0.2 mls
KOH	0.15 mls	0.15 mls
0.2 mls of substrate equals 5 μM. of 2-ketogluconate		

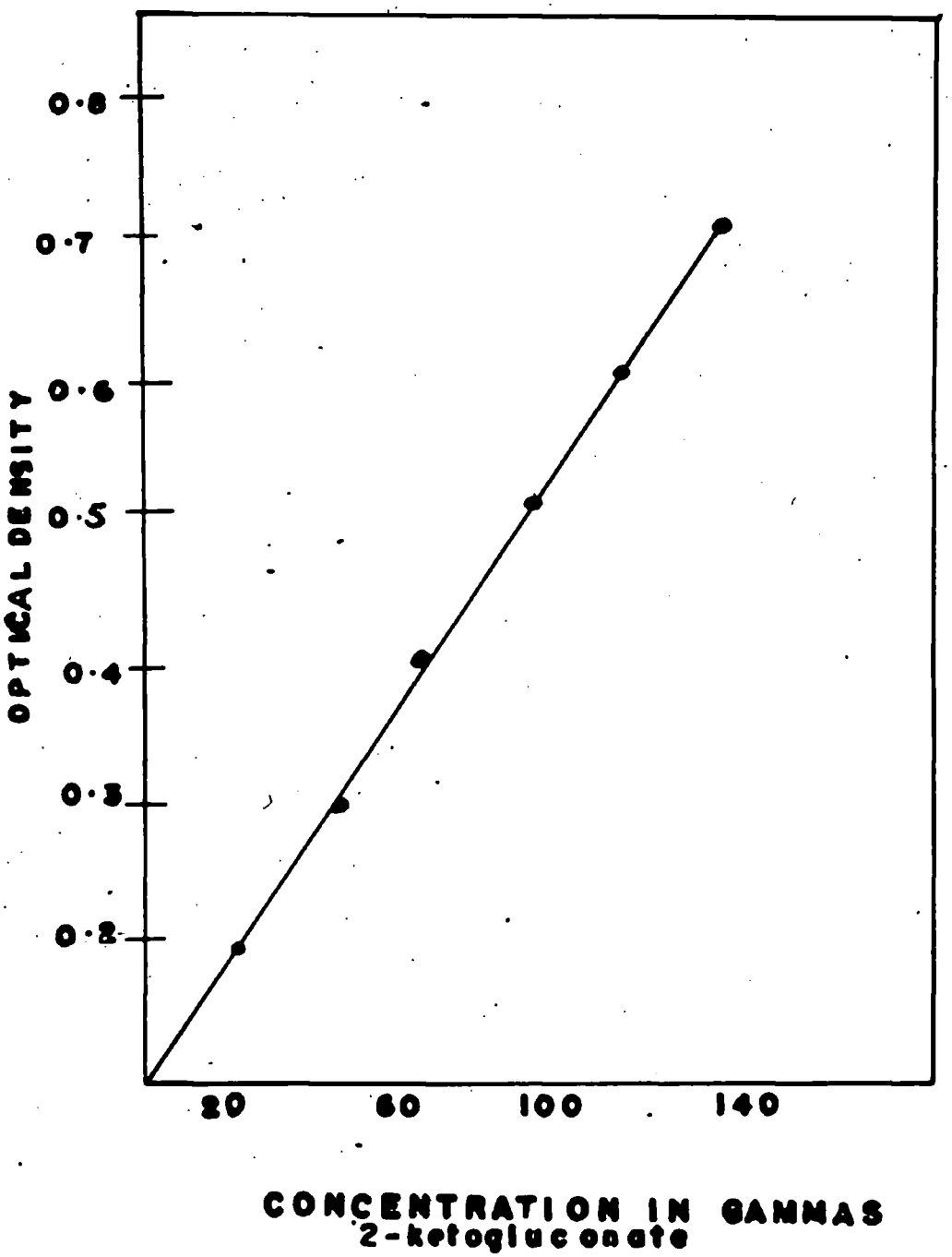


Figure IV A standard curve to measure pure 2-ketogluconate. To 1 ml of reagent (15 mgs of o-phenylenediamine per ml of 0.25N HCl) was added 2 mls of a neutral solution containing 2-ketogluconate in the following concentrations:

(a) 12.1 micrograms.
(b) 24.2 "
(c) 36.3 "
(d) 48.4 micrograms
(e) 60.5 "
(f) 72.6 "

The formation of hydroxyquinoxalines, by the reaction, was measured spectrophotometrically at 330mμ. The curve was interpolated beyond 72.6 micrograms of 2-ketogluconate.

Cells were weighed out on an analytical balance and suspended in distilled water at a rate of 40 mgs per ml. The cells were brought into suspension by use of a Van Potter Homogenizer. After the reaction was completed the cup contents were treated with 2 mls of a 30% perchloric acid and left overnight to deproteinize. After deproteinization the denatured protein was removed by gravity filtration. The supernatant fluid was then brought to neutrality with 10 N NaOH and made to volume in a 25 ml volumetric flask. In addition to volumetric flasks containing the endogenous and the reacted 2-ketogluconate, a third volumetric flask containing 1 ml of boiled dried cells plus 1.5 mls of pH 7.4 M/15 phosphate buffer and 5 μM of 2-ketogluconate was made to volume. The third flask was employed in order to determine the ability to recover 2-ketogluconate in the presence of dried cells. After treating 2 mls of each volumetric flask as previously described the solutions were read in a Beckman Model B spectrophotometer and the results are shown in Table III. The endogenous flask was used as a reagent blank:

TABLE III

RESULTS OF O-PHENYLENEDIAMINE TECHNIQUE

Substance	Optical Density at 330 mμ	Optical Density at 560 mμ	Ratio
Reacted 2-ketogluconate	0.36	0.55	0.65
Boiled cells plus 2-ketogluconate	0.72	0.51	1.44
2-ketogluconate	0.75	0.50	1.50

From table III it will be seen that the boiled cells give a proper ratio of 1.4 which is close to that of pure 2-ketogluconate which is 1.5. In addition this flask when calculated according to the standard curve proved to contain 4.9 micromoles of 2-ketogluconate. On the other hand, the reacted 2-ketogluconate gave a ratio of 0.65 which appears to show that the dried cell preparation had acted upon 2-ketogluconate and oxidized it to a new compound. In addition the compound fluoresced a light amber color under ultra violet in contrast to the unmistakable greenish-grey fluorescence of pure 2-ketogluconate. Although this method worked well with pure compounds the fact that new compounds were formed which masked any unreacted 2-ketogluconate made

it impossible to tell quantitatively the extent of 2-ketogluconate oxidation. Many attempts at varying deproteinizing solutions made no difference upon the technique with regard to the apparent formation of a new compound. This method was abandoned since it was inaugurated as a quantitative method to measure the disappearance of 2-ketogluconate. The new compound masked any residual 2-ketogluconate and since a 100 per cent conversion of this substrate was highly unlikely the method appeared unworkable. An outstanding feature of this technique, after many attempts, was the reproducibility of the standard curve when employing pure 2-ketogluconate. This reproducibility warrants further experimentation with cell preparations before it could become a useful tool in enzyme studies.

(b) Dialysis work with whole cells. It was decided that dialysis of 2-ketogluconate grown cells might remove any excess iron. This free iron could be responsible for catalyzing the oxidation of sulfhydryl groups. Cells were harvested at 18 hours and dialyzed against ice water. During this process the cells were stirred vigorously by a motor driven propellor for 8 hours. Aliquots were taken at various intervals from the dialysis sack and after being made to 57% transmission

in a Fisher electrophotometer were used in manometric studies at the rate of 0.5 mls per cup. Initial results were very encouraging but when dialyzed cells were employed against whole undialyzed cells the results were identical and it appeared that dialysis had no effect on whole cells.

Work on fuel gas dried cells was continued in order to obtain reproducible results with these preparations. It was observed during manometric studies that when the substrate was tipped into the cup after equilibration a white curdy precipitate was formed. Since the calcium salt of 2-ketogluconic acid was being employed as a substrate it was felt that the calcium was forming an insoluble precipitate with the phosphate buffer. IRC-50 resin was conditioned as previously described and the calcium 2-ketogluconic acid was shaken with the resin for several hours to remove the calcium. Although this treatment prevented precipitation it had no beneficial effect on the activity of the preparation.

(c) Work with Streptomycin. The factor which was to a large measure responsible for the unreproducible results of fuel gas dried cells was the survival of the small number of live cells. Streptomycin inhibits the formation of adaptive enzymes and therefore the synthesis of cell

protein but does not prevent oxidation. It was, therefore, felt that when used in conjunction with fuel gas dried cells it would prove very useful by preventing cell multiplication without inhibiting oxidation.

A solution of streptomycin containing 37.5 mgs/ml was made up and 0.1 mls was used for each Warburg cup. Cells were harvested and subjected to fuel gas. After a series of 20 Warburg trials the outcome was consistently as depicted in (figure V.) It will be noticed from (figure V) that the endogenous activity was reduced by the streptomycin treatment and that the oxidative ability was also reduced accordingly. Since a great number of unknown factors played a prominent role in this technique it was felt that it would be postponed until further work could be accomplished.

The work with co-factors was also carried out as for monoxide dried cells but again the results were negative and did not affect the preparations stability. Despite this fact a chromatographic survey was carried out but it failed to reveal any new compounds in the pathway of 2-ketogluconate breakdown.

Cell Free Preparations

The limiting factor in the utilization of many

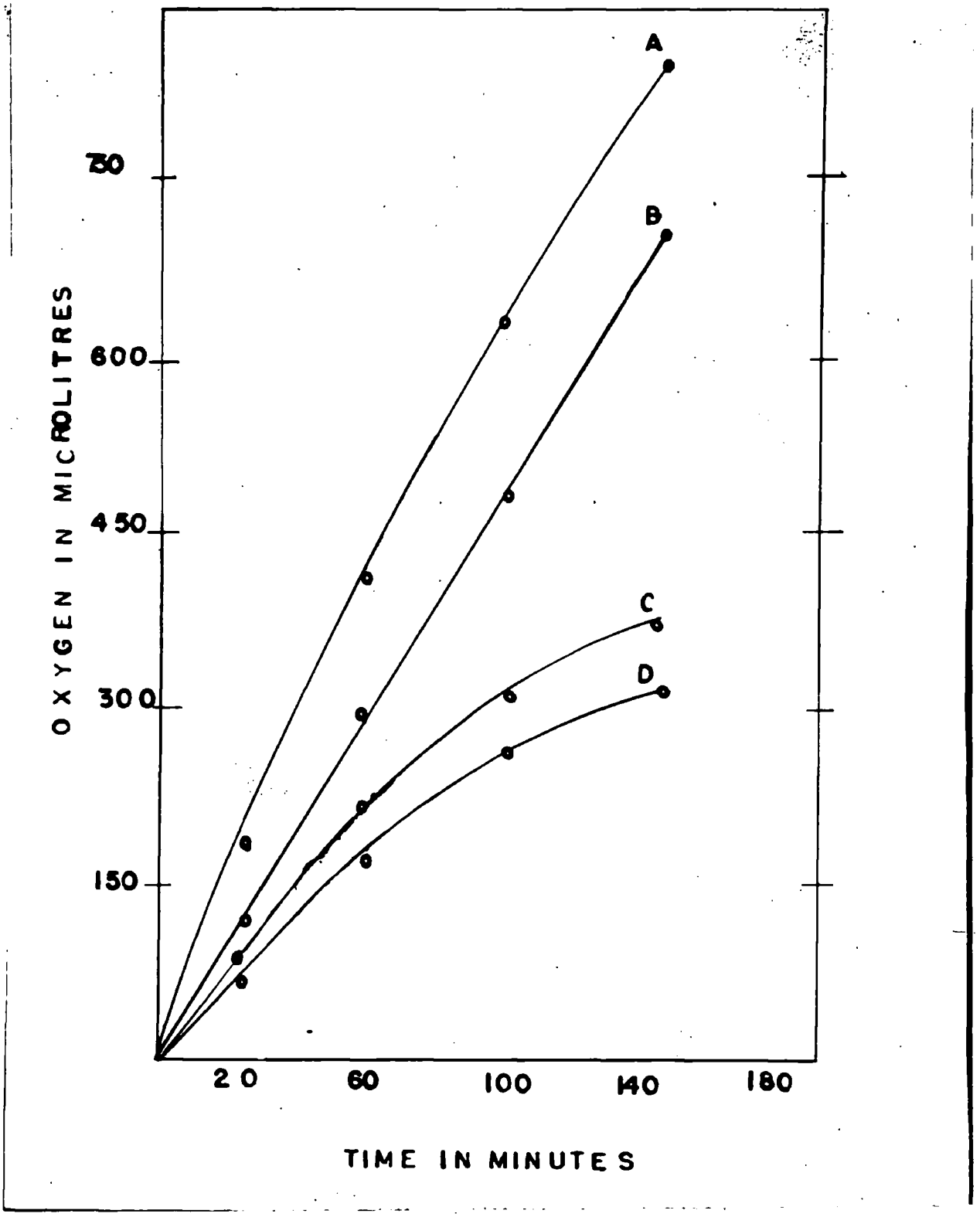


Figure V The effect of streptomycin on fuel gas dried cells. Oxygen uptake is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4. Each Warburg cup contained 20 mgs of dried cells.

A - 2-ketogluconate and fuel gas dried cells.
B - Endogenous
C - 2-ketogluconate, fuel gas dried cells and 3.75 mgs of streptomycin.
D - Endogenous plus 3.75 mgs of streptomycin.

intermediates is the permeability of the cell membrane. Campbell and Stokes (7) found that resting cells harvested from a growth medium containing acetate as the sole source of carbon had no ability to oxidize citrate, cis-aconitate, isocitrate, alpha ketoglutarate, succinate or fumarate without a period of adaptation. But when these cells were dried they had the ability to oxidize these compounds immediately illustrating the breakdown of this permeability barrier.

Many techniques have been advanced and many of these embrace the rupturing of the cell wall so that the bacterial contents may be released into a suitable medium and further studied. Hence, intracellular enzymes may be purified and studied without the interfering permeability factor. A valid criticism of isolated intracellular enzyme systems is that enzymes obtained in this manner are being studied in an environment which is completely foreign to them with regard to pH etc. Despite this criticism, which may be applied to many biological studies, a great deal of the work today has accrued from studies with cell free extracts. Successful work has been carried out in this laboratory with the gluconate oxidizing enzyme obtained from the cell by sonic vibration and

employed in a pure state with various hydrogen acceptors. Unfortunately, all previous attempts of applying this technique to the 2-ketogluconate enzyme have been unsuccessful. It was felt that a preparation obtained in this manner would be a big step towards solving the major problem of 2-ketogluconate degradation. The last portion of this thesis will be concerned with the trials attempted in order to obtain a cell free preparation of aeruginosa which would maintain its activity against 2-ketogluconate.

(a) Adapting the pressure crushing technique to enzyme work. A technique had been devised whereby frozen cells subjected to great pressure melt and burst open during this thawing process. This technique had been employed for studying the structure of bacterial nucleic acids and it appeared to be readily adaptable to enzyme studies. In particular the 2-ketogluconate enzyme being of an unstable character, might remain active with this technique. Glucose grown cells were harvested after 18 hours and washed once with distilled water. The cells were then suspended in distilled water at a concentration of 300 mgs/ml, slowly added to a cold steel cylinder and quickly frozen in a bath of dry ice. A steel piston was then inserted into the cylinder and subjected to 12000 pounds pressure

from a Carver Laboratory press. After centrifuging at 10°C. the preparation was employed at the rate of 1 ml/cup in manometric studies. (Figure VI) shows the promising nature of this technique with regard to its limited ability to oxidize glucose, gluconate, and 2-ketogluconate. But from (Figure VII) it will be seen that the results are inconsistent and unreproducible. Also, it would appear that live cells are multiplying since the reaction was not completed even after 3 hours. (Figure VIII) shows an excellent preparation with a good ability to oxidize 2-ketogluconate with the reaction completed after 140 minutes. But, again, it was not possible to reproduce this.

Table 4 shows the various methods used in an attempt to preserve the 2-ketogluconate enzyme during crushing:

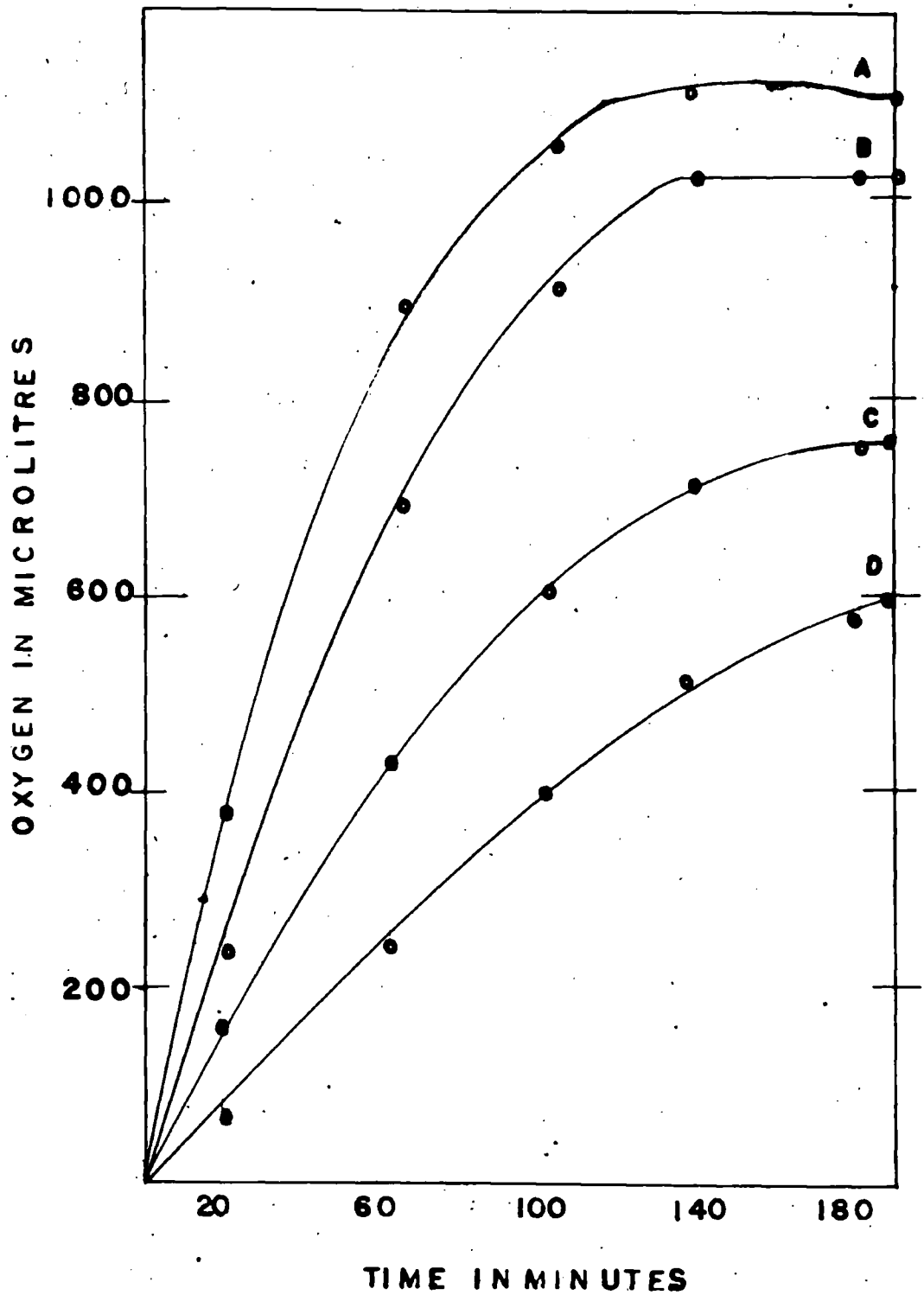


Figure VI A pressure crushed cell preparation showing activity on various substrates.
Oxygen uptake is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4. Resting cells were frozen 20 minutes before being subjected to 12000 pounds pressure.

A - Glucose
B - Gluconate
C - 2-ketogluconate
D - Endogenous

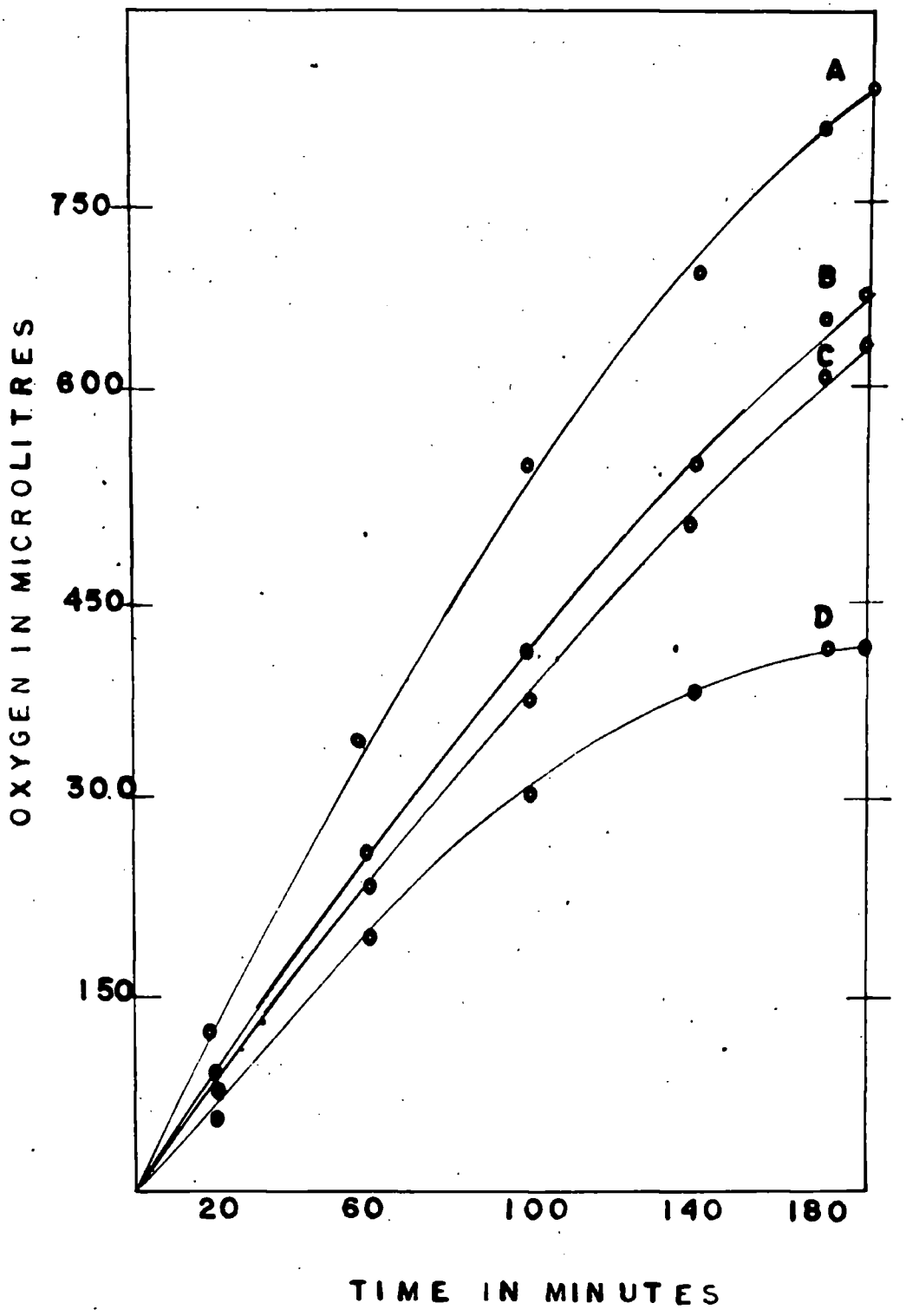


Figure VII - A pressure crushed cell preparation showing activity after 3 hours.
Incomplete oxygen uptake data is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4. Resting cells were frozen 20 minutes before being subjected to 12000 pounds pressure.

A - Glucose
B - Gluconate
C - 2-ketogluconate
D - Endogenous

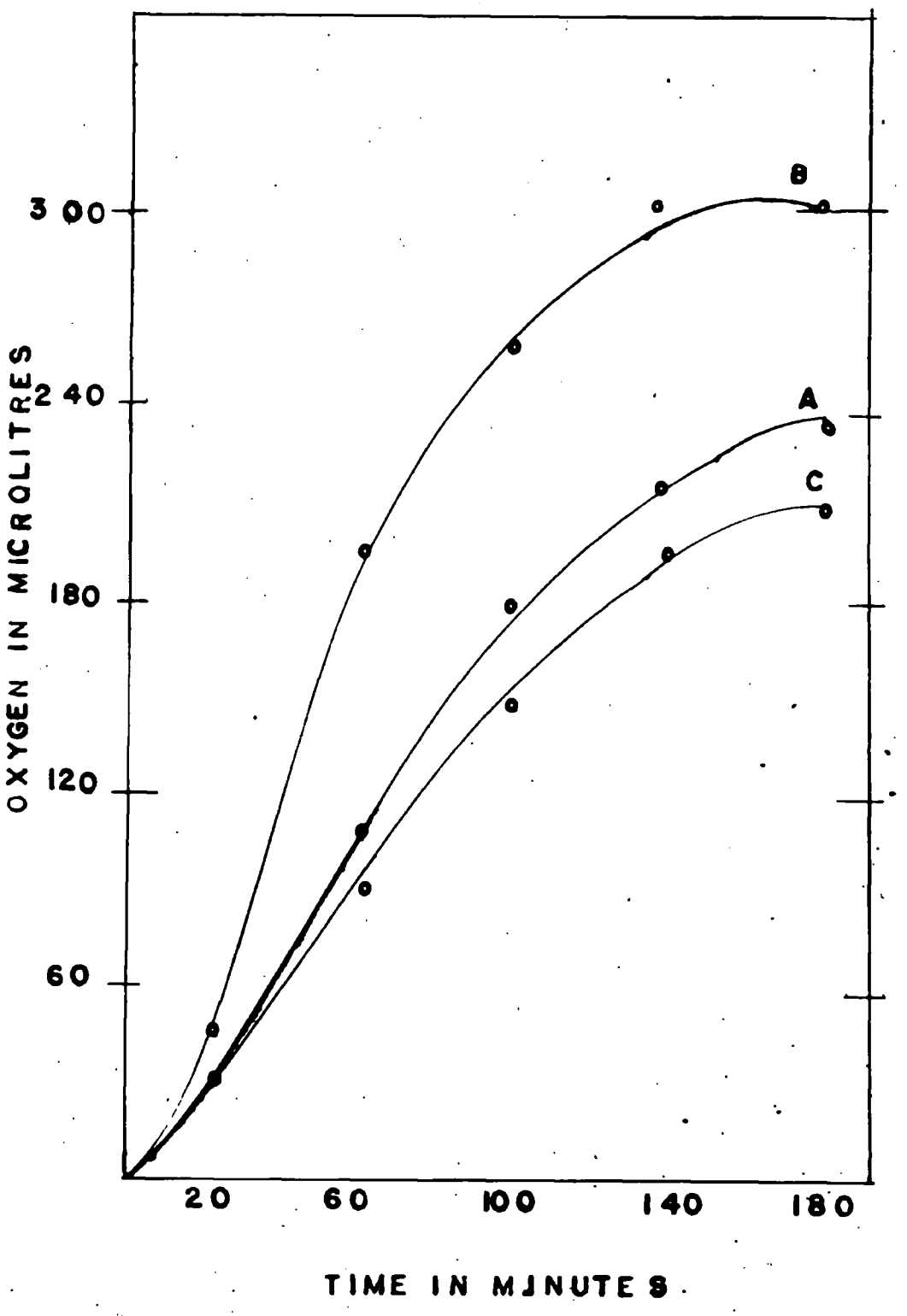


Figure VIII - A pressure crushed cell preparation.
Net oxygen uptake data is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4.

A - Glucose
B - Gluconic
C - 2-ketogluconate

TABLE 4

METHODS TO OBTAIN REPRODUCIBLE

2-KETOGLUCONATE ACTIVITY

Age of Cells	Conc.	Protective Substrate	Nature of Freezing	Result on 2-ketoglu-conate
18 and 24 hours	300 mgs/ml	7% sucrose	frozen 1½ hrs and crushed at 12000 pounds	No Activity
18 and 24 hours	300mgs/ml	0.3% Cysteine-Hydro-chloride	frozen 20 mins. crushed at 12000 pounds	No Activity
18 and 24 hours	300mgs/ml	2% lact-albumin	frozen 20 mins. crushed at 12000 pounds	No Activity

Another difficulty with the procedure was that whole uncrushed cells adhered to the gummy supernatant material and produced very erratic results. Many methods were attempted to correct this fault and the whole procedure may be summed up in Table V.

TABLE 5

RESULTS OF VARIOUS TRIALS

WITH CRUSHING TECHNIQUES

Carbon Source	Age of cells	Treatment	Results
(1) Glucose	18 hours and 24 hours	Centrifuged twice at 5000 rpm's @ 10°C.	Neglible activity
(2) Glucose	18 hours and 24 hours	Agitated gently with chilled Van Potter pestle and centrifuged twice at 5000 rpm's at 10°C.	No activity
(3) Glucose	18 hours and 24 hours	Diluted preparation with one volume of M/30 pH 7.0 Phosphate Buffer Centrifuged once @ 5000 rpm's @ 10°C.	No activity
(4) Glucose	18 hours and 24 hours	As above but in addition used 2,6-dichlorophenolindophenol as a hydrogen acceptor	No activity
(5) Glucose	18 hours and 24 hours	Cells frozen in ethyl alcohol dry ice bath	No activity
(6) 2-ketogluconate	18 hours and 24 hours	As for methods 1 to 6 inclusive	No activity

(b) Sonic Preparations. As stated previously all efforts to obtain sonicates active on 2-ketogluconate had met with failure. In this laboratory a Raytheon sonic oscillator vibrating at 10 kilocycles is employed. A laminated nickel rod is made to vibrate at 10 kcs. per second and this in turn moves a diaphragm up and down in a bacterial suspension and the cells are reportedly crushed by tremendous pressures due to cavitation. The cell wall bursts resulting in what is referred to as the crude sonicate. This sonicate is then centrifuged at 25000 xg for 30 - 60 minutes at -10°C. This removes all cell debris allowing the use of a cell extract in manometric studies. Preliminary studies using oscillating times of from five to eight minutes on a chilled preparation of 2-ketogluconate or glucose grown cells showed a very limited oxidation of 2-ketogluconate with a maximum uptake of 28 microlitres of oxygen. Positive results were infrequent, but work was continued and various agents were added to the preparation prior to sonicing. Cysteine or glutathione was added in varying amounts to protect any sulfhydryl enzymes, but repeated trials of different concentrations proved negative. For example, one trial in 20 might give an encouraging result. Variations in the suspending

medium for the cells were explored and various buffers from M/15 to M/50 and from pH 7.0 to 7.4 again gave negative results. Another resort was to vary the sonicing times from 5 to 8 minutes in place of the usual 15 minutes, and in this manner rupture sufficient cells to provide limited activity on 2-ketogluconate. The results varied from 10 to 28 microlitres of oxygen uptake and provided encouragement for continuation of these studies. The use of 8% sucrose was not successful but Saito (30) working on the biochemistry of vision had used 45% sucrose as his only means of obtaining a pure protein preparation. In addition Koepsell (20) reported that gluconate grown cells went completely past 2-ketogluconate to alpha ketoglutarate, whereas, glucose grown cells accumulated 2-ketogluconate and only slowly utilized it to alpha-ketoglutarate after a period of fourteen to eighteen days. Koepsell's work applied to fluorescens, but it was felt that gluconic grown cells of aeruginosa washed in a buffer and suspended in 45% sucrose might retain their activity against 2-ketogluconate. Figure (IX) shows the good activity on 2-ketogluconate with the preparation levelling off at almost 1 atom of oxygen. These results were easily duplicated and in

addition lost little activity during storage overnight in the frozen state at -10°C.(Figure X). The results appeared valid and work was continued on this method.

The crude preparation was tested for activity on sucrose. 10 micromoles of sucrose were employed as a substrate and no activity could be detected manometrically, during the same period that this preparation had taken up and levelled off at 38 microlitres of oxygen, when 2-ketogluconate was used as a substrate. Glucose grown cells were subjected to the same treatment (Figure XI) and also showed activity on 2-ketogluconate up to 1 atom of oxygen. In all cases the endogenous was very high so it was necessary to show the curves without the endogenous respiration.

(c) Respiratory Quotients. The respiratory quotient (RQ) is defined as the ratio of CO_2 produced to oxygen consumed. This is a valuable tool in determining the extent of decarboxylation of a specific substrate, for example, an RQ of 3 for 2-ketogluconate would indicate 3 molecules of CO_2 liberated for every oxygen taken up.

RQ's were carried out according to the method of Umbreit et al. (33) and the typical protocol for such a procedure is seen in Table VI.

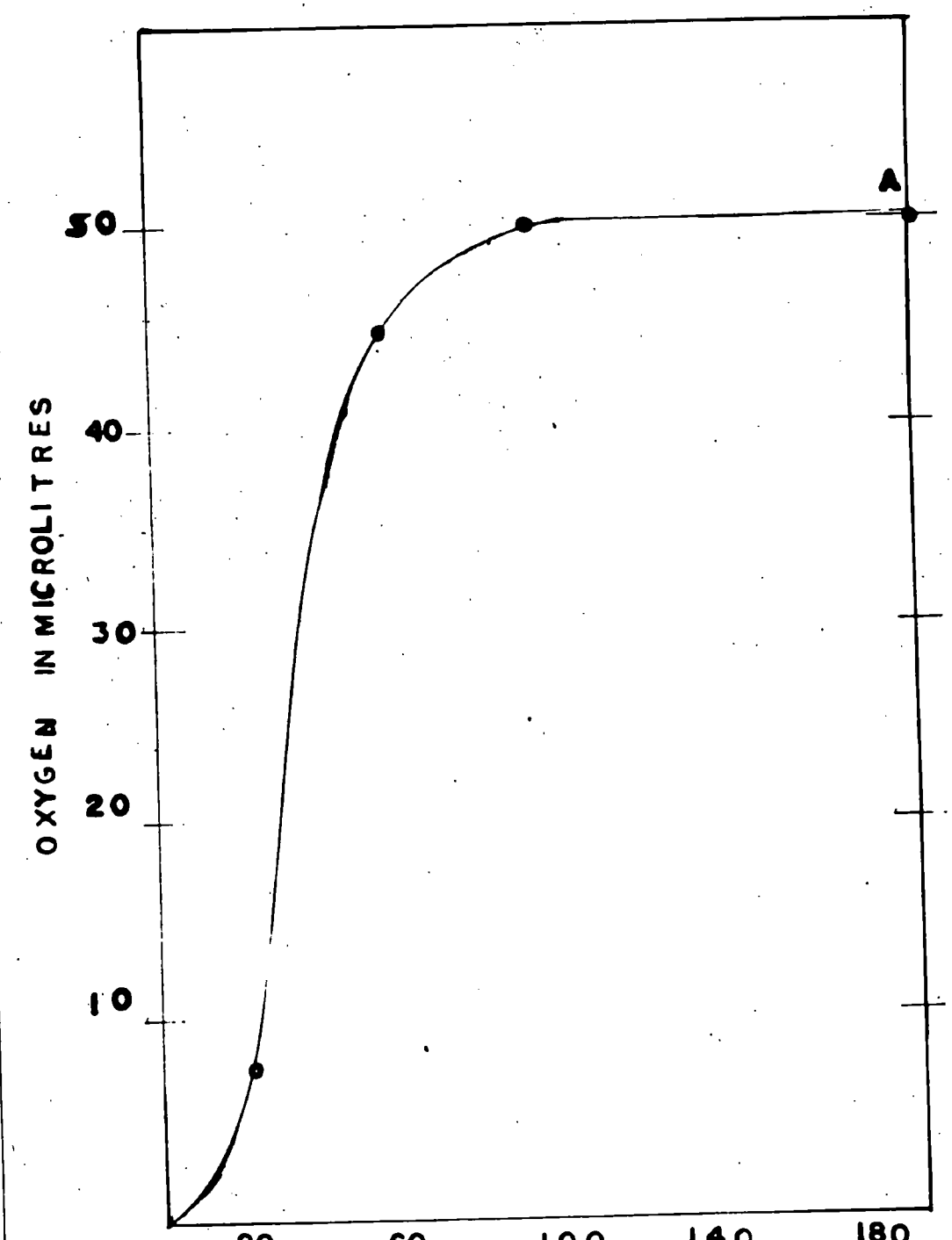


Figure IX - Gluconate grown cells soniced in the presence of 45% sucrose.
Net oxygen uptake data is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4. Soniced cells were used at the rate of 1 ml per Warburg vessel.
A - 2-ketogluconate.

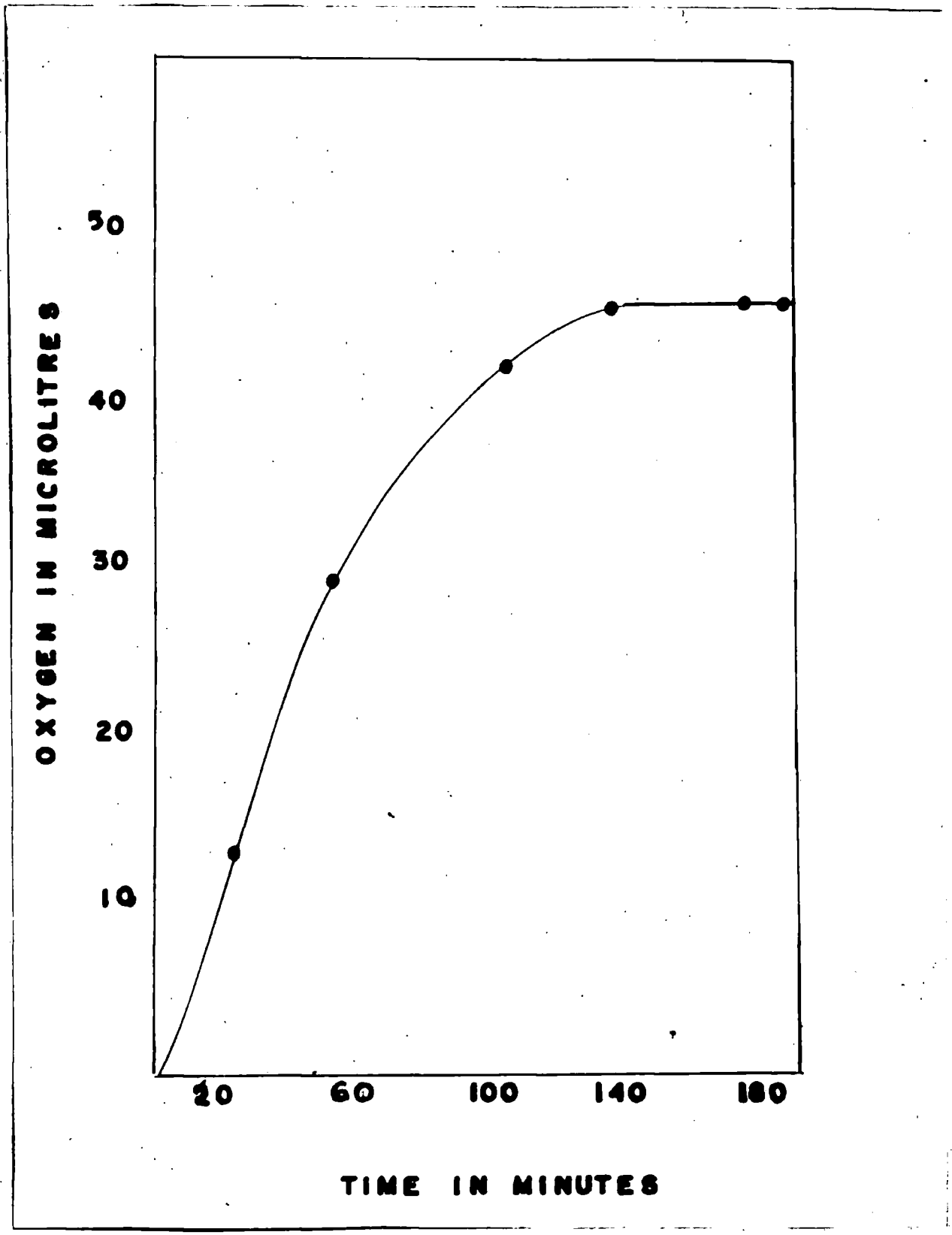


Figure X - Sucrose soniced preparation stored overnight in the frozen state.

Net oxygen data is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4.

A - 2-ketogluconate.

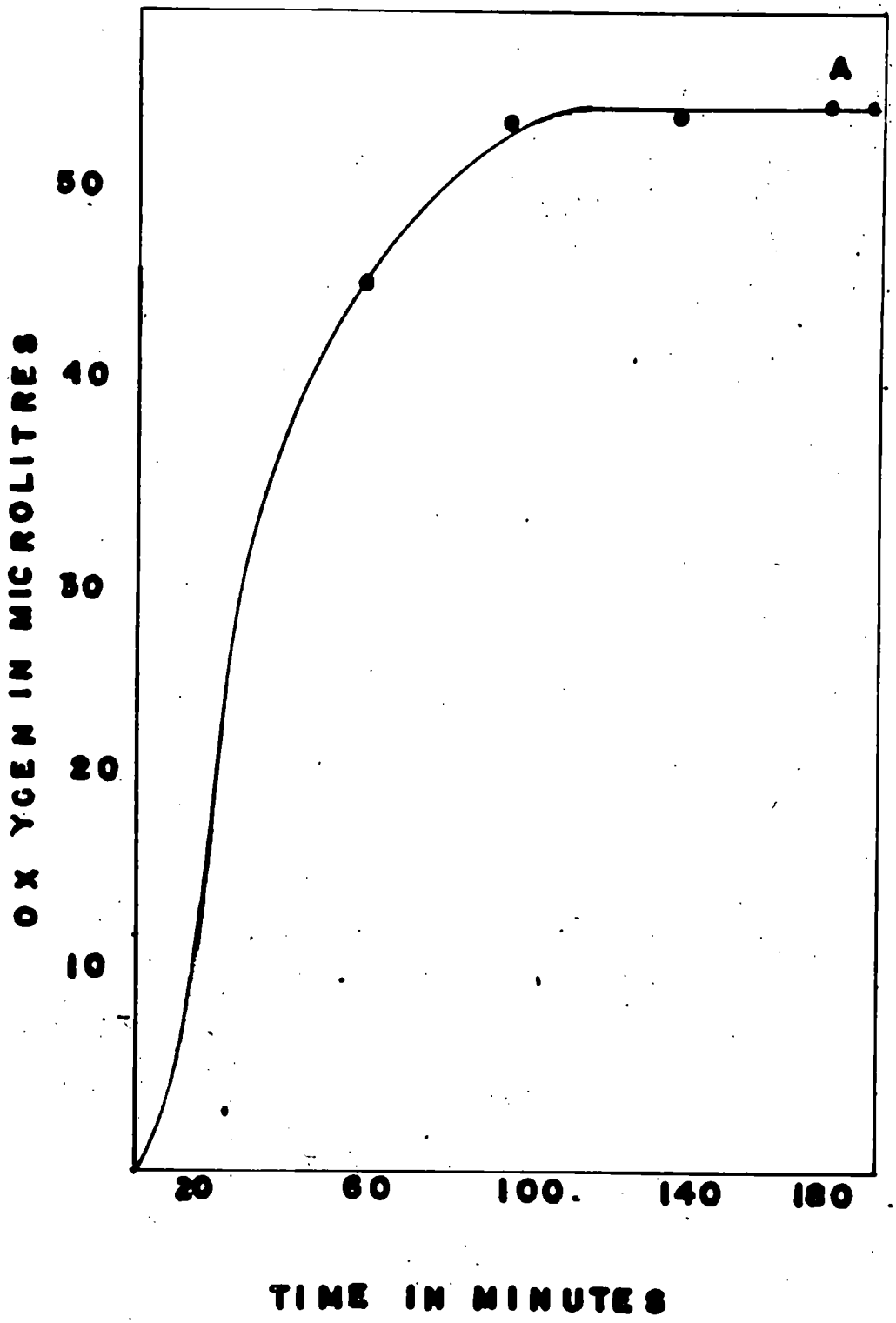


Figure XI - Glucose grown cell soniced in the presence of 45% sucrose.
Net oxygen uptake data is shown using 5 micromoles of substrate. The reaction was carried out at 31°C. and pH 7.4. Soniced cells were used at a rate of 1 ml per Warburg vessel.

A - Glucose

TABLE VI

PROTOCOL FOR DETERMINING RESPIRATORY QUOTIENTS

	Endogenous	2-keto gluconate	End.	2-keto gluconate	2-keto gluconate
M/15 pH7.0 Phosphate buffer	1.5 mls.	1.5 mls.	1.5 mls.	1.5 mls.	1.5 mls.
Water	0.5 mls	0.3 mls.	0.5 mls.	0.3 mls.	1.45mls.
Sonicate	1.0 mls.	1.0 mls.	1.0 mls.	1.0 mls.	-
Substrate	-	0.2 mls.	-	0.2 mls.	0.2mls.
KOH	0.15 mls.	0.15 mls.	-	-	-

The last cup shows the amount of CO_2 present in the reagents.

After the run was completed an equation was provided for calculating the RQ.

$$X_{CO_2} = (h - h'k_{O_2}'/K_{O_2})K_{CO_2}$$

h	= corrected reading of CO_2 evolved	h = -4.5
h'	= reading of manometer with KOH	h' = -40.3
k'_{O_2}	= constant of manometer with KOH	k'_{O_2} = 13.74
K_{O_2}	= constant of manometer without KOH	k_{O_2} = 17.61
K_{CO_2}	= constant of manometer without KOH	K_{CO_2} = 19.59

Endogenous CO_2

$XCO_2 = -.45-(-40.3 \times 13.74/17.61)\ 19.59 = 526.9$ of CO_2

CO_2 due to 2-keto. = 785.8 µl of CO_2
CO_2 due to reagents = 50 µl of CO_2
Amount of CO_2 due to 2-ketogluconate = 735.8-526.9=208.9
O_2 consumed = 80 µl

$$R.Q. = \frac{CO_2}{O_2} = \frac{208.9}{80} = 2.6$$

Similarly, an R.Q. with a new preparation was as follows:

CO_2 due to endogenous = 338 μl

CO_2 due to 2-ketogluconate = 530 μl

O_2 uptake = 57 μl

$$R.Q. = \frac{192}{57} = 3.3$$

The R.Q. was then averaged and accepted as 3.

(d)Optimum pH activity. Double strength veronal buffer was employed but failed to maintain its pH throughout the Warburg run. Instead, a 1M Tris buffer solution was substituted and worked very well. Figure XII shows the pH curve using increments from 6.8, 7.3, 7.6, 7.9, 8.3 and 8.7 with the optimum appearing at pH 7.4. The pH was tested at the completion of the Warburg run in a Beckman pH meter. Unlike the veronal buffer which showed great utilization, the Tris buffer maintained its pH throughout.

(e)Attempts to demonstrate phosphorylation. ATP had no stimulatory effect on the preparation at a final concentration of 100 micromoles, in the presence or absence of magnesium. A valuable technique of Barker and Lipmann (1), which measures acid-stable (ester) phosphate or acid formation, either aerobically or anaerobically, was employed and proved negative. In addition there was no inhibition by 2.5×10^{-2}M sodium fluoride. Of various substrates employed

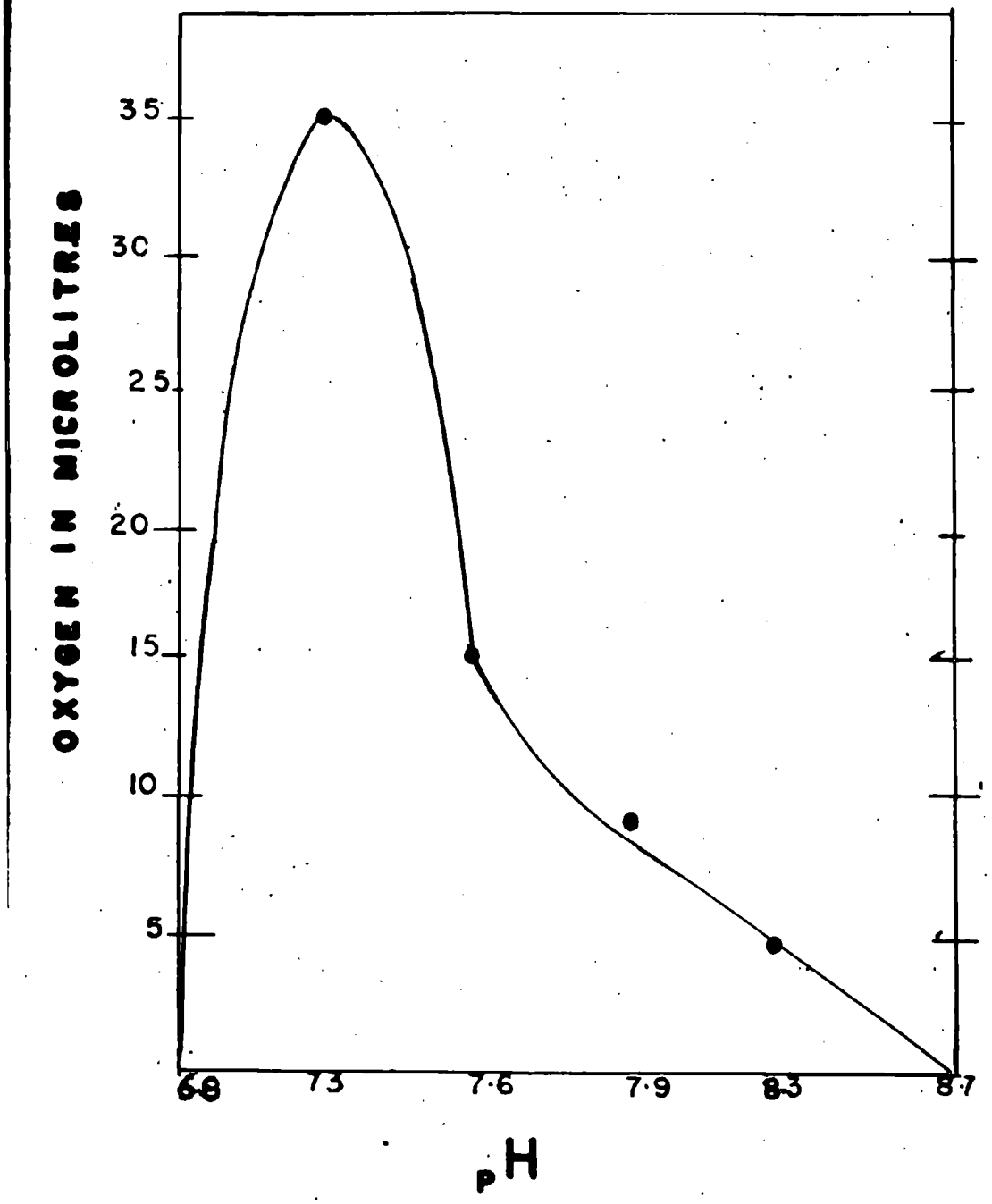


Figure XII - pH curve

The Warburg cups contained 1 ml crude sucrose sonicate, 0.2 mls 2-ketogluconate (5 micromoles), distilled water and 1.0 mls of 1 M Tris buffer.

Net oxygen uptake in 1.5 hours.

ribose-5-phosphate, arabinose and ribonic acid showed no activity when used with a sonicate which was active on 2-ketogluconate.

(f)Anaerobic studies with 2-ketogluconate. There was no stimulation by ATP during aerobic studies and no phosphorylated compounds had been identified using methods 6 and 7 of Table VII. Barker and Lipmann's technique was negative both aerobically and anaerobically. It was then decided that a crude sonicate in the presence of ATP and magnesium ions might as for Wood (27) and DeLey (10) produce a phosphorylated compound if anaerobic conditions were adopted. In particular it was felt that this compound could be 2-keto-D-gluconate-6-phosphate and this avenue still remained open to further work.

Gluconic grown cells were harvested at 18 hours and washed twice with M/50 pH 7.0 phosphate buffer. The cells were suspended in M/50 pH 7.0 phosphate buffer at the rate of 250 mgs/ml and glutathione was added at the rate of 20 mgs/100 mls. This resultant suspension was soniced for 15 minutes and then centrifuged for one hour at 34°F.

Warburg studies were carried out on the crude sonicate using strict anaerobiosis. Six cups containing M/15 pH 7.4 phosphate buffer, magnesium ions, ATP

to a final concentration of 0.03M and water, served as the endogenous. Six additional cups were set up as previously described with the exception that each contained 4 micromoles of 2-ketogluconate. White phosphorous was placed in the center well of each Warburg Vessel in order to absorb all dissolved oxygen. Nitrogen was allowed to pass through each cup while they equilibrated 7 minutes at room temperature. After being closed off all vessels were allowed to equilibrate in the Warburg bath. The substrate was then tipped into the reaction vessel and the whole allowed to react for 2 hours at 31°C.

As soon as 180 minutes had elapsed the cups were removed and all the cups making up the endogenous were pooled. Those cups containing 2-ketogluconate were treated similarly. This was necessary to keep within the phosphate range of Hanes and Isherwood's specifications for the chromatography of phosphate esters (as in method 6, Table 1). Calculations were based on a 20% conversion of the 2-ketogluconate to the phosphorylated compounds. It was necessary to take into consideration that the combined 6 cups containing 2-ketogluconate be halved before concentration, since one portion was to be hydrolyzed. In addition it was felt a capillary pipette would deliver

1-2 microlitres.

Deproteinization of both the endogenous and the 2-ketogluconate cups was carried out by adding 0.4 mls of 0.4M acetate buffer at pH 3.8 and heating for 3 minutes in a boiling water bath. Denatured protein was removed by gravity filtration. The endogenous was concentrated to 2-3 drops in vacuo at 25°C. The other portion containing the reacted 2-ketogluconate was halved. One half was vacuumed distilled to several drops and the other brought to 1 N with HCl. The latter half was heated in a boiling water bath for 30 minutes. This latter portion containing any hydroyzable phosphate esters was also concentrated to several drops.

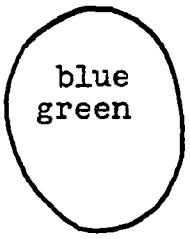
Chromatographic analysis using Hanes and Isherwood's method as previously described, revealed no phosphorylated compounds. From a reproduction of a chromatograph in figure (13) it will be seen that the hydrolysis was effective since the pyrophosphate was cleaved to inorganic phosphate. The method is also valid as evidenced by adenylic acid which was employed in the range of that calculated previously.

Chromatographic analysis

In order to study the products chromatographically the reaction was carried out in a 125 ml capacity

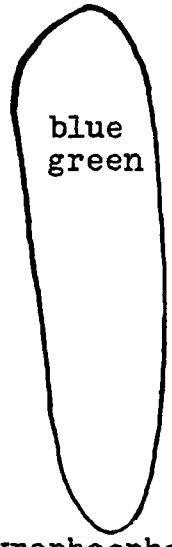
CHROMATOGRAPHIC ANALYSIS
FOR
PHOSPHORYLATED COMPOUNDS

FIGURE XIII
Hanes and Isherwood's method.

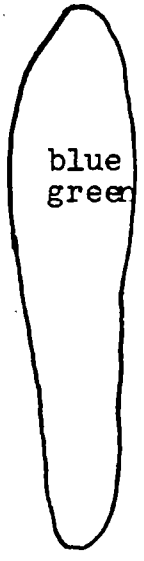


Inorganic Phosphorous

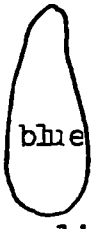
Ultra violet absorbing



Pyrophosphate
from
Phosphate buffer



Pyrophosphate
from
Phosphate buffer



Adenylic acid

Reacted 2-keto-gluconate

Hydrolyzed 2-keto-gluconate

Endogenous

AMP

TABLE VIII

SOLVENTS AND SPRAYS EMPLOYED DURING IDENTIFICATION OF UNKNOWN

Solvent	Spray and treatment	Purpose
(1) 0.08M Acetate buffer pH 3.5 in 4 vols. ethanol	5% ammoniacal $AgNO_3$	Ribose-5-PO_4 Ribulose-5-PO_4
(2) Ethyl acetate-2 Pyridine -1 Water -2	m-phenylene-diaminedihydro-chloride in 76% ethanol 5 mins. @ 105°C.	Sugar spots especially ketohexonic acids show a well defined fluorescence in ultra violet.
(3) Phenol (or) Butanol -80 H_2O -20 Ethanol -20	Aniline hydrogen oxalate Aniline - 0.93gms. Ethanol -50.00mls. mix, with equal vol. 0.2M oxalic acid(aq.)	A red color with, aldohexoses, uronic acids, pentoses.[1]
(4) Butanol -80 H_2O -20 Ethanol -10	Aniline hydrogen phthalate Aniline - 0.93gms. phthalic acid - 1.66gms. H_2O sat. - 100mls. butanol 5 minutes @ 105°C.	A bright red color, aldohexoses, desoxy sugars, uronic acids give various shades of red and brown.[1]
(5) Ethanol -45 Methanol -45 H_2O -10	0.1N $AgNO_3$ in 5N NH_4OH	Hexoses, hexonic and ketohexonic acids, Keto acids.[2]
(6) Isopropanol -2 $(NH_4)_2SO_4$ -1 (1% solution). $(NH_4)_2SO_4$ washed paper.	Hanes and Isherwood spray (16). Heat 7 mins. steam 15 mins. reduce with H_2S.	Separation of Phosphoric esters.[3]
(7) TCA -5gms. propanol -15mls. H_2O -25mls. NH_3 0.25mls.	Hanes and Isherwood spray. Heat 2 mins. Ultra violet 7 mins. (Bandurski & Axelrod)	Separation of Phosphoric esters.

1 Both these sprays gave red spots(positive)with 2-ketogluconate.

2 This spray and solvent confirmed the presence of pyruvate.

3 Sucrose gave a blue spot(positive) after spraying and heating at 75-85°C.

Warburg reaction vessel containing 25 micromoles of 2-ketogluconate (1 ml), 7.5 mls of a pH 7.4 M/15 phosphate buffer, 5 mls of a sonicate from gluconate grown cells and 4 mls of distilled water. The rate of oxidation was followed with a conventional Warburg system containing one-fifth of the above constituents plus potassium hydroxide in the center well. When the reaction was completed the large cup was deproteinized by the addition of 1 ml of a solution containing 6 gms of $ZnSO_4.7H_2O$ and 0.1 gms of $CuSO_4.5H_2O$ to 2 mls. of cup content and left overnight. After gravity filtration to remove denatured protein the whole was concentrated to approximately 1 ml in vacuo at 25°C.

A large cup was run using the crude sucrose sonicate and chromatographic analyses was carried out as for Table VIII. Every solvent and spray in that table was employed to no avail. The difficulty was that the 45% sucrose used during the sonic process had become very concentrated during the vacuum distillation. Hence, every attempt at chromatographic analyses was masked by the heavy concentration of sucrose which obscured any products. Acid-base chromatography had revealed an unknown acidic spot. If this product was acidic, then it could be adsorbed to a basic ion-exchange resin and the sucrose being neutral could be washed free.

The resin could then be made basic and the recovered product could be chromatographed. Such was the case.

Several resins were tried but held the acidic compound too tightly to be eluted by dilute or strong NaOH. IR-4-B an amberlite ion-exchange resin proved satisfactory. The resin was conditioned 12 hours in a 1/4 solution of concentrated ammonium hydroxide. The deproteinized contents were passed through the resin very slowly. The whole was washed free of sucrose with 5 litres of distilled water and the column was made basic with 50 mls of 0.01N NaOH. The resultant eluent was concentrated to 1 ml by vacuum distillation.

The product was ascertained to be pyruvic acid by acid-base chromatography. This detection was made possible by the use of butanol saturated with 4N formic acid as a solvent. Unknown and known compounds at a final concentration of 5 mgs/ml were spotted on Whatman No. 1 filter paper by means of a capillary pipette. The whole was allowed to develop overnight at room temperature in a descending chromatographic tank 28 cms in diameter and 61 cms in heighth. The tank was left sealed for 24 hours prior to chromatographing in order to allow equilibration of

the solvent. After the chromatograph had developed it was sprayed with 0.02% chlorophenol red dissolved in 0.01 N NaOH. The presence of pyruvic acid was substantiated by technique 5 on Table VII.

DISCUSSION

In the past, extreme instability of the 2-ketogluconate enzyme has made it impossible to chart the route of 2-ketogluconate degradation. The proposed pathway of glucose degradation by way of gluconate and 2-ketogluconate depends upon the fate of the latter compound if this route is to be proven functional in P. aeruginosa. McDonald's method of obtaining a dried cell preparation with a limited ability to oxidize 2-ketogluconate was the first positive progress made in 3 years. The necessity of pursuing and clarifying this technique in order to solve 2-ketogluconate oxidation was considered of prime importance in the study of the intermediate metabolism of P. aeruginosa.

In order that any intermediates may be identified in metabolic studies, it is necessary to obtain a stable, reproducible cell preparation. Such a preparation must be obtained in large amounts and on different occasions. An inactive or unstable preparation will waste several weeks or months of preliminary work. Thus a great deal of time was spent in attempts to obtain consistently reproducible preparations.

A difficulty encountered when drying cells under

carbon monoxide was the method of production of the gas. The large number of traps made this work hazardous and the methods used to produce the gas made it somewhat expensive. Thus, when fuel gas was found to substitute successfully for carbon monoxide the results were very encouraging. Unfortunately, however, all attempts to acquire a reproducible preparation gave discouraging results. Despite these results fuel gas could be a very important medium in future work with the 2-ketogluconate enzyme.

The pressure crushing technique was new to enzyme work but the results were very encouraging. Many variables still remained to be explored, e.g., varying the freezing time as well as the freezing medium and employing protamine sulphate to precipitate all nucleic acids. When the nucleic acids have been removed it is felt that live cells will be easily removed by centrifugation.

The results with sonic preparations were the most encouraging. The fact that a reproducible preparation was obtained with a good ability to oxidize 2-ketogluconate is a step toward the solution of the problem on hand. Although the respiratory quotient of three was substantiated by the presence of pyruvate in the reaction mixture the problem is by no means solved. For we do not understand the mechanism

whereby the three carbon atoms are lost. No other compounds were detected by the chromatographic methods employed.

An outstanding problem is the high endogenous respiration. It appears that the optimum pH for endogenous activity is identical to that for 2-ketogluconate oxidation, namely, 7.4. In addition, any attempts to reduce the high endogenous activity reduces the activity on 2-ketogluconate accordingly. When glucose or gluconate grown cells were disintegrated, the endogenous of the extract was extremely low. But when sonicates of 2-ketogluconate grown cells were employed the endogenous was just as high as fuel gas or monoxide dried cells. It remains for tracer techniques to clarify this picture.

Lastly, the absence of phosphorylation either aerobically or anaerobically does not conform to the work of DeLey (10) or Wood (27).

Again, it is impossible to generalize and state that P. aeruginosa is unique in its metabolism since phosphorylation does not occur at the 2-ketogluconate level. It may well be that the phosphorylated product is extremely labile or is decomposed into unidentifiable

phosphate esters.

The data have provided several means of obtaining preparations active against 2-ketogluconate. In addition several new techniques have been introduced.

SUMMARY

1. McDonald had obtained an active cell preparation of *P. aeruginosa* (26). This preparation was obtained by drying resting cells in a partial vacuum over calcium chloride, in the presence of carbon monoxide, and in the absence of light. Work was continued on this preparation.

2. The preparation had a limited ability to oxidize 2-ketogluconate but results were not reproducible.

3. The monoxide-dried preparation had an extremely high endogenous respiration. It would appear that endogenous activity could not be reduced without destroying 2-ketogluconate oxidizing activity.

4. The following attempts to provide monoxide-dried cells, which would produce a stable preparation active against 2-ketogluconate, were unsuccessful.

   (a) Cells were dried in the presence of hydrogen and monoxide gas.

   (b) Cells were dried in the presence of white phosphorous in order to prevent inactivation of the 2-ketogluconate enzyme by oxidation.

   (c) Cells were dried in the presence of varying amounts of substrate on the premise that an enzyme is more stable in the presence of its substrate.

(d) Cells were treated with bile salts in an attempt at solubilizing the 2-ketogluconate enzyme.

(e) Various co-factors were added to the cell preparation in the event that it had lost its ability to synthesize coenzymes during the drying process.

(f) Cells were gassed with carbon monoxide in the complete absence of light, to ensure that inactivation of the enzyme by light, did not occur during the gassing process.

5. Fuel gas was successfully substituted for carbon monoxide but, again the preparation was not reproducible.

6. It was hypothesizedthat fuel gas bound the iron of cytochrome into an iron-monoxide complex reversible by light. Any of the excessive iron then acted as a catalyst for the oxidation of sulfhydryl groups during the drying process. Work with chemically clean glassware and varying levels of iron supported this hypothesis.

7. The following chemical methods were adopted to measure the disappearance of 2-ketogluconate and substantiate oxygen uptake data.

(a) Folin Malmros reducing sugar method (33).

(b) Friedman and Haugen modified 2,4-dinitrophenylhydrazine method (15).

(c) Lanning and Cohen's method which makes use of the condensation of o-phenylenedianine with ketohexonic acids to form 2-hydroxyquinoxaline (21).

8. When 2-ketogluconate was substituted for glucose as the growth substrate, the results with fuel gas dried cells, were identical to those obtained when glucose grown cells were employed.

9. Resting cells were dialyzed to remove any excess iron in the event that this iron could be responsible for catalyzing the oxidation of sulfhydryl groups. The results were identical to those obtained with undialyzed cells.

10. Streptomycin was employed in order to reduce the hazard of live cells appearing in the fuel gas preparation. Although the endogenous respiration was reduced the 2-ketogluconate activity was reduced accordingly.

11. A technique was employed whereby frozen cells, subjected to great pressure, burst open during the thawing process. The presence of some live cells made all attempts to obtain a workable preparation impossible.

12. Resting cells suspended in 45% sucrose were soniced by a Raytheon 10 KC. sonic oscillator. This produced a sonicate with a good ability to oxidize 2-ketogluconate, the reaction being completed at 1 to 2 atoms of oxygen.

13. pH 7.4 was found to be the optimum pH for this sonic preparation.

14. The preparation had a respiratory quotient of 3.0.

15. The preparation was not stimulated by ATP and showed no inhibition by 2.5×10^{-2} M sodium fluoride.

16. No phosphorylation could be detected either aerobically or anaerobically by measuring acid stable (ester) phosphate or acid formation.

17. Chromatographic studies with a crude sonicate in the presence of ATP and magnesium under anaerobic conditions revealed no phosphorylated products.

18. The reaction product of 2-ketogluconate oxidation was determined to be acidic by acid-base chromatography and was finally identified chromatographically as pyruvic acid.

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