THE METABOLISM OF 2-KETOGLUCONATE BY *PSEUDOMONAS AERUGINOSA*

by

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ABSTRACT

The non-phosphorylated oxidative pathway of glucose dissimilation has been confirmed in Pseudomonas aeruginosa using whole cells and cell-free extracts. The oxidation of glucose to 2-ketogluconate was complete and stoichiometric in cell-free extracts and cell-free extracts of glucose grown cells were shown to be incapable of metabolizing 2-ketogluconate.

It was shown that whole cells completely degraded 2-ketogluconate and quantitatively accumulated pyruvic acid in the presence of specific inhibitors. The initial step involved in 2-ketogluconate dissimilation was found to be exceptionally labile to the effects of a variety of metabolic inhibitors.

The metabolism of 2-ketogluconate was demonstrated to involve the initial phosphorylation with adenosine triphosphate (ATP) as the phosphate donor. The resultant intermediate, 2-keto-6-phosphogluconate, was identified and was shown to undergo reduction by a nicotinamide adenine dinucleotide phosphate linked reductase to 6-phosphogluconate which, in turn, was metabolized to pyruvate by enzymes of the Entner-Doudoroff pathway.

Radioactivity from 2-ketogluconate-C^{14} was rapidly incorporated into cellular constituents, primarily protein, by washed cell suspensions of P. aeruginosa, but oxidation

of 2-ketogluconate did not involve the accumulation of keto-acid intermediates.

The role of 2-ketogluconic acid as a key intermediate for the conservation of excess carbon under conditions where nitrogen is limiting was discussed.

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INTRODUCTION

The non-phosphorylated oxidation of glucose by Pseudomonas aeruginosa has been well documented (Norris and Campbell, 1949; Stokes and Campbell, 1951; Claridge and Werkman, 1953), but the metabolic role of 2-keto-gluconate, the apparent end product of these oxidative reactions, has remained obscure. All previous attempts to elucidate the dissimilatory scheme involved in 2-keto-gluconate metabolism in this organism have been unsuccessful.

Pseudomonas fluorescens also oxidizes glucose by the non-phosphorylated route, but with this organism the degradation of 2-ketogluconate has been shown to proceed solely via the Entner-Doudoroff pathway. (Kovachevich and Wood, 1955; Narrod and Wood, 1956).

It was the object of this endeavor to study the metabolism of this organism, establish the degradative route of 2-ketogluconate catabolism and to, if possible, determine why the key enzymes have resisted demonstration so tenaciously.

LITERATURE REVIEW

The formation of 2-ketogluconate (2-KG) during the oxidation of glucose was initially observed in growing cultures of Acetobacter gluconicum. (Bernhauer and Gorlick, 1935). The oxidation of 2-KG was found to be inhibited by either high aeration or by incubating the cells at relatively low temperatures for 30 to 60 days.

I. The Non-phosphorylated Oxidation of Glucose

The formation of gluconate and 2-KG as transient intermediates during glucose dissimilation by Pseudomonas aeruginosa was demonstrated qualitatively by Norris and Campbell (1951), by the isolation of products from cultures growing in a glucose-ammonium-phosphate medium. From this evidence and quantitative manometric data observed during the oxidation of glucose both by whole cells and by dried cell preparations it was concluded that gluconate and 2-KG were intermediates, and that most of the glucose, if not all, was metabolized via the non-phosphorylated route.

Using cell-free extracts of P. aeruginosa Claridge and Werkman (1953), substantiated the quantitative observations of Stokes and Campbell and assumed that hexose phosphates were not intermediates in these reactions. This oxidative system proved to be sensitive to cyanide and it was concluded that metal linked carriers were operative in

in the transport of electrons.

Non-phosphorylated hexose oxidation has been recognized in a great variety of microorganisms, principally in bacteria. Koepsall (1950), demonstrated that glucose and gluconate were rapidly oxidized to 2-KG and that this intermediate was utilized slowly in growing cultures of Pseudomonas fluorescens. Wood and Schwert (1952), corroborated these observations using cell-free extracts of P. fluorescens which oxidized glucose and gluconate quantitatively to 2-KG and attempts to demonstrate phosphorylated hexose oxidation were unsuccessful. The oxidation of glucose, gluconate and 2-KG by Serratia marscesens required 3.0, 2.5, and 2 μm of oxygen per μM of substrate respectively, and 2-KG accumulated as a transitory intermediate (Wasserman, Hopkins, and Seibles, 1956). Katznelson (1959), surveyed 19 species belonging to Xanthomonas, Pseudomonas, Agrobacterium, Erwinia and Corynebacterium genera for the ability to oxidize glucose, gluconate, and 2-KG. All species oxidized glucose and gluconate but only two species were capable of oxidizing 2-KG.

Certain Aerobacter species have been shown to utilize the non-phosphorylated route of glucose oxidation similar to the pathway established in Pseudomonas. (DeLey, 1955). However, DeLey and Cornut (1951) demonstrated that this enzyme system was not formed preferentially

under aerobic growth conditions but was active in anaerobically cultured bacteria. They postulated that the aerobically grown cells had cytochrome systems which allowed alternate pathways of glucose dissimilation to be utilized selectively.

In glucose grown cells and cell-free extracts of the acetic acid bacterium, Gluconobacterium liquefaciens, the oxidation of glucose proceeded not only through gluconate to 2-KG but also to the more oxidized intermediate 2,5-diketogluconate (2,5-KG) via 5-ketogluconate (5-KG) (Stouthamer, 1961). The Acetobacter were found to oxidize glucose in a similar manner through gluconate to 2- and 5-KG. (Fewester, 1956; Schramm, Gromet, and Hestrin, 1957). Katznelson, Tanenbaum, and Tatum (1953), have reported that Acetobacter melanogenum produces 2,5-KG from glucose and Schramm and Racker (1957), have reported a rapid oxidation of glucose to 2-KG in Acetobacter xylinum with only a minor proportion of glucose being phosphorylated directly.

Direct non-phosphorylated oxidation of glucose has also been found in Waring-blended mycelial suspensions of the fungus Caldariomyces fumago which normally does not utilize 2-KG--the oxidation end product--due to a toxic accumulation of H_2O_2, however, this inhibition was eliminated by the subsequent addition of catalase (Ramachandran and Gottlieb, 1963).

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotidephosphate (NADP) have been shown to act as hydrogen acceptors in the oxidation of glucose-6-phosphate (G-6P) to 6-phosphogluconate (6-PG) by yeasts, bacteria, and animal tissues. However, a number of bacteria have been reported to oxidize glucose to gluconic acid with an enzyme system linked to particulate structures of the cell-free extract. These bacteria include Acetobacter suboxydans (King and Cheldelin, 1957), P. flourescens (Wood and Schwert, 1953), Pseudomonas pseudomonallei (Dowling and Levine, 1956), Pseudomonas quercito-pyrogallica (Bentley and Schlecta, 1960), Aerobacter aerogenes (Dalby and Blackwood, 1955), Azotobacter vinelandii (Brodie and Lipmann, 1955), and Bacterium anitratum (Hauge, 1960). No evidence has been found to indicate either NAD or NADP participation in the dehydrogenation process. It has been hypothesized that these oxidative enzymes are linked to the respiratory chain of the microorganisms and, as such, they appear bound to the particulate fraction of all cell-free extracts. In some soluble aldose dehydrogenase systems, NAD and NADP do play an important role (Harrison 1931; Wiemberg, 1961). Cell-free extracts of Corynebacterium creatinovorans were found to differ from whole cells in their ability to oxidize glucose to 2-KG, but the addition of flavine adenine dinucleotide (FAD) to the cell-free extracts permitted quantitative oxidation

of glucose to gluconic acid (Ghiretti and Guzman-Barron, 1954). The particulate aldose dehydrogenase of Rhodopseudomonas spheroides has been shown to be dependent upon an unidentified, heat-stable, soluble cofactor for its activity (Szymona and Doudoroff, 1960). Niederpruem and Doudoroff (1965), have shown that this cofactor is present in the particulate fraction of aerobically grown cells and also in the soluble fraction, but only in the latter after the cessation of exponential growth. The cofactor was absent from anaerobically grown cells and from P. flourescens, Escherichia coli, yeast, and mouse liver.

Glucose oxidation in Bacillus subtilis was shown to require NAD and hydrogen peroxide was formed. In addition, the oxidation of glucose by spore extracts of Bacillus cereus was found to be catalyzed by a soluble NAD requiring system. Thus the glucose, and possibly the gluconate, dehydrogenases of Gram-positive organisms appeared to differ from those of Gram-negative organisms. (Halvorson and Church, 1955).

In P. aeruginosa, the oxidation of glucose to gluconic acid was shown to proceed via an apparent non-NAD or -NADP requiring oxidative step to gluconolactone and the subsequent enzymic hydrolysis of the lactone to gluconate was found to be magnesium dependent (Gronlund, 1961). Gluconic dehydrogenase, which catalyzes the oxidation of gluconic acid to 2-KG without concurrent phosphorylation, was partially

purified by Ramakrishnan and Campbell (1955), and examined for substrate specificity and for an electron acceptor requirement, but the reaction had no pyridine nucleotide requirement and pyocyanine functioned optimally as an electron acceptor. DeLey and Stouthamer (1959), investigated the gluconate dehydrogenases of A. suboxydans and A. melanogenum and showed that separate soluble NADP specific dehydrogenases existed for 2-KG and 5-KG formation and that a particulate, possibly cytochrome linked, gluconate oxidase yielding 2-KG existed in these organisms.

Campbell, Linnes, and Eagles (1954), concluded from molar growth yield experiments, using limiting equimolar amounts of glucose, gluconate, or 2-KG as the sole carbon source, that no energy was gained from the oxidation of glucose to 2-KG since almost identical amounts of growth of P. aeruginosa, as measured by turbidity and protein content, were obtained. Later, however, Strasdine, Campbell, Hogenkamp, and Campbell (1961), followed radioactively labelled inorganic phosphorous (P^{32}) incorporation into resting cells and found an inverse relation between the degree of substrate oxidation and P^{32} incorporation thereby indicating that phosphorylation was coincident with the passage of electrons to oxygen through the electron transport chain.

The enzymic degradation of 2-KG in microorganisms is, in almost all cases, readily inhibited by intense aeration which allows nearly quantitative accumulation of

2-KG from glucose and therefore this procedure has value as a commercial source of the compound. Recoveries of 75-85% have been achieved with shake cultures of P. aeruginosa (Hill, 1952), P. fluorescens (Asai and Ikeda, 1948), and Gluconobacter species (Ikeda, 1960); 90-97% yields have been reported with P. putida (DeLey and Cornut, 1951), Cyanococcus chromospirans (British patent, 1951), and recently with Serratia marscesens (Misenheimer and Anderson, 1964).

II. Pathways of 2-Ketogluconate Dissimilation

DeLey (1953), demonstrated an enzyme, 2-keto-gluconokinase, in alumina ground or Hughes' press cell-free extracts of 2-KG adapted cells of Aerobacter cloacae. This new enzyme affects the transphosphorylation of 2-KG in the presence of adenosine triphosphate (ATP) and magnesium ions. Subsequent partial purification of the enzyme permitted the isolation of the phosphorylated acid 2-keto-6-phosphogluconate (2-K-6-PG), which was identified by the spectral characteristics of the free acid and its quinoxaline derivative. Aerobacter aerogenes, grown on 2-KG, contained a specific inducible kinase and the enzyme was purified 124 fold... (Frampton and Wood, 1961). Ghiretti and Guzmann-Barron (1954), showed that the phosphorylation of 2-KG by cell-free extracts of C. creatinovorans was a step in the pathway of glucose

dissimilation in this organism; the end product of the transphosphorylation with ATP was not isolated but was assumed to be 2-K-6-PG. Narrod and Wood (1956), demonstrated, and separated the induced enzyme 2-keto-gluconokinase from the constitutive enzyme gluconokinase using cell-free extracts of P. fluorescens and the phosphorylated intermediate 2-K-6-PG was identified. DeLey and Vandamme (1955), extended their early observations on inducible kinases in Aerobacter species to a wider spectrum of organisms and demonstrated 2-ketogluconokinase in the genera Pseudomonas, Xanthomonas, Escherichia, Aerobacter, Paracolobacterum, Serratia, Erwinia, and Bacillus.

Fewster (1957), demonstrated 2-ketogluconokinase and a new enzyme, 5-ketogluconokinase, in sonic extracts of A. suboxydans by manometric measurements of acid production during transphosphorylation reactions and postulated, and later confirmed, that 2-K-6-PG was decarboxylated to arabonic acid.

DeLey and Stouthamer (1959), were unable to detect 5-ketogluconokinase in their strain of A. suboxydans. Cell-free preparations of A. melanogenum grown with glucose as the carbon source readily phosphorylated 2-KG as measured manometrically by the release of CO_2 from a bicarbonate buffer (Fewster, 1957). DeLey's strain of this organism could not be shown to phosphorylate either 2-KG or 5-KG, but rather the metabolism of both ketogluconates was shown

to consist of a reduction to gluconate which was subsequently phosphorylated to 6-PG (DeLey and Stouthamer, 1959).

A decarboxylase for 2,5-diketogluconate (2,5-KG), a product of 2-KG dissimilation in A. melanogenum, was purified from the organism and the decarboxylation product of the reaction was isolated as the o-nitrophenylhydrazone. The apparent inability to identify the reaction product was due to the extreme lability of the free intermediate and to a lesser degree the hydrazone derivative (Datta, Hochster, and Katznelson, 1958). The identity of this intermediate has not been reported to date.

Fermentation product analyses and isotope tracer data from specifically labelled substrates have been used to ellucidate 2-KG degradative pathways in certain lactic acid bacteria. Blackwood and Blakley concluded that the fermentations of glucose, gluconate, and 2-KG by Leuconostoc mesenteroides were similar (Blackwood and Blakley, 1956). A specific adaptive 2-KG kinase was isolated from these cells when grown on 2-KG. The enzyme was partially purified and was active in the presence of magnesium ions with either ATP, ITP, or GTP acting as the phosphate donor. The product of the kinase reaction, 2-K-6-PG, was isolated and identified. Cifferi and Blakley (1959), demonstrated that the metabolism of 2-KG in L. mesenteroides proceeded according to the following pathway:

2-KG ⟶ 2-K-6-PG ⟶ 6-PG ⟶ ribulose-5-phosphate + CO_2 ⟶ xylulose-5-phosphate ⟶ acetyl phosphate + glyceraldehyde-3-phosphate. The reduction of 2-K-6-PG to 6-PG required either $NADH_2$ or $NADPH_2$. Goddard and Sokatch (1963) showed that Streptococcus faecalis converted 2-KG to pentose phosphate, but by a primary decarboxylation. Pentose-phosphate was then fermented to pyruvate through a sedoheptulose diphosphate variation of the pentose phosphate pathway found in this organism.

DeLey and DeFloor (1959) demonstrated and characterized 2-ketogluconoreductase activity in the 2-KG adapted bacterium Corynebacterium helvolum, the yeast Debaromyces hansenii and the mold Aspergillus nidulans, and it was shown that the enzyme was 20 times more active with $NADPH_2$ than with $NADH_2$. Sonic extracts of P. fluorescens have been shown to have reductase activity in both glucose and 2-KG grown cells (Frampton and Wood, 1961).

Metabolic inhibitor and C^{14} tracer studies with intact cells and crude enzyme preparations of Pseudomonas saccharophila proved to Entner and Doudoroff (1952), that glucose and gluconic acid were utilized in such a manner as to yield 2 molecules of pyruvic acid per molecule of substrate. The carboxyl groups of one-half of the pyruvate molecules were derived from carbon atom one of glucose instead of carbon atoms 3 and 4 which would have been the result if the Embden-Meyerhof pathway was operative. These

workers therefore proposed an oxidative pathway involving a 3:3 split by the cleavage of 6-PG, or an isomer of the compound, to provide triose phosphate and pyruvic acid. It was hypothesized that 6-PG underwent an enzymic dehydration and rearrangement to 2-keto-3-deoxy-6-phosphogluconate (2-K-3-D-6-PG), which was subsequently split by an aldolase-type of reaction. McGee and Doudoroff (1954), separated two enzymatic activities from cell-free extracts of P. saccharophila; one converted 6-PG to the phosphorylated keto-acid-identified as the previously postulated 2-K-3-D-6-PG; and one split this keto-acid into pyruvic acid and glyceraldehyde-3-phosphate.

Extracts of P. fluorescens were shown to carry out a rapid oxidation of G-6-P and 6-PG but lacked the complete spectrum of enzymes necessary for glycolysis (Wood and Schwert, 1954). The enzyme, 6-PG dehydrase, was subsequently purified from dried cells and 2-K-3-D-6-PG was isolated as the product of the reaction of the purified enzyme with 6-PG (Kovachevich and Wood, 1955). A second enzyme was obtained which had no activity with 6-PG, but which catalyzed the cleavage of 2-K-3-D-6-PG (Kovachevich and Wood, 1955;a).

Specific kinases for the non-phosphorylated analogue 2-keto-3-deoxygluconic acid (2-K-3-DG) have been demonstrated in E. coli grown with glucuronic or galacturonic acids (Cynkin and Ashwell, 1960), and in Rhodopseudomonas

spheroides grown in glucose (Szymona and Doudoroff, 1960).

Hughes' press cell-free extracts of P. aeruginosa were demonstrated to contain a weak 2-K-6-PG reductase (Reid, 1959), and a very potent 2-K-3-D-6-PG aldolase (Gronlund, 1961), suggesting that this organism possesses, at least in part, pathways of glucose dissimilation similar to those established in other Pseudomonas species. Claridge and Werkman (1953), concluded that cell-free extracts of P. aeruginosa do not metabolize 2-KG, but radioautographic analyses of whole cell incubation mixtures with 2-KG showed the presence of a tricarboxylic acid cycle and of functioning assimilation mechanisms.

Since the establishment of the Entner-Doudoroff pathway in 1954, there has been growing evidence that the formation of 2-keto-3-deoxy sugar acids represents a ubiquitous metabolic mechanism in the bacterial utilization of carbohydrates. Thus, the metabolism of such diverse compounds as D-galactose (DeLey and Doudoroff, 1957), D-arabinose (Ashwell, 1957), L-arabinose (Palleroni and Doudoroff, 1956), D glucosamic acid (Merrick and Roseman, 1958), D-ribose-5-phosphate (Weissbach and Hurwitz, 1959), D-arabinose-5-phosphate (Levin and Racker, 1959), D-glucuronic and mannonic acids (Ashwell, Wahba, and Hickman, 1950; Hickman and Ashwell, 1960; Smiley and Ashwell, 1960; Cynkin and Ashwell, 1960), and alginic acid (Preiss and Ashwell, 1962), have been shown to involve the formation of

analogous intermediates, all possessing this common structural feature.

In two separate instances evidence has been produced that 2-keto-3-deoxy-sugar acids provide an intermediate available for alternate biosynthetic pathways. Shikimic acid has been shown to result from the cyclization of 2-keto-3-deoxy-heptonic acid (Weissbach and Hurwitz, 1959), similarly, α-ketoglutarate has been found to arise from L-arabinose through the intermediate 2-keto-3-deoxy-L-arabonic acid (Weimberg, 1959).

III. Oxidative Assimilation

The early concept of the phenomenon of oxidative assimilation was considered to be the incorporation of strictly non-nitrogenous organic compounds into endogenous storage products during the oxidation of carbon compounds (Barker, 1936; Macrae and Wilkinson, 1958). However, the restriction of this concept to the incorporation of non-nitrogenous substances was shown to be limiting when it was found that ammonia was a product of endogenous respiration in P. aeruginosa and that reincorporation of this product into cellular material was induced by the addition of an oxidizable carbohydrate (Warren, Ells and Campbell, 1960). This observation was extended to other microorganisms (Gronlund and Campbell, 1961) and was suggested as a general phenomenon occurring with all bacteria that have the ability

to utilize ammonia as a nitrogen source.

Rather than accumulate carbonaceous material intracellularly, P. aeruginosa was shown to accumulate α-ketoglutarate extracellularly during the oxidation of glucose-U-C^{14} by resting cells. The utilization of the extracellular α-keto-acid coincided with the incorporation of ammonia and radioactivity into cellular constituents--principally protein (Duncan and Campbell, 1962). This work has been extended to include a variety of bacteria, and although α-ketoglutarate accumulation during glucose oxidation would seem to be a widespread phenomenon (Asia, Aida, Sugisaki and Yakeishi, 1955; Perry and Evans, 1960). Tomlinson and Campbell (1963), have shown that the general assimilation pattern may be similar but the accumulating intermediate need not be α-ketoglutarate, and the failure to accumulate an oxidizable intermediate results in low levels of C^{14} assimilation in certain strains of Acetobacter and Azotobacter studied (Tomlinson and Campbell, 1963).

The assimilation of hexonic acids has received little attention although Claridge and Werkman (1953), have acknowledged the existence of assimilation mechanisms in P. aeruginosa oxidizing 2-KG-U-C^{14}.

MATERIALS AND METHODS

I. Organism and Media

Pseudomonas aeruginosa ATCC 9027, an obligate aerobe, was the organism used throughout this study. Stock cultures were maintained in a glucose-ammonium salts medium at 6 C after a twenty-four hour growth period and these were streaked at regular intervals onto glycerol-peptone agar and examined for typical colonial and cellular morphology as well as for the ability to produce this organism's characteristic pigment, pyocyanin.

Cells required for experimental procedures were routinely grown in Roux flasks using a 1% fresh twenty-four hour inoculum in a medium containing 0.3% $NH_4H_2PO_4$, 0.2% K_2HPO_4, 0.5 ppm iron as $FeSO_4$. The pH was adjusted to 7.4 and glucose and $MgSO_4 \cdot 7H_2O$ were added after sterilization, from 10% stock solutions, to give final concentrations of 0.2% and 0.1% respectively. When required in the medium 2-KG, at final concentrations of 0.2 or 0.6%, was substituted for glucose. The keto-acid was sterilized by passing a 10% stock solution through a 0.3 type PH Millipore filter.

II. Preparation of Washed Cell Suspensions

Cells were harvested by centrifugation at 3000xg at 6 C and washed twice with 0.85% saline pH 7.4. and the washed cell suspensions were resuspended to exactly ten times the growth concentration in tris-(hydroxymethyl)aminomethane buffer(Tris) pH 7.4. This corresponded to an optical density (O.D.) reading of 13.5/ml. at 650mμ in a Beckman model B spectrophotometer and approximately 5 mg dry weight of cells/ml.

III. Preparation of Cell-free Extracts

1. Sonic oscillation

Harvested washed cells were resuspended in $\frac{M}{5}$ Tris buffer (pH 7.4) to a final concentration of 40 times growth (approximately 20 mg. of dry weight of cells per ml). This concentrated cell suspension, of not less than 10 ml, was subjected to 5 minutes of sonic oscillation in a 10 Kc. Raytheon oscillator which was followed by centrifugation at 6 C for 10 minutes at 3000xg to remove whole cells. The resultant supernatant fluid was removed and kept on ice.

2. French press

Cells were harvested and washed as described previously and resuspended at 40 times growth concentration in $\frac{M}{5}$ Tris buffer (pH 7.4). Three to four drops of a commercial deoxynuclease (DNase), solution (1 mg/ml) were

mixed with the cells and the cylinder of the French pressure cell (Milner, Lawrence, and French, 1950) was charged with the cell suspension. An applied pressure of 15-18000 pounds p. s. i. was affected with a Carver hand-operated hydraulic press. The resulting pressate was centrifuged at 3000xg for 10 minutes to remove whole cells and the supernatant fluid was removed and stored on ice.

3. Hughes' press

Washed cell pellets were packed into open-ended pyrex vials of 11 mm in diameter. The vials were tightly sealed with rubber stoppers and these cells were quick-frozen in an ethanol dry-ice bath for 30 minutes. The cell-free extracts were prepared immediately by crushing the cells in a Hughes' press (Hughes, 1951), previously cooled to -22 C, by applying a pressure of approximately 12,000 pounds p. s. i. with a Carver hydraulic press. The pressate was diluted with cold $\frac{M}{5}$ Tris buffer (pH 7.4) at a ratio of 1:6 v/v to yield an approximate concentration of 200 mg wet weight of cells/ml. Approximately 3-4 drops of DNase were added to each preparation prior to homogenizing in a pre-chilled Potter homogenizer. After homogenizing, the suspension was subjected to centrifugation at 3000xg for 10 minutes at 5 C and the resulting supernatant fluid was kept on ice.

All enzyme assays were carried out immediately after preparation of the cell-free extracts.

IV. Manometric Measurements

Respirometry was carried out on washed cell suspensions and pressates in $\frac{M}{20}$ Tris buffer (pH 7.4) at 30 C by means of a conventional Warburg apparatus. Experiments at 20 C were performed with a refrigerated Warburg respirometer. A representative Warburg reaction mixture is presented as follows:

	Endogenous	Test
Cell suspension (10x growth)	1.00 ml	1.00 ml
M/20 Tris buffer (pH 7.4)	1.00 ml	1.00 ml
Substrate (25 μM/ml)	-	0.20 ml
Distilled H_2O	1.00 ml	0.80 ml
20% KOH	0.15 ml	0.15 ml

Variations in procedure, such as the addition of co-factors or metabolic inhibitors, were accompanied by an appropriate decrease in the volume of the distilled water. In experiments involving metabolic inhibitors, a volume of water equal to the substrate volume was added to the sidearm of the endogenous control cup so that inhibitor concentrations in the cups prior to substrate oxidation would be identical. All substrates and inhibitors were prepared at concentrations of 25 μM/ml or 50 μM/ml and were adjusted to pH 7. Calcium was removed from gluconate or 2-ketogluconate with Dowex 50 H^+ prior to neutralization.

V. Spectrophotometric Enzyme Assays

Enzyme assays involving the oxidation and reduction of pyridine nucleotides were followed by measuring changes in optical density at 340 mμ with a Beckman model DU spectrophotometer or a Beckman model DB spectrophotometer equipped with a Varicord linear/log recorder.

With limiting pyridine nucleotide concentrations and excess substrate the reactions were initially zero order, the rate of the reaction being dependent on enzyme concentration. All enzyme activities were expressed as change in O.D. per minute per mg protein of the cell-free extract $x10^2$. Reaction mixtures were prepared in quartz cuvettes with a 1 cm light path and a total volume of 1 ml.

1. 2-Keto-6-phosphogluconate reductase

2-K-6-PG-reductase activity was determined by measuring $NADPH_2$ oxidation during the conversion of 2-K-6-PG[1] to 6-PG. The method of Reid (1959) was used as modified by Gronlund (1961).

A typical assay was as follows:

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[1]2-K-6-PG was kindly supplied by Dr. F. J. Simpson, Prairie Regional Laboratory, Saskatoon, Saskatchewan.

	enzyme control	substrate control	Test
$\frac{M}{5}$ Tris buffer pH 7.4	0.20 ml	0.20 ml	0.20 ml
$MgCl_2$ (100μM/ml)	0.10 ml	0.10 ml	0.10 ml
2K6PG (25μM/ml)	-	0.05 ml	0.05 ml
Pressate	0.01 ml	-	0.01 ml
Distilled H_2O	0.67	0.63 ml	0.62 ml
TPNH 0.005 M.	0.02 ml	0.02 ml	0.02 ml

All constituents, with the exception of the substrate, were added to the cuvette and the instrument "zeroed" on the reaction mixture at an O.D. of 0.500. The substrate, 2-K-6-PG, was added at zero time, the reaction mixture shaken, and the decrease in O.D. recorded.

2. 2-ketogluconokinase

The procedure used to measure this enzyme was dependent on the presence of 2-K-6-PG reductase. The substrate 2-KG replaced 2-K-6-PG in the 2-K-6-PG-reductase assay and 0.01 ml of adenosine triphosphate (ATP) (25μM/ml) was included in the reaction mixture. The ATP dependent oxidation of $NADPH_2$ at a rate significantly greater than that of the control would indicate a 2-KG kinase activity.

3. 2-keto-3-deoxy-6-phosphogluconate aldolase

This enzyme was measured indirectly by the conversion of pyruvic acid, a product of the aldolase reaction, to

lactic acid accompanied by $NADH_2$ oxidation in the presence of an excess of commercial lactic dehydrogenase (Kovachevich and Wood, 1954). The rate of activity was dependent on formation of pyruvic acid from 2-K-3-D-6-PG[2] and any interference by glyceraldehyde-3-phosphate dehydrogenase was inhibited with iodoacetate. The following reaction mixtures were used:

	enzyme control	substrate control	Test
M/5 Tris buffer pH 7.4	0.20 ml	0.20 ml	0.20 ml
$MgCl_2$ (50μM/ml)	0.05 ml	0.05 ml	0.05 ml
2-K-3-D-6-PG (25μM/ml)	-	0.05 ml	0.05 ml
Iodoacetate (10 mg/ml)	0.10 ml	0.10 ml	0.10 ml
Lactic dehydrogenase (100 mg/ml)	0.02 ml	0.02 ml	0.02 ml
$NADH_2$ 0.005 M	0.02 ml	0.02 ml	0.02 ml
Pressate	0.01 ml	-	0.01 ml
Distilled water	0.60 ml	0.56 ml	0.55 ml

All components, with the exception of 2-K-3-D-6-PG, were added to the cuvette and the instrument was "zeroed" at an O.D. reading of 0.500. The recording of the rate of O.D. decrease began at zero time immediately following reaction initiation by the addition of the substrate.

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[2] 2K3D6PG was the generous gift of Dr. W. A. Wood, Dept. of Agricultural Chemistry, Michigan State University.

4. 6-phosphogluconate dehydrase

Enzyme activity was measured by using the previously described assay for 2-K-6-PG aldolase with 6-PG as the substrate and 0.05 ml of pressate. The rate of activity was found to be dependent on the initial dehydration of 6-PG to 2-K-3-D-6-PG.

VI. Assimilation Experiments

1. Incubation with 2-KG

Experiments involving the oxidative assimilation of C^{14} labelled 2-KG were carried out in Warburg respirometer vessels in order that oxygen consumption could be followed. A typical manometric reaction mixture has been described, and all experiments were carried out at 30°C. Reactions were terminated at specific time intervals by pipetting the contents of the vessels into ice cold Pyrex centrifuge tubes and the tubes were immediately centrifuged at 6 C. The supernatants were removed and kept on ice or frozen at -22 C. Cell pellets were fractionated immediately.

2. Fractionation of whole cells

The chemical fractionation procedure used was essentially that of Roberts et al (1955), with the modification by Clifton and Sobek (1961) incorporated and the hot trichloracetic acid (TCA) soluble fraction was prepared by heating the sample in 5% TCA for 20 minutes at 90°C rather

than at 100°C for 30 minutes. The cold TCA soluble fraction represents the transient cell intermediates, amino-acids, and possibly carbohydrate metabolism intermediates; the acid-ethanol fraction represents lipids, phospholipids and alcohol soluble protein; the hot TCA soluble fraction contains nucleic acids; and the hot TCA insoluble material contains the bulk of the cellular protein.

VII. Electrophoretic and chromatographic methods

1. Electrophoresis

Paper electrophoresis was carried out with a water-cooled electrophoresis apparatus similar to a Resco model E-800-2B equipped with a Resco model 1911 power supply. The buffer system routinely used was 0.1 M ammonium carbonate ($NH_4HCO_3 \cdot NH_2COONH_4$-Analar) pH 8.6 and samples were spotted on Whatman #4 filter paper. Current was applied for 1.5 - 2 hours at maximum voltage which varied between 700 and 750 volts depending on the area of the paper involved.

2. Chromatography

a. anion exchange column chromatography

Formic acid-ammonium formate elution from Dowex 1 formate columns was used to separate radioactive metabolic intermediates. The column was prepared in the following manner:

the fines were decanted from an aqueous suspension of the resin; the resin was packed into a 1 x 15 cm column and washed with 500 ml of distilled water; the packed column was washed with several liters of 0.5 N NaOH until only weakly positive for Cl^-, then washed with water; the column was repacked in, and washed with, 2.0 N formic acid until the concentration of the eluate was the same as the wash acid; the column was washed with distilled water until the pH was constant (pH 4.5). After use, the columns were regenerated with 4 N formic acid followed with distilled water until the eluate was pH 4.5.

Reaction supernatants or perchloric acid (PCA) extracts were adjusted to pH 5.0 prior to charging the column. The mixture was washed into the column with 10 ml of distilled water and then elution commenced. Gradient elutions from 0.0 - 1.0 N formic acid (Bartlett, 1958), and with 4 N formic acid and 0.4 N ammonium formate (Hurlbert, Schmitz, Brumm, and Potter, 1954), were used to separate intermediates. Flow rates of approximately 1 ml per minute were affected and approximately 10 ml fractions were collected with a Gilson fraction collector model T-10.

b. paper chromatography

Paper chromatography was routinely performed on reaction mixtures and supernatant fluids by spotting

samples on Whatman No. 4 filter paper and running them by the descending technique. The solvent systems commonly employed for carbohydrates were ethylacetate-pyridine-saturated aqueous boric acid (60:25:20 v/v) (Grado and Ballou, 1961), and acetone-pyridine-water (2:1:1 v/v) (Szymona and Doudoroff, 1960). Amino acids were separated with butanol-acetic acid-water (60:15:25 v/v) and with phenol-ammonia (200:1 v/v) (Smith, 1960).

Keto-acid 2,4-dinitrophenylhydrazones were prepared by reaction with 2,4-dinitrophenylhydrazine in 2 N HCl (2 mg/ml) at 37 C for 30 minutes and the derivatives were extracted into several volumes of ethylacetate. These hydrazones were then extracted into 1 M Tris pH 11, made acidic, and extracted back into ethylacetate for chromatography.

The removal of phosphate from phosphorylated intermediates was carried out by incubation with a commercial bacterial alkaline phosphatase in pH 8 $\frac{M}{20}$ Tris buffer at 37 C.

c. detection reagents

Carbohydrates were detected by dipping chromatograms in 0.12% m-periodate in acetone and spraying with 0.18% benzidine in acetone (Cifonelli, 1954). Reducing sugars were detected by dipping chromatograms in 0.5% $AgNO_3$ in acetone and developing in 0.5 N NaOH in 70% ethanol and the background colour was removed with 5%

aqueous $Na_2S_2O_3 \cdot 5H_2O$ (Smith, 1960). Organic acids were detected by spraying with 0.1% brom-cresol green in ethanol diluted to 1/5 concentration in acetone. Keto-acids were detected by spraying the chromatograms with 2% orthophenylene-diamine in 0.05 N HCl, heating the chromatograms at 70-80°C, and observing the quinoxaline derivatives as characteristic coloured compounds and by their fluorescence when irradiated with ultra-violet light (Lanning and Cohen, 1951). The following colours were observed:

standards		visible	ultra violet
pyruvate	-	pink	fluorescent
α-ketoglutarate	-	yellow	fluorescent
2KG	-	yellow	fluorescent
2K6PG	-	purple	no fluorescence
2K3D6PG	-	yellow-green	fluorescent

Keto-acid 2,4-dinitrophenylhydrazone derivatives were observed as ultra-violet absorbing spots or as colours obtained on spraying the chromatograms with 0.5 N NaOH in ethanol (Smith, 1960). Phosphate esters were detected by spraying with 1% ammonium molybdate in acetone and developing the colour under ultra-violet light (Smith, 1960).

VIII. Isotopic Methods

Uniformly-labelled glucose-C^{14}, glucose-1-C^{14}, and glucose-6-C^{14} were obtained from Merck

and Co. Ltd. and were diluted with non-radioactive glucose solutions so that 5 μM of glucose with a specific activity of 3.5 μ curie per 5 μM was obtained (Duncan and Campbell, 1962). The preparation of variously labelled 2-KG is described in the text.

Aliquots of reaction mixtures, cell suspensions, cell fractions, and chromatographic eluates were plated, in duplicate, on stainless steel planchets and dried under an infra-red lamp. Radioactive CO_2 was determined by quantitatively removing the filter paper and KOH from the Warburg reaction vessel and rinsing the center well with several volumes of distilled water (Gronlund and Campbell, 1964). The combined rinsings, KOH, and paper were made up to 5 ml in a volumetric flask. The paper and rinsings were then mixed in a test tube with a glass rod by means of a vortex mixer. Aliquots were plated in quadruplicate and dried as above. Planchets were counted at infinite thinness using a Nuclear-Chicago scaler model 181A equipped with an automatic gas-flow counter having a thin-end-window Geiger tube. Corrections were made for background. To reduce statistical deviation at least 1000 counts were recorded when possible.

Radioactive chromatograms were scanned by running approximately one-inch strips through a Nuclear-Chicago Model C 100 B Actigraph II with the gas flow counter and a Model 1620 B Analytical Count ratemeter equipped with a chart recorder.

IX. Analytical Methods

1. Ammonia determinations

The ammonia in the supernatant fluids from reaction mixtures was determined by the Conway microdiffusion method (Conway, 1950). Under the conditions employed, values from 0.0 - 0.75 μM per sample could be measured.

2. Glucose determinations

Glucose concentrations in reaction mixtures were measured by the "glucostat" method of Worthington Biochemical Corp. The enzymatic reaction of added glucose oxidase with substrate glucose resulted in peroxide production which was measured spectrophotometrically in the presence of ortho-dianisidine and peroxidase. Glucose was measured in the range 0.1 - 1.0 μM per sample.

3. Chemical determination of 2-KG

2-KG was measured in reaction supernatants by the method of Lanning and Cohen (1951), which is based on the formation of the quinoxaline derivative on reaction with o-phenylenediamine. The assay permits a measurement of 10 - 100 μgs per sample. Corrections for glucose, pyruvate, and α-ketoglutarate interference were made when these compounds were present.

4. Enzymatic determinations of keto-acids

a. Lactic dehydrogenase assay for pyruvate

Pyruvate was measured directly by the quantitative decrease in O.D. at 340 mμ caused by the oxidation of $NADH_2$ in the presence of commercial lactic dehydrogenase. Under the conditions employed 0.02 - 0.10 μM of pyruvic acid per sample could be measured.

b. Glutamic dehydrogenase assay for α-ketoglutarate

Glutamic dehydrogenase was used as a spectrophotometric assay for α-ketoglutarate in the presence of excess ammonia, limiting α-ketoglutarate and $NADH_2$. The enzyme was purchased from Nutritional Biochemical Co. and was found to be readily inactivated in dilute solution so that 0.005 ml of concentrated enzyme was used. The sensitivity of the assay was the same as that of the lactic dehydrogenase assay for pyruvate and both methods were very specific.

5. Protein determinations

Protein was determined with the Folin-phenol reagent as described by Lowry et al (1951), and it is specific for substituted aromatic rings, hence for tyrosine, phenylalanine, and tryptophane residues of protein. The protein standard used was egg albumin.

EXPERIMENTAL RESULTS AND DISCUSSION

I. The Non-phosphorylated Oxidative Pathway of Glucose Dissimilation

1. Identification of products of glucose oxidation

The accumulation of 2-KG from glucose by aerobic organisms such as P. aeruginosa under conditions of intense aeration (Lockwood, Tabenkin, and Stodola, 1941; Ney, 1948; Hill, 1951), demonstrated that a non-phosphorylated oxidative mechanism was available to the cells. However, as 2-KG is an endproduct under these adverse, specific conditions it does not necessarily follow that the keto-acid is an intermediate of glucose dissimilation under normal conditions. Norris and Campbell (1951), demonstrated that P. aeruginosa oxidized glucose by way of both gluconate and 2-KG when growing under normal physiological conditions. These intermediates accumulated in the growth medium over an eight hour growth period. Resting cells of P. aeruginosa were shown to have a strong constitutive ability to oxidize glucose, gluconate, and 2-KG.

The evidence that 2-KG is a transient intermediate during glucose dissimilation by growing cultures was corroborated in this investigation. As an alternative to the sampling of growing cultures over a period of time, 20 hour carbon limited cultures of P. aeruginosa (stationary phase cells) were pulsed with 0.2% glucose, gluconic, or 2-KG

and permitted to oxidize the carbon source for 1 hour. At the end of this time, the cultures were centrifuged, the supernatant fluids treated batchwise with Dowex 50 H^+ and reduced to a small volume (approximately 10 mls) by flash evaporation at 40 C. The supernatant fluids were analyzed by paper electrophoresis and paper chromatography. Gluconate and 2-KG were shown to be intermediates in glucose dissimilation and 2-KG was identified as an intermediate in gluconic acid dissimilation; no other compounds were found to accumulate with any of the three carbon sources (Table 1). Washed

Table 1. Intermediates of glucose and gluconic acid dissimilation by growing cultures.

Substrate	Paper chromatography	Paper electrophoresis
glucose	2-KG, gluconate.	only gluconate or 2-KG were
Gluconate.	2-KG, gluconate.	detected by electrophoresis
2-KG	2-KG	

suspensions of glucose grown cells actively metabolize these three substrates (Fig. 1). Glucose and gluconic acid were metabolized by washed-cell suspensions at the same rate but 2-KG was initially oxidized more slowly primarily due to a 5-10 minute lag period.

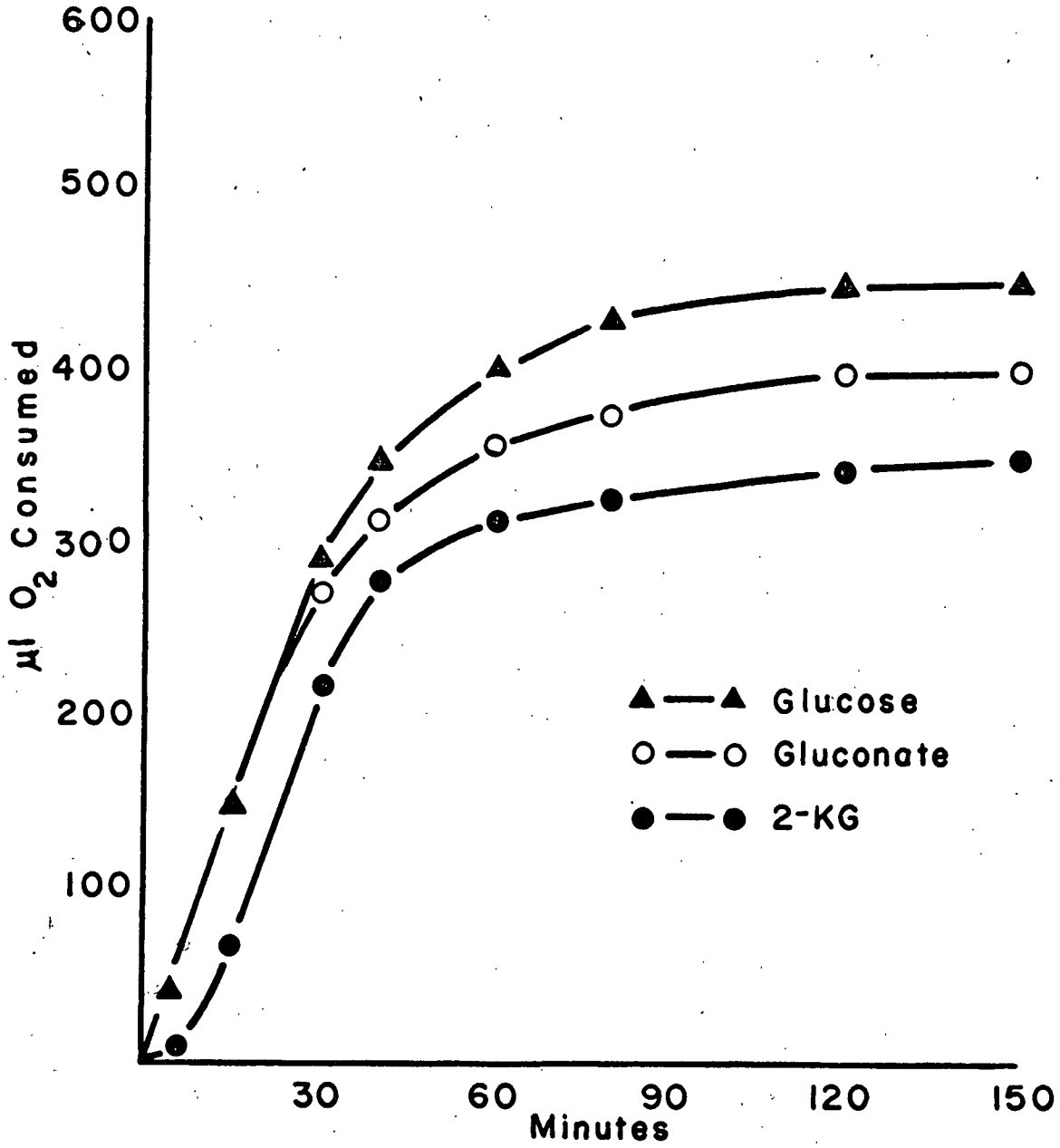

Fig. 1. Oxygen uptake by P. aeruginosa with 5µM of glucose, gluconic acid or 2-ketogluconic acid.

The non-phosphorylated oxidation of glucose to 2-KG by dried cell-free preparations was first demonstrated in P. aeruginosa by Stokes and Campbell in 1951. These cell preparations were shown to oxidize glucose with the uptake of 2 μatoms of oxygen, and gluconic acid with the uptake of 1 μatom of oxygen per μM of substrate. In each case, 2-KG was identified, by paper chromatography as the only end-product of the reactions thus suggesting quantitative accumulation under these conditions. Identical manometric data has been obtained using cell-free extracts prepared by sonication (Claridge and Werkman, 1963), and also using cell-free extracts prepared with a Hughes' press (Hogenkamp 1956).

Hogenkamp also showed that the oxidation of gluconolactone to 2-KG was accompanied by 1 μatom of oxygen uptake per μM of substrate and this was substantiated by Gronlund (1961), who demonstrated that the enzymic conversion of the lactone to the acid was magnesium dependent. These latter observations agreed with the findings of Brodie and Lipmann (1954), who showed that sonicated cell-free extracts of Azotobacter vinelandii oxidized glucose to gluconic acid by two distinct enzymic reactions; the oxidation of the aldehyde to the lactone; and its subsequent enzymic hydrolysis to the free acid. Ramakrishnan and Campbell (1955), partially purified gluconic dehydrogenase from sonicated cells and the enzyme was found to catalyze the conversion

of gluconic acid to 2-KG without concurrent phosphorylation. Neither NAD nor NADP functioned in this oxidation.

The formation of 2-KG from glucose and gluconic acid has been substantiated during the course of this investigation during various attempts to detect intermediates of 2-KG metabolism and to correlate these with glucose and gluconate metabolism. The reactions were carried out in large Warburg vessels using cell-free extracts, 25μM of glucose, gluconate, or 2-KG, and 50μM of Mg^{++}. One reaction mixture, with glucose as the substrate, was incubated without added magnesium.

The reactions were allowed to proceed for 90 minutes with the exception of the magnesium deficient mixture which was stopped at 20 minutes. All reactions were terminated with PCA and following the removal of protein, nucleic acids and excess PCA the resulting clear supernatant fluids were treated batchwise with Dowex 50 H^{+} and analyzed by electrophoresis and paper chromatography. From the results (Table 2) it can be seen that gluconolactone, gluconic acid, and 2-KG were formed from glucose by a cell-free pressate and that 2-KG was the end-product of the reaction. These results are analogous to those obtained by Stokes and Campbell (1951), and by Gronlund (1961).

Table 2. Products of glucose, gluconate and 2-KG dissimilation by cell-free extracts

Substrate	Paper chromatography	Paper electrophoresis
Glucose	2-KG	only gluconate or 2-KG were detected by electrophoresis
Glucose (no magnesium)	2-KG, gluconolactone, gluconate	
Gluconate	2-KG	
2-KG	2-KG	

2. Preparation and identification of 2-KG-C^{14}

Consequently, an investigation into the time course and quantitation of the conversion of glucose to 2-KG by cell-free pressates of P. aeruginosa was carried out prior to the enzymic preparation of 2-KG-C^{14} from glucose-C^{14}. A cell-free pressate was incubated on the Warburg apparatus with 5μM glucose, Tris buffer (pH 7.4), and 10μM of $MgCl_2$ in each of several Warburg vessels. Reactions were terminated at various time intervals by the addition of 0.2 ml of 1.4N PCA to the incubation mixture, and analyses for glucose, 2-KG, pyruvate and α-ketoglutarate were performed on the neutralized supernatant fluids. The results are graphically demonstrated (Fig. 2) and it can be seen that under these conditions the conversion of glucose to 2-KG was rapid and essentially quantitative thus providing an excellent means for the preparation of radioactive 2-KG from radioactive glucose.

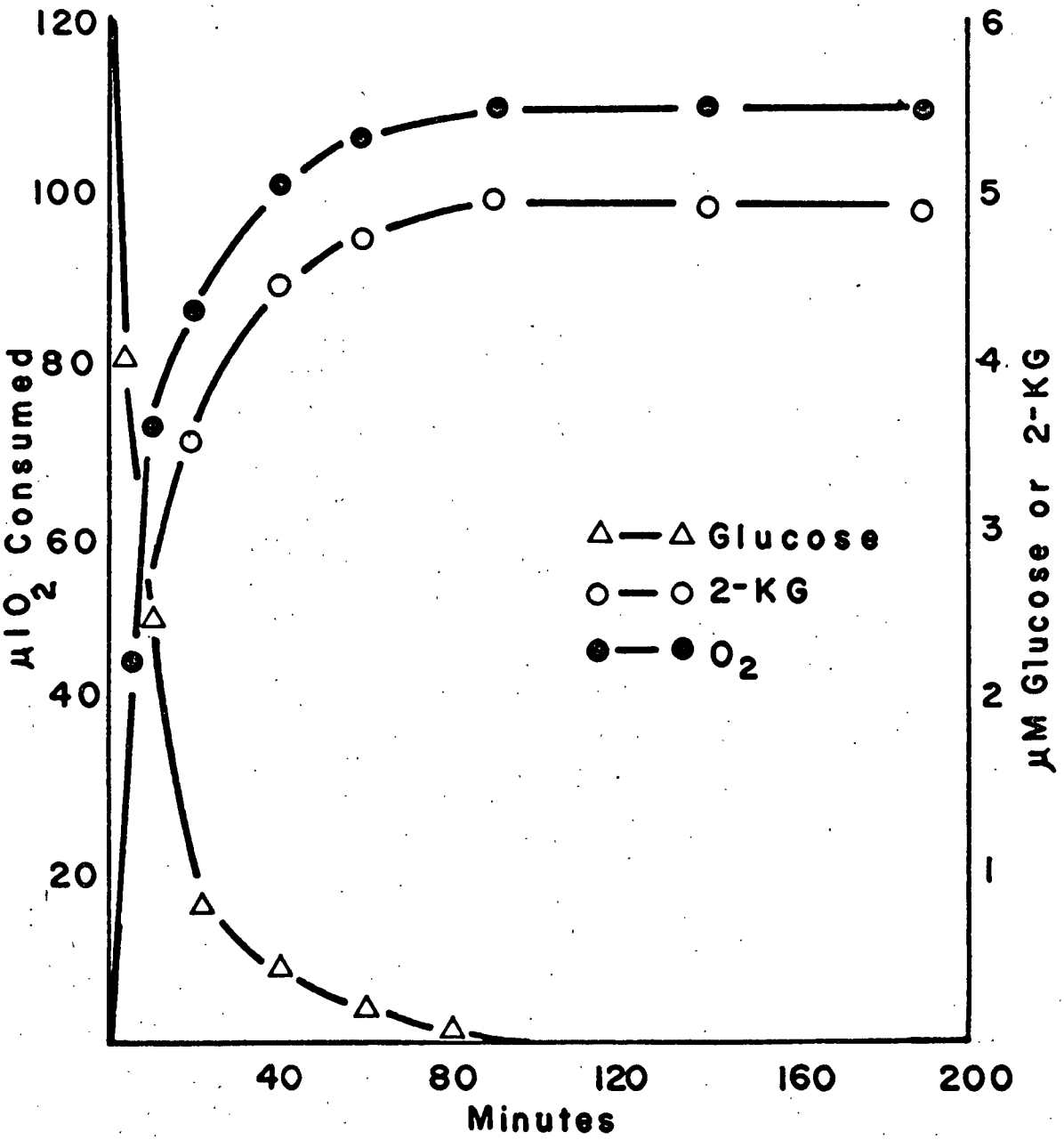

Fig. 2. Disappearance of glucose and formation of 2-KG in a cell free extract of P. aeruginosa

Frampton and Wood (1961), used a particulate fraction obtained from a cell-free preparation of P. fluorescens to prepare a 2-KG-1-C^{14} and 2-KG-6-C^{14} from glucose-1-C^{14} and glucose-6-C^{14} respectively. Their reactions were terminated after 1μM of oxygen/μM of substrate had been consumed, and the particulate matter was removed by centrifugation. The clear supernatant liquid was heated and used directly as the source of labelled 2-KG and no attempt at confirmatory identification was mentioned.

The procedure used in this work employed a cell-free pressate of P. aeruginosa and reaction mixtures were prepared and treated as described previously. The resultant protein-free neutralized supernatant fluid was applied to a Dowex 1-formate column which was eluted by the linear gradient technique with 500 ml of distilled water and 500 ml of formic acid. Ten ml aliquots were collected and assayed for radioactivity and UV absorbing material (Fig. 3), and the appropriate tubes, which in almost all cases constituted the only major radioactive peak, were pooled and evaporated to dryness several times at 40 C with a flash evaporator to remove the formic acid. The residue was resuspended in water, extracted with several volumes of ethyl-ether to remove any traces of formic acid, evaporated to dryness, resuspended in distilled water, and diluated to the desired specific activity with non-radioactive 2-KG. Negligible amounts of radioactivity were extracted into the ether. Radioactivity

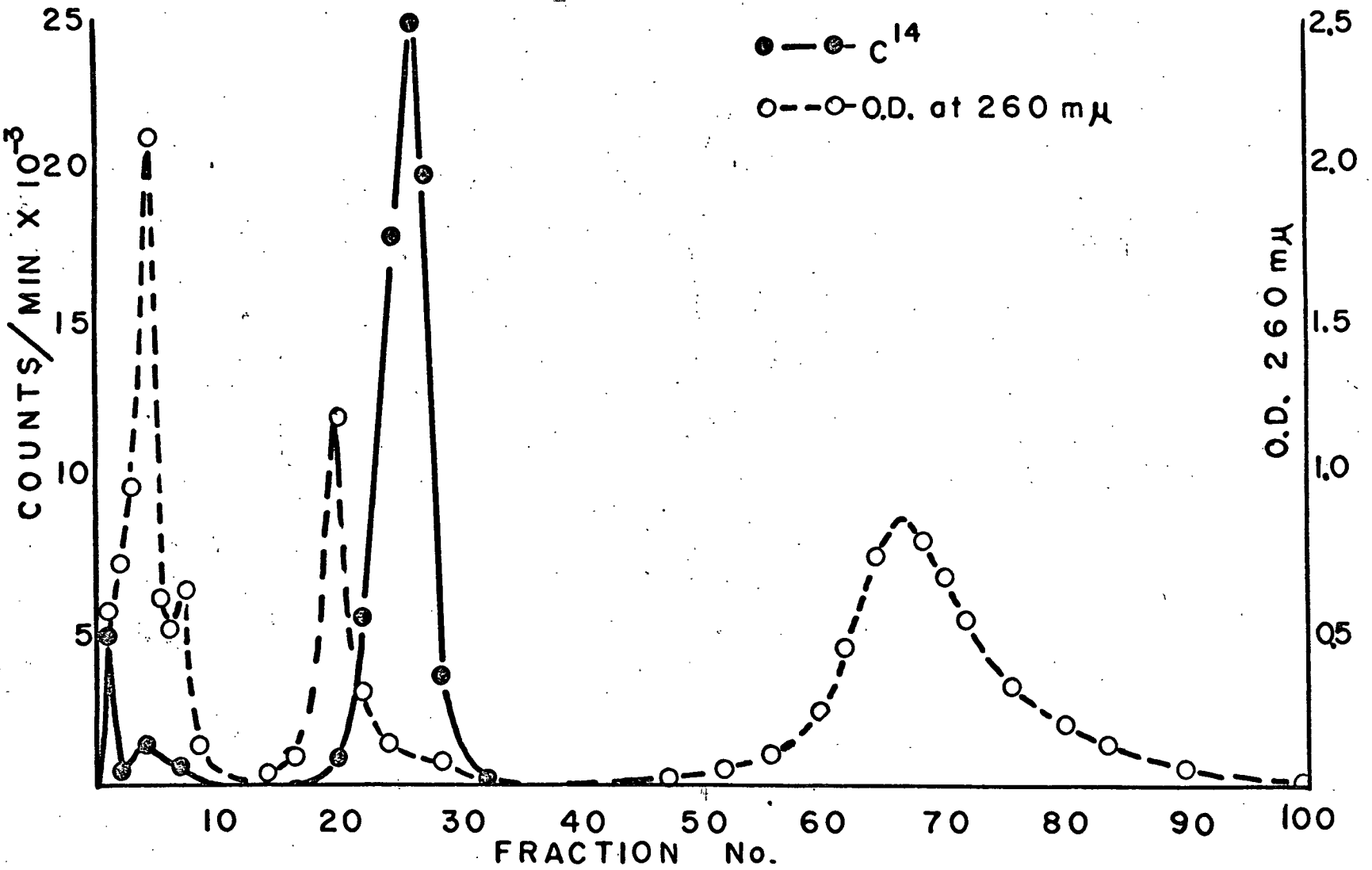

Fig. 3. Elution sequence of C^{14} compounds and U.V. absorbing material from a Dowex 1-formate anion exchange column

recoveries were 95-100% and 2-KG-C^{14} yields varied from 85-95% depending largely upon the pressate used. The radioactive product was identified conclusively as 2-KG by its position of elution from the column, electrophoretic mobility at pH 8, and by paper chromatography of both the free acid and its 2,4-dinitrophenylhydrazone derivative (Table 3).

II. Oxidative Assimilation of 2-Ketogluconate

The oxidative assimilation of glucose by P. aeruginosa has undergone thorough investigation by Duncan and Campbell (1962), who showed, using glucose-U-C^{14}, that a large proportion of assimilated C^{14} was incorporated into proteinaceous material via the reincorporation of accumulated α-ketoglutarate and endogenously supplied ammonia. In the presence of added ammonia it was found that assimilation was greatly increased, no α-ketoglutarate accumulated in the supernatant and the time course of C^{14} incorporation was markedly shortened. The tenability of these observations was reinforced by the extension of this work to other species of Pseudomonas and also Achromobacter species but attempts to reveal other key intermediate substances that were "pacemakers" for glucose oxidation and assimilation in P. aeruginosa were unsuccessful. Von-Tigerstrom and Campbell (1965), have demonstrated that pyruvate also accumulated in the Warburg supernatant fluid although at a much earlier time than α-ketoglutarate.

Table 3. Identification of 2-KG-U-C^{14} as the end-product of the enzymic oxidation of glucose-U-C^{14}

Compound	Paper chromatography Rg.	B.P.	O.P.D.	2,4-DNPH(Rf)	Electrophoresis (Re)
Glucose	1.00	+	absorbing	-	0
2-KG	0.51	+	fluorescent	0.41	0.19
Gluconate	0.31	+	-	-	0.19
Gluconol-actone	0.36	+	-	-	0.19
G-6-P	0.12	+	absorbing	-	0.23
6-P-G	0.09	+	-	-	0.33
Ribose-5-phosphate	0.10	+	-	-	0.26
Pyruvate	1.00	-	fluorescent	0.72,0.56	-
α-keto-glutarate	0.53	-	fluorescent	0.23	-
Reaction product	0.51	+	fluorescent	0.41	0.19

B.P. - Benzidene-periodate

O.P.D. - Orthophenylenediamine

2,4-DNPH - 2,4-Dintrophenylhydrazone

Rg - relative to glucose

Re - relative to the length of the electrophoretogram

Pyruvate, unlike α-ketoglutarate, did not appear to be important as a primary assimilation compound because it also accumulated in the presence of added ammonia and during growth in non-nitrogen limiting media.

Since 2-KG, pyruvate and α-ketoglutarate all have a common structural feature, it was of interest to determine whether or not 2-KG could serve in a capacity similar to α-ketoglutarate during oxidative assimilation. It was also of interest to compare the assimilation of 2-KG-U-C^{14} with the well established pattern obtained with glucose-U-C^{14} in an attempt to demonstrate possible divergences in glucose metabolism relative to 2-KG metabolism. The rate of 2-KG disappearance, oxygen uptake, and the appearance of endogenously produced ammonia during the oxidation of 2-KG by washed cell suspensions were measured (Fig. 4a). Pyruvate and α-ketoglutarate were measured enzymically but negligible amounts accumulated in the supernatant fluids. In comparison with the pattern established by Duncan and Campbell (1962), it may be seen that the disappearance of 2-KG was not complete until approximately 60 minutes, whereas glucose was found to disappear by 30 minutes. Of more importance is the apparent inability of the organism to accumulate keto-acids, specifically α-ketoglutarate, under these conditions. This observation sharply contrasts the findings of Von-Tigerstrom and Campbell (1963), who found that approximately 1.5 μM of α-ketoglutarate and 1.0 μM of pyruvate accumulated

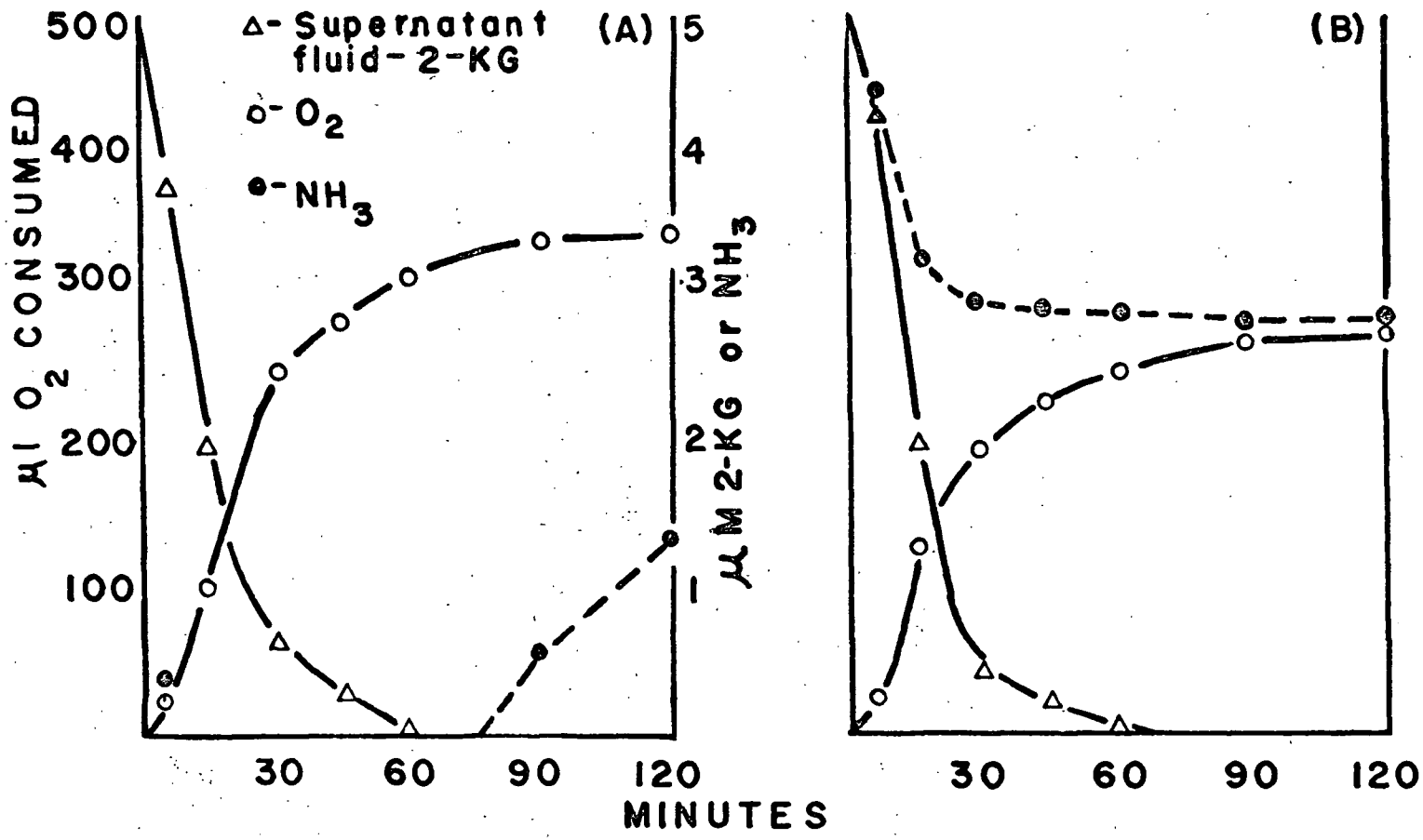

Fig. 4. Time course of 2-KG disappearance, oxygen uptake, and NH_3 release or disappearance with resting cells of P. aeruginosa. (A) without added NH_3 (B) with added NH_3.

in the Warburg supernate during the rapid oxidation of glucose. The failure to accumulate keto-acids from 2-KG would suggest that there was a supply of ammonia available which would permit immediate cellular utilization of α-ketoglutarate as it was formed. Experiments with added ammonia appeared to increase oxidative assimilation as evidenced by a decrease in O_2 uptake but did not enhance 2-KG disappearance, and again, neither α-ketoglutarate nor pyruvate were found to accumulate in the Warburg supernatant fluids (Fig. 4B). The failure to accumulate α-ketoglutarate in the presence of ammonia is analogous to the situation during glucose oxidation (Duncan and Campbell, 1962), but the reason for the inability to accumulate pyruvate is not obvious since pyruvate accumulation is unaffected by added ammonia during glucose oxidation by this organism (Von-Tigerstrom and Campbell, 1965).

Analogous experiments were carried out with 2-KG-U-C^{14} and the radioactivity was found in all major cell fractions (Table 4). The cold TCA soluble fraction appeared to be important during early stages of assimilation, and most of the substrate appeared to be assimilated into nitrogenous components but considerably earlier than was observed with glucose-U-C^{14} (Duncan and Campbell, 1962). After 60 minutes incubation, the radioactivity in the cells decreased with a concomitant increase in $C^{14}O_2$ (Fig. 5A). The decrease in cellular C^{14} was, for the most part, due to a loss of radioactivity from the protein fraction. Thirty-five percent of the radioactivity of this fraction was lost during the

Table 4. Incorporation of C^{14} from 5μM of 2-KG-U-C^{14} into washed cells

Time min.	Cold TCA soluble	Acid alcohol-soluble	Hot TCA soluble	Residual protein	Total in fractions
	% of total C^{14}				
20	3.10	-	-	-	13.50
30	4.90	6.20	4.25	11.90	27.25
60	5.45	8.00	4.64	15.20	33.29
90	3.87	8.44	3.19	11.71	27.16
120	2.64	6.28	2.78	9.80	20.50
	% of total C^{14} incorporated into cell fractions				
20	23.0	-	-	-	100
30	18.0	22.8	15.6	43.6	100
60	16.4	24.0	13.9	45.7	100
90	13.5	29.4	11.1	45.0	100
120	12.9	30.7	13.9	48.9	100

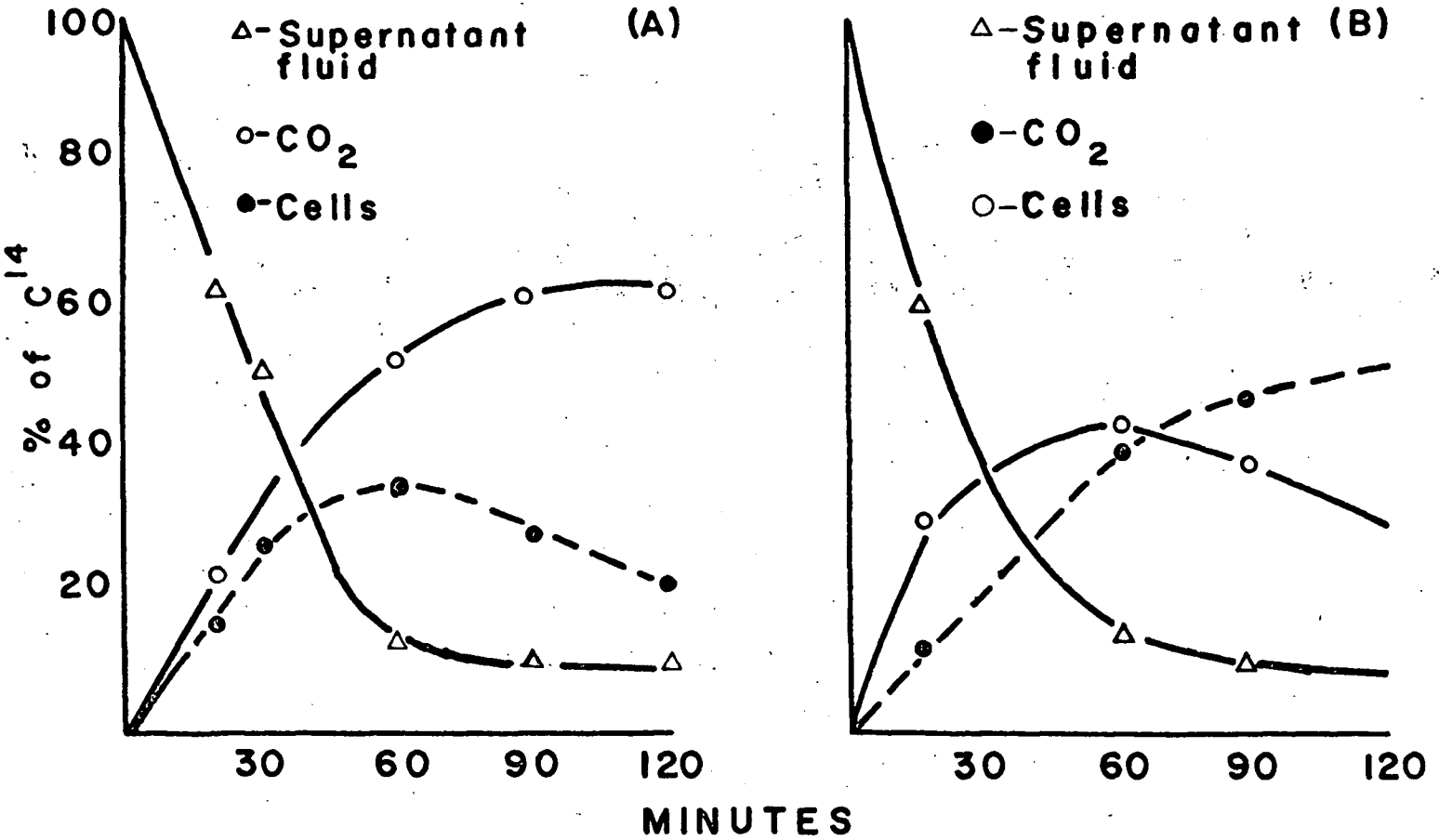

Fig. 5. Time course of the distribution of C^{14} added to washed cell suspensions as 2-KG-U-C^{14}. (A) In the absence of added NH_3. (B) In the presence of 5μM added NH_3.

60-120 min interval. The cold TCA soluble fraction and, to a lesser extent, the hot TCA soluble fraction also decreased in radioactivity at this time. Duncan and Campbell (1962), observed similar losses from the protein and cold TCA fractions but not from the hot TCA fraction. In general, a much greater incorporation of radioactivity into cellular constituents was observed with 2-KG-U-C^{14} than with glucose-U-C^{14}. In fact, the data obtained by Duncan and Campbell (1962), concerning the effects of 5μM of added ammonia on glucose-U-C^{14} assimilation closely corresponds to that achieved with 2-KG-U-C^{14} in the absence of added ammonia. This is considered to be further evidence for the availability of ammonia during the oxidation of 2-KG by P. aeruginosa. The quantity of radioactivity incorporated into the cell fractions was twice as great at 60 minutes as the maximum achieved by Duncan and Campbell (1963), at 120 minutes, and this increase was manifested as a general increase in radioactivity of all cellular fractions. The incorporation of radioactivity into the acid-alcohol soluble fraction (lipid, phospholipid, and alcohol soluble protein) was accumulative whereas with glucose-U-C^{14} experiments (Duncan and Campbell, 1962), the radioactivity was slowly reduced.

When 5μM of ammonia were added to the reaction vessels, there was an increase of 36% in the total quantity of assimilated radioactivity and almost 100% of this increase appeared in the protein fraction. An even more pronounced effect of exogenous ammonia was the rapidity of C^{14} incorporation (Table 5). Duncan and Campbell (1962),

demonstrated a 110% increase in assimilation of radioactivity from glucose-U-C^{14} in the presence of 5μM exogenous ammonia. The lower increase of assimilated radioactivity from 2-KG-U-C^{14} in the presence of 5μM added ammonia emphasizes the probability of the greater availability of ammonia in the presence of 2-KG.

These observations may be explained, in part, by the failure to accumulate α-ketoglutarate under these conditions. If α-ketoglutarate had been immediately incorporated into cellular material then the probability of complete substrate oxidation via the tricarboxylic cycle would have been reduced. Duncan and Campbell (1962), have demonstrated that chloramphenicol markedly reduced C^{14} incorporation into cellular material, and explained this as a manifestation of the inability of the cell to synthesize a specific permease necessary for the reincorporation of accumulated α-ketoglutarate. However, chloramphenicol had a much lesser effect on oxygen uptake with 2-KG thereby supporting the observation that α-ketoglutarate does not accumulate during the oxidation of 2-KG (Table 6). The uncoupling agent, sodium azide, had more than twice the stimulatory effect on oxygen uptake with 2-KG than with glucose. This substantiates the greater quantitative assimilation observed with 2-KG-U-C^{14} relative to glucose-U-C^{14}.

The postulation that more ammonia is available during 2-KG oxidation than glucose oxidation by washed cell suspensions has been supported by indirect evidence.

Table 5. Incorporation of C^{14} from 5μM 2-KG-U-C^{14} into washed cells in the presence of 5μM NH_4Cl

Time	Cold TCA soluble	Acid alcohol-soluble	Hot TCA soluble	Residual protein	Total in fractions
	% of total C^{14}				
15	5.93	8.52	2.91	13.20	30.56
60	4.64	8.85	4.54	27.55	45.30
90	4.16	9.32	4.09	22.05	39.62
	% of total C^{14} incorporated into cell fractions				
15	19.4	27.9	9.5	43.2	100
60	10.3	19.5	10.0	60.7	100
90	10.4	23.5	10.30	55.7	100

Table 6. The effect of chloramphenicol and sodium azide on the oxygen consumption by washed cell suspensions in the presence of 5μM glucose or 2-KG

Substrate 5μM	Assimilation inhibitor	% change in O_2 uptake based on 2-KG*
Glucose	chloramphenicol 6.2×10^{-4} M	-14.5
2-KG	chloramphenicol 6.2×10^{-4} M	- 7.8
Glucose	NaN_3 5×10^{-3} M	+ 4.8
2-KG	NaN_3 5×10^{-3} M	+12.2

* This calculation was based on the assumption that all glucose was metabolized via 2-KG and therefore 2 atoms of oxygen/μM of glucose was subtracted prior to calculation of % change.

Protein and RNA have been established as substrates of endogenous respiration in P. aeruginosa (Gronlund and Campbell, 1963), and when these cellular components were specifically labelled the label was shown to appear as $C^{14}O_2$ during incubation of the cells in the absence of exogenous substrate. The effects of various exogenous substrates on the release of $C^{14}O_2$ from specifically labelled cells was determined (Gronlund and Campbell, 1965). It was found that 2-KG, as an exogenous substrate, stimulated the endogenous $C^{14}O_2$ evolution by 150% over the value obtained with exogenous glucose and presumably 1.55times more NH_3 was made available. However, all substrates including glucose, α-ketoglutarate, aspartate, 2-KG, and adenosine suppressed degradation of cellular RNA to various degrees at 30, 60, 90, and 120 minutes, but 2-KG increased RNA oxidation above the endogenous level at 10 minutes - the interval corresponding to the 2-KG oxidation lag. At this time from 35-123% more $C^{14}O_2$ was evolved with 2-KG as the substrate relative to other substrates tested. These data were interpreted as meaning that 2-KG stimulated RNA degradation as well as protein but the RNA degradation was probably associated with the lag in oxygen uptake.

The depression of oxygen consumption by exogenous ammonia was more pronounced with 2-KG than with glucose as can be seen from the manometric data in Table 7. Approximately 300% more oxygen consumption depression

Table 7. Inhibitory effect of ammonia on oxygen consumption by washed cell suspensions in the presence of various substrates

Substrate 5μM	Time minutes	% Inhibition	% Inhibition based on O_2 uptake of 2-KG
Glucose	60	16.6	18.0
	120	12.5	12.0
	180	3.8	4.0
2-KG	60	23.8	-
	120	27.0	-
	180	13.5	-

occurred with 2-KG than with glucose at 180 minutes. These data support the greater C^{14} incorporation into cellular constituents observed with 2-KG-U-C^{14} reactions in the presence of added ammonia. Trace amounts of pyruvate and α-ketoglutarate were detected by chromatography of their 2,4-dinitrophenyl-hydrazones thus demonstrating that 2-KG is, in fact, degraded via TCA intermediates as is glucose. No other detectable intermediates, such as glutamic or glucosamic acid which would be expected as a result of the amination of α-ketoglutarate or 2-KG respectively, were present in the supernatant fluids after hydrazone extraction.

III. Intermediates of 2-KG Metabolism

A study of the pathways of metabolism of a compound must be made with extreme care and with as many different techniques as is necessary to conclusively substantiate the validity of intermediates and/or of functioning enzyme systems. The fact that a living cell does possess and utilize a particular pathway should be demonstrated by the isolation and identification of intermediates, and this approach may be facilitated by the use of various available metabolic inhibitors. The requisite enzymes or enzyme systems should be demonstrated in cell-free extracts or, if possible, in whole cells and the relative activities measured. Finally, labelled substrates

and the isolation of key intermediates may be used to obtain information on the route through which the different intermediates have passed. The use of only one of these approaches may give erroneous answers as Racker (1954), has emphasized. The previous application of these basic principles to the problem of 2-KG metabolism in P. aeruginosa has met with relatively little success. Reid (1959), using cell-free extracts prepared with a Hughes' press, was unsuccessful in finding intermediates and key enzymes involved could not be demonstrated, with the exception of a relatively weak 2-K-6-PG reductase. Claridge and Werkman (1953), concluded that cell-free extracts of this organism were incapable of metabolizing 2-KG and therefore investigated this problem with whole cells and 2-KG-U- C^{14}. However, no key six carbon intermediates were identified but various TCA intermediates and amino acids were isolated and identified. Glucose-6-phosphate was isolated and its existence was justified by the presumption of either a reversal of direct oxidation, which is very unlikely, or by a reversal of glycolysis. Gronlund (1961), confirmed the presence of the enzyme 2-K-6-PG reductase, and isolated glucose-6-phosphate from anaerobic 2-KG reactions with cell-free extracts and ATP, and suggested that the substrate has been dissimilated via the pentose phosphate pathway. Two other compounds were isolated and were tentatively identified as 3-phosphoglyceraldehyde and 3-phosphoglyceric acid from their mobilities on electrophoresis. The

existence of these trioses as intermediates of 2-KG metabolism was considered feasible in light of the identification of G-6-P and the existence of the pentose phosphate pathway.

1. Identification of intermediates of 2-KG dissimilation with whole cells

Since cell-free extracts have proved to be relatively inert toward 2-KG it was decided to continue the investigation using whole cell suspensions. Reactions were carried out in large Warburg cups with 25μM of 2-KG as the substrate. Cups were removed at various time intervals and the reactions were terminated by pipetting the cup contents into ice-cold centrifuge tubes and the Warburg supernatant fluids were obtained by centrifugation. Hydrazones were prepared and the supernatant fluids were concentrated and subjected to chromatographic and electrophoretic analysis. Anaerobic experiments were carried out similarly, under an atmosphere of N_2. In one set of reaction mixtures the reaction was carried out in a neutralized hydrazine sulfate buffer in order to trap any short-lived transient ketone or aldehyde intermediates (Meyerhof and Junowicz-Kocholaty, 1943), and the 2,4-dinitrophenylhydrazone derivatives were prepared and analyzed. The results are tabulated in Table 8. No intermediates other than pyruvate and α-ketoglutarate could be demonstrated and, in the case of reactions containing the trapping agent, hydrazine, large amounts of these

Table 8. Products of 2-KG dissimilation by washed cell suspensions of P. aeruginosa

Reaction time minutes	Paper Chromatography Supernatant	Hydrazone	Paper Electrophoresis
Endogenous	-	-	-
10	2-KG	pyruvate	*2-KG or gluconate
20	"	"	" "
30	"	pyruvate, α-keto-glutarate	" "
40	"	pyruvate, α-keto-glutarate	" "
Anaerobic			
10	2-KG	-	2-KG or gluconate
20	"	trace of pyruvate	" "
Hydrazine			
10	2-KG	pyruvate	2-KG or gluconate
20	"	pyruvate, α-keto-glutarate	" "

*Gluconate and 2-KG have the same electrophoretic mobility under the conditions employed.

keto-acids accumulated. Traces of pyruvate were detectable in the anaerobic reaction mixtures but it could not unequivocally be concluded that a series of non-oxidative steps were functioning to give rise to these intermediates due to the possibility of small amounts of O_2 contamination. From these experiments it was concluded that any intermediates that form during the dissimilation of 2-KG must be extremely transient and do not accumulate extracellularly. Therefore special techniques had to be employed to promote the accumulation of intermediates.

2. Identification of intermediates of 2-KG dissimilation in the presence of inhibitors

Although the assumption that one can prove or disprove the existence of operable metabolic pathways by the use of inhibitors has been open to much criticism (Racker, 1954), the use of these agents has found useful application in carbohydrate biochemistry. Entner and Doudoroff (1951), found that the substrate assimilation mechanisms of P. saccharophila could be inhibited by relatively weak concentrations of iodoacetate (2×10^{-3}M), dinitrophenol (2.5×10^{-4} M), and arsenite (2×10^{-3} M) and, in the cases of the latter two poisons, quantitative accumulation of pyruvic acid from glucose and gluconic acid substrates was achieved. When gluconate-1-C^{14} or gluconate-6-C^{14} were used as a substrate the

label was found in the carboxyl and methyl carbons respectively of pyruvate, thereby suggesting a 3:3 split mechanism, completely different from the established Embden-Meyerhof pathway. A similar approach led Frampton and Wood (1961), to the conclusion that under conditions of arsenite poisoning a crude cell-free extract of P. fluorescens could convert variously labelled 2-KG quantitatively to pyruvate, and subsequent degradation studies also indicated the presence of a 3:3 split mechanism. Katznelson (1958), studying hexose phosphate metabolism in A. melanogenum, found that arsenite and fluoride treated sonicates allowed quantitative pyruvate accumulation when 6-PG was used as a substrate, but relatively little pyruvate accumulated when a pentose phosphate was used as a substrate. From this experiment they concluded that 6-PG was more likely to be degraded by the Entner-Doudoroff route rather than by the pentose-phosphate cycle.

Several inhibitors were investigated in the course of this work for two reasons: firstly that an enzyme involved in 2-KG degradation might be selectively inhibited thereby allowing the accumulation of at least chromatographic amounts of a key intermediate, and secondly that perhaps enough pyruvate could be accumulated to allow insight into the mechanism of 2-KG degradation. The effect of various concentrations of each inhibitor was followed manometrically and pyruvate accumulation was

determined quantitatively at each concentration employed.

a) sodium arsenite

Arsenite proved to be a potent inhibitor of 2-KG oxidation with maximum effect at concentrations greater than 10μM/cup as can be seen from Figure 6A. The maximum yield of pyruvate, 6μM, (Fig. 6B) was achieved with 3μM of arsenite/cup and although quantitative accumulation was not achieved, subsequent analysis of the supernatant fluid showed that some 2-KG still remained unreacted and was not likely to react under these inhibited conditions as oxygen uptake had ceased. These data suggested the existence of some type of a 3:3 split mechanism.

Of considerable importance was the observation that no oxygen consumption occurred with concentrations of inhibitor exceeding 6.0μM but some pyruvate was still found. These results very strongly suggested that a mechanism for the degradation of 2-KG to pyruvate without oxidation must be operative.

b) bromopyruvic acid

Meloche (1965), studied the effect of the product analogue, bromopyruvate, on the enzyme 2-K-3-D-6-PG aldolase and found it to be a potent inhibitor. From kinetic studies, it was seen that a reversible enzyme-bromopyruvate complex was formed prior to enzyme inactivation.

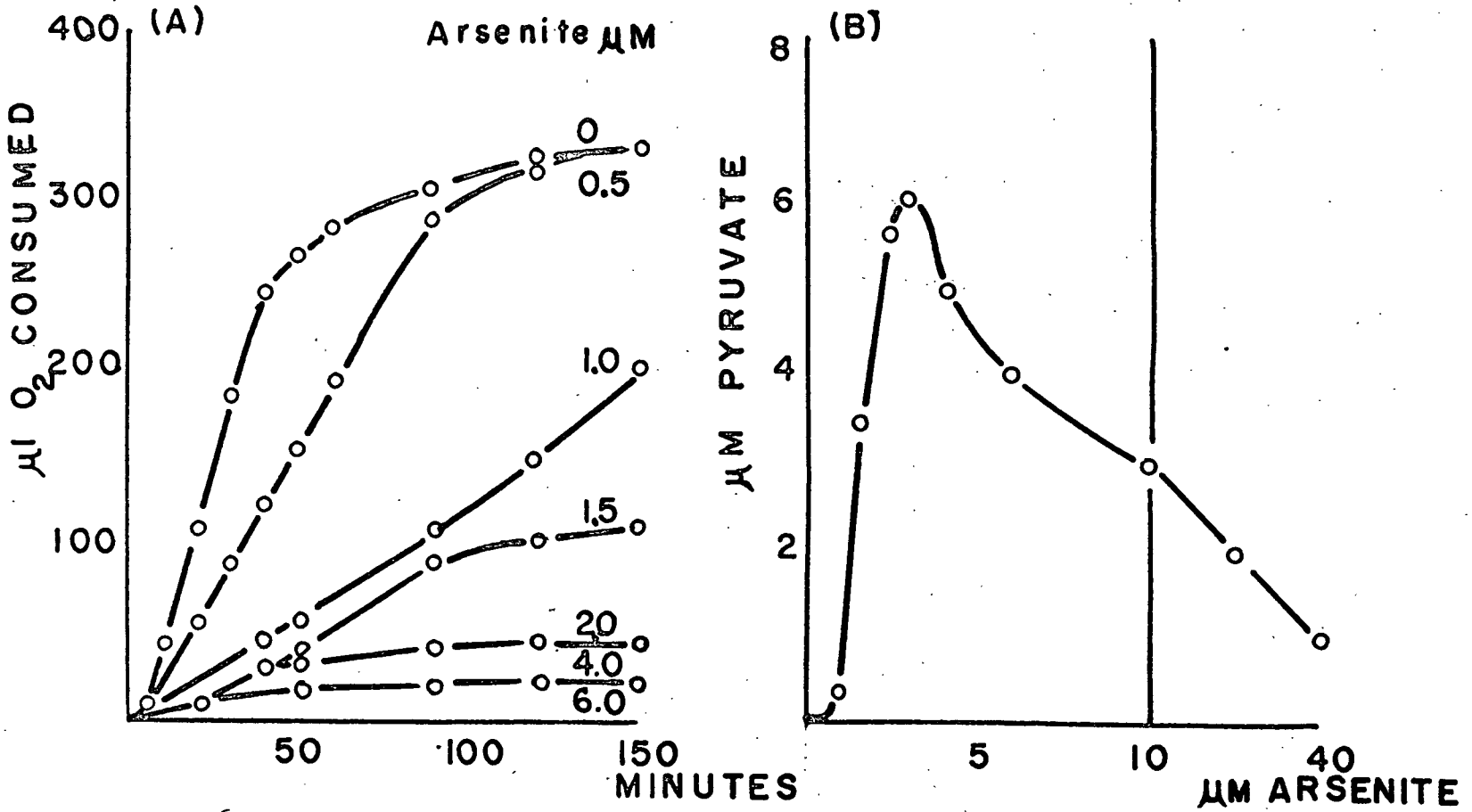

Fig. 6A. Oxygen consumption by washed cell suspensions in the presence of arsenite and 5µM 2-KG. Endogenous values have been subtracted.

Fig. 6B. Pyruvate accumulation from 5µM 2-KG by washed cell suspensions in the presence of various concentrations of arsenite.

Because of Meloche's work bromopryuvate was thought to be an ideal inhibitor of 2-K-3-D-6-PG aldolase in whole cells of P. aeruginosa due to its probable specificity. Inhibitory concentrations were determined (Fig. 7A) and pyruvate accumulation was measured (Fig. 7B). Although the pyruvate determinations were hindered somewhat by the reactivity of the analogue, bromopyruvate, with lactic dehydrogenase, a fairly accurate determination was carried out. At the concentrations of bromopyruvate which allowed sufficient oxygen consumption to account for the oxidation of triosephosphate to pyruvate, almost quantitative pyruvate accumulation, 2μM/μM 2-KG, was detected. These results may be explained by the functioning of an active Entner-Doudoroff pathway as has been demonstrated in this organism (Gronlund, 1961).

c) sodium selenite

Enzymes susceptible to the inhibitory effects of selenite have been widely investigated (Rosenfeld and Beath, 1964) and it has been suggested that selenite functions by combining with an active group in the enzyme, presumably a sulfhydryl group. In this investigation of 2-KG metabolism, selenite was found to be a strong inhibitor of 2-KG oxidation (Fig. 8A), exhibiting considerable inhibition at levels greater than 2.5μM/cup and complete inhibition of oxygen consumption at 20μM. However, as in the case of arsenite inhibition, pyruvate was produced

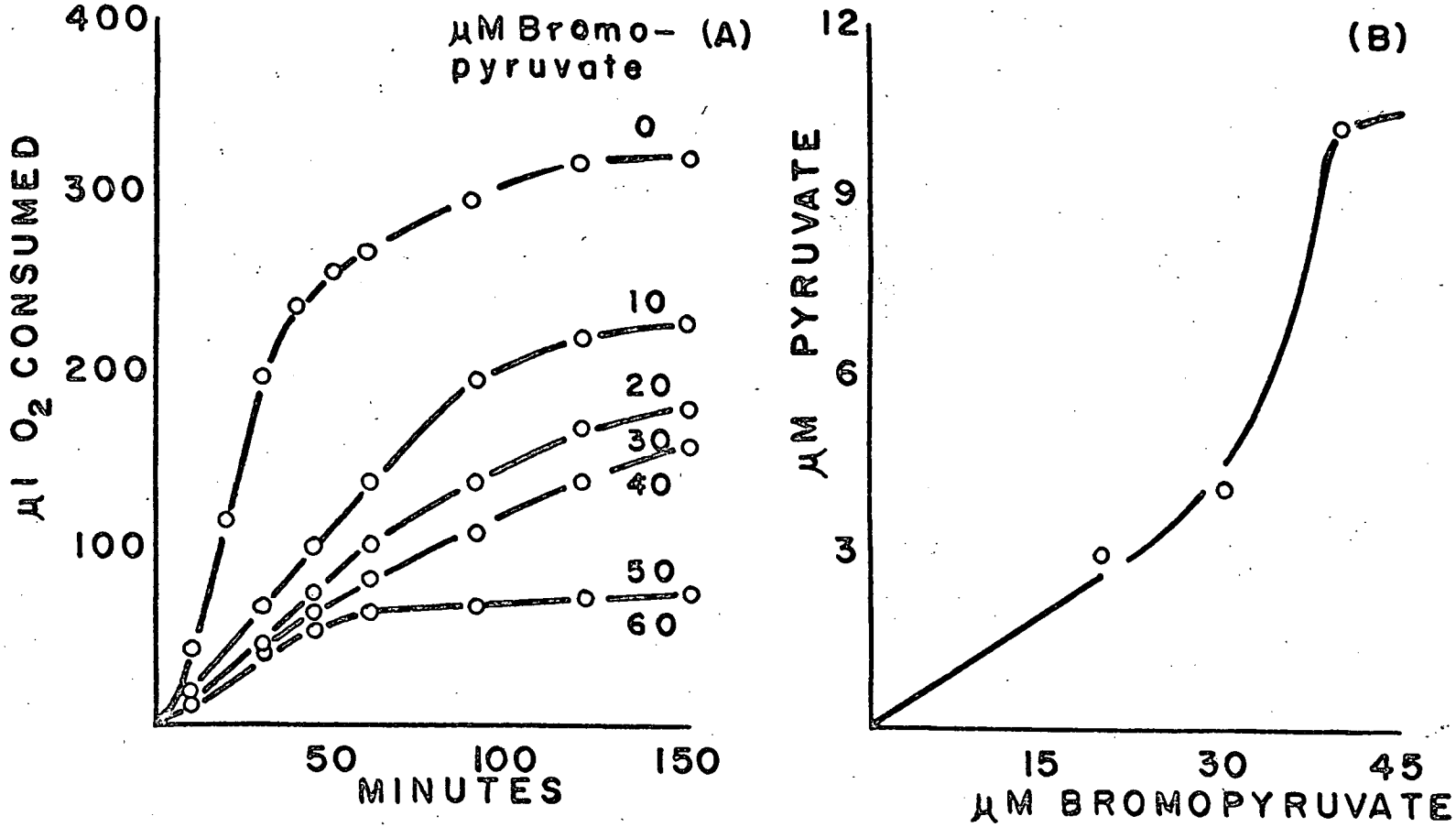

Fig. 7A. Oxygen uptake by washed cell suspensions in the presence of selenite and 5µM 2-KG. Endogenous values have been subtracted.

Fig. 7B. Pyruvate accumulation from 2-KG by washed cell suspensions in the presence of various concentrations of bromopyruvate.

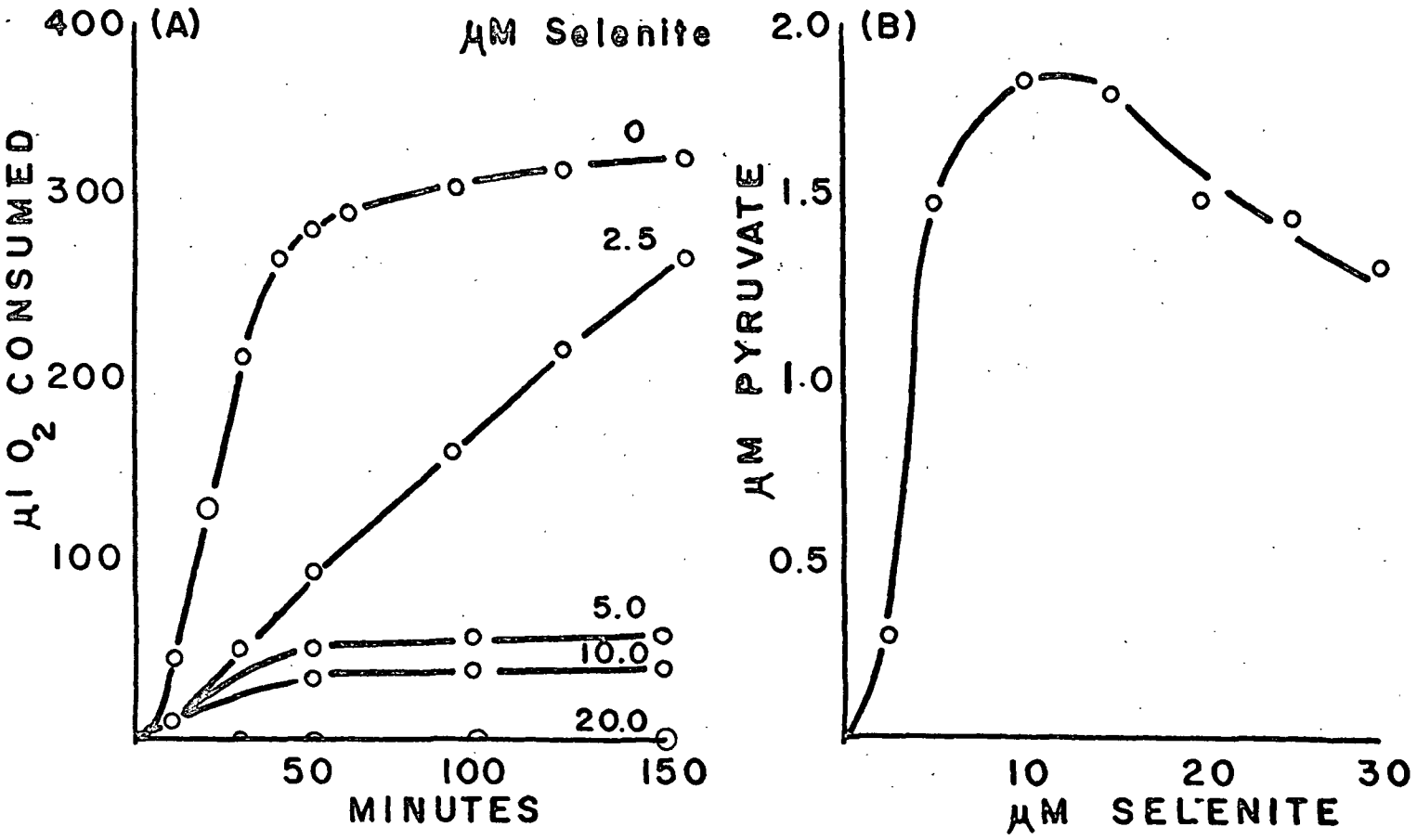

Fig. 8A. Oxygen uptake by washed cell suspensions in the presence of selenite and 5μM 2-KG. Endogenous values have been subtracted.

Fig. 8B. Pyruvate accumulation from 2-KG by washed cell suspensions in the presence of various concentrations of selenite.

without concomitant oxygen consumption substantiating the existence of an non-oxidative metabolic route from 2-KG to pyruvate.

d) sodium fluoride and iodoacetamide

Much information has been made available by the judicious use of sodium fluoride and iodoacetamide from the point of view of intermediate accumulation in the presence of these substances and as a guide to an assessment of pathway involvement, particularly glycolysis (Hochster and Quastel, 1963). In this study, neither sodium fluoride nor iodoacetamide caused a significant accumulation of pyruvic acid, suggesting inhibition of 2-KG metabolism at an earlier stage. Concentrations of 900μM sodium fluoride were necessary to completely inhibit oxygen uptake from 2-KG (Fig. 9). Conversely, iodoacetamide proved to be a potent inhibitor in that 10μM per cup reduced oxygen consumption to 14% of the control value (Fig. 10). Complete inhibition of oxygen uptake with iodoacetamide was a difficult task requiring high concentrations of the inhibitor. Oxygen uptake values between 50 and 60μl were constantly encountered with concentrations up to 20μM/cup suggesting that perhaps no inhibition at the level of triosephosphates took place.

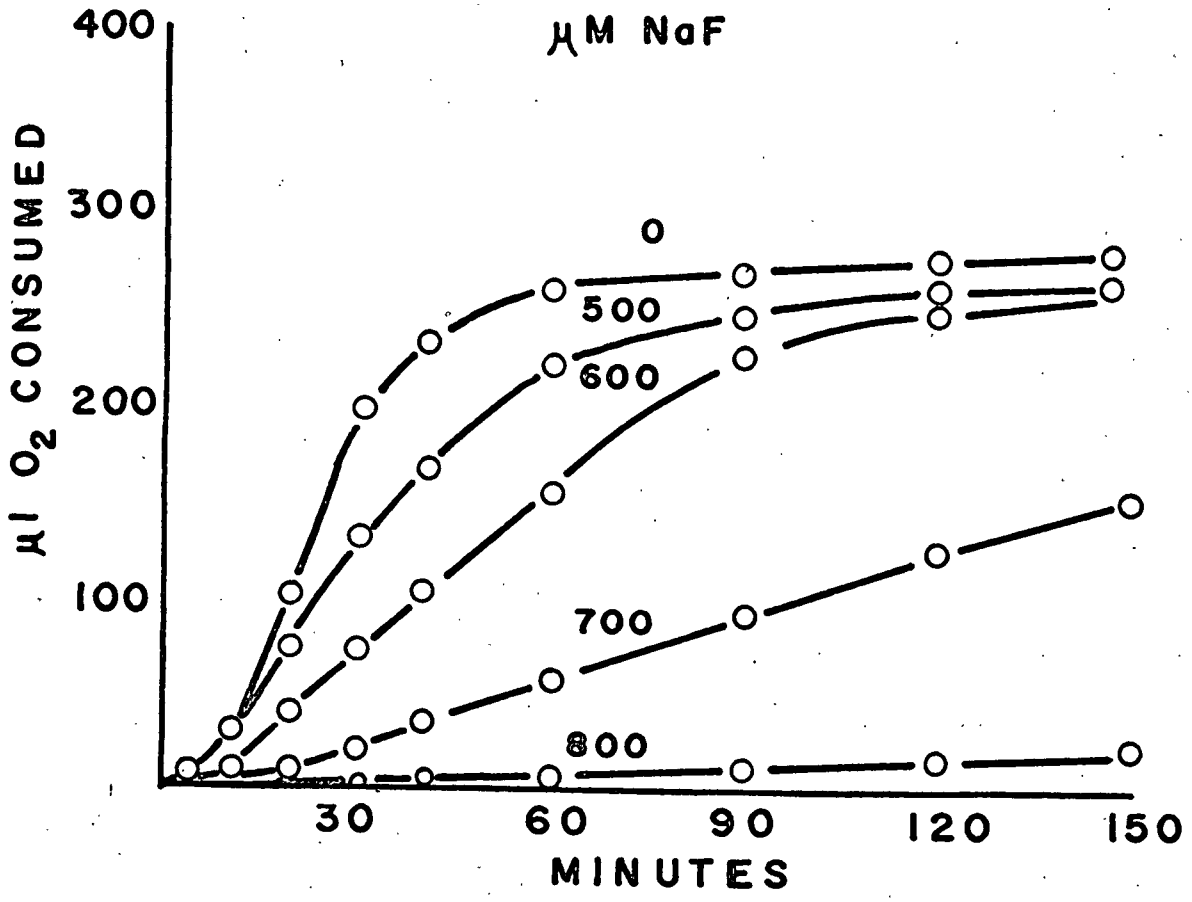

Fig. 9. Oxygen uptake by washed cell suspensions in the presence of sodium fluoride and 5μM 2-KG. Endogenous values have been subtracted.

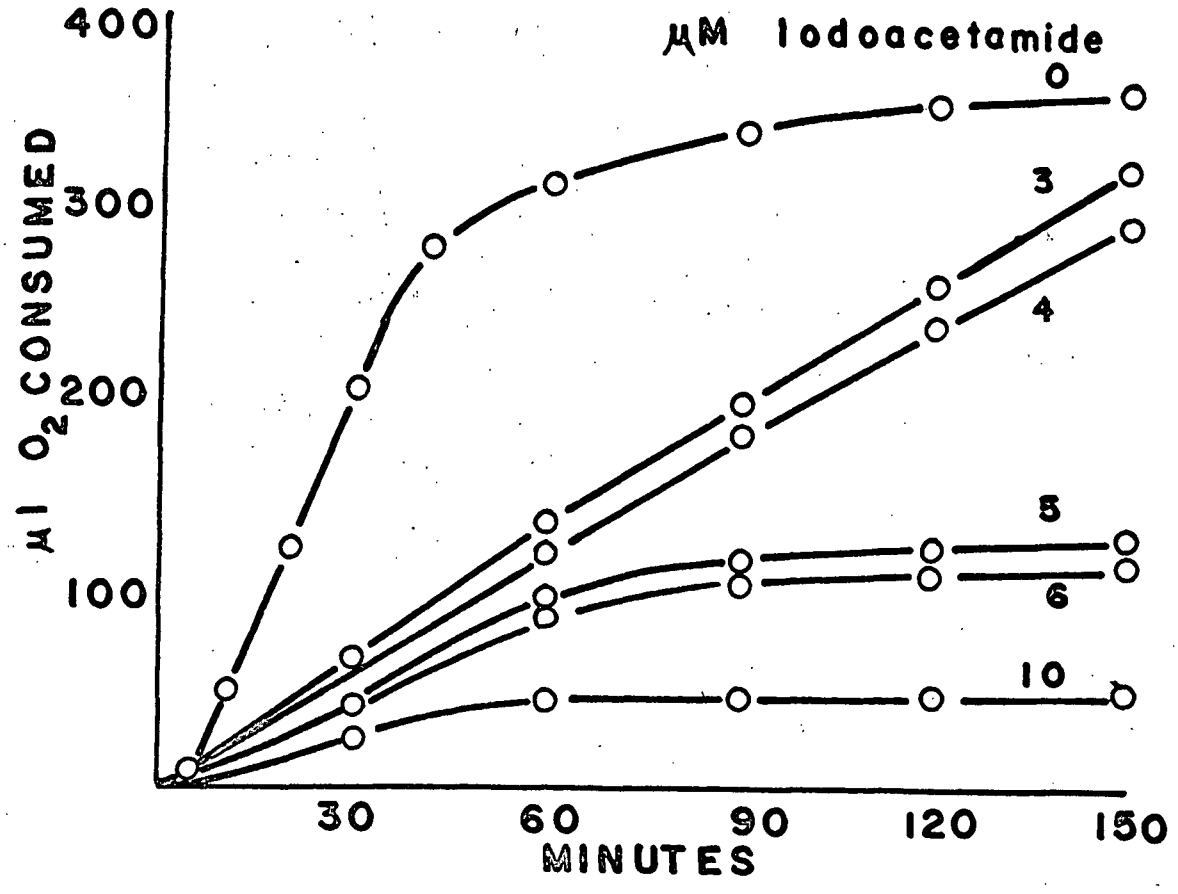

Fig. 10. Oxygen uptake by washed cell suspensions in the presence of iodoacetamide and 5μM 2-KG. Endogenous values have been subtracted.

e) ethylenediaminetetracetic acid (EDTA)

EDTA was also used as a metabolic inhibitor in an attempt to isolate metabolic intermediates. The inhibitory action of EDTA on oxygen consumption was consistently unusual in that the inhibition increased rapidly with increasing EDTA concentrations between 5 and 20μM/cup, but then a reversal of inhibition occurred at higher concentrations (Fig. 11). No explanation has been found for this observed phenomenon.

During the investigation of the effect of metabolic inhibitors on 2-KG metabolism, it was observed that with every inhibitor used, with the exception of EDTA, the oxygen uptake achieved with glucose as the substrate was always 1.0μM/μM substrate greater than when 2-KG was the substrate. This suggested that, under inhibited conditions, glucose was oxidized quantitatively to 2-KG. Subsequent analyses demonstrated that 2-KG was an end product of the inhibited reactions with glucose. However, comparative analyses of pyruvate accumulation (Table 8(a)) showed that consistently greater pyruvate yields were achieved when glucose was used as the substrate. This demonstrates that alternate routes of glucose metabolism other than the non-phosphorylated oxidative pathway are utilized by P. aeruginosa. These pathways are probably either via phosphorylation of glucose by hexokinase, by phosphorylation of gluconate by the gluconokinase, or by both pathways (Gronlund, 1961).

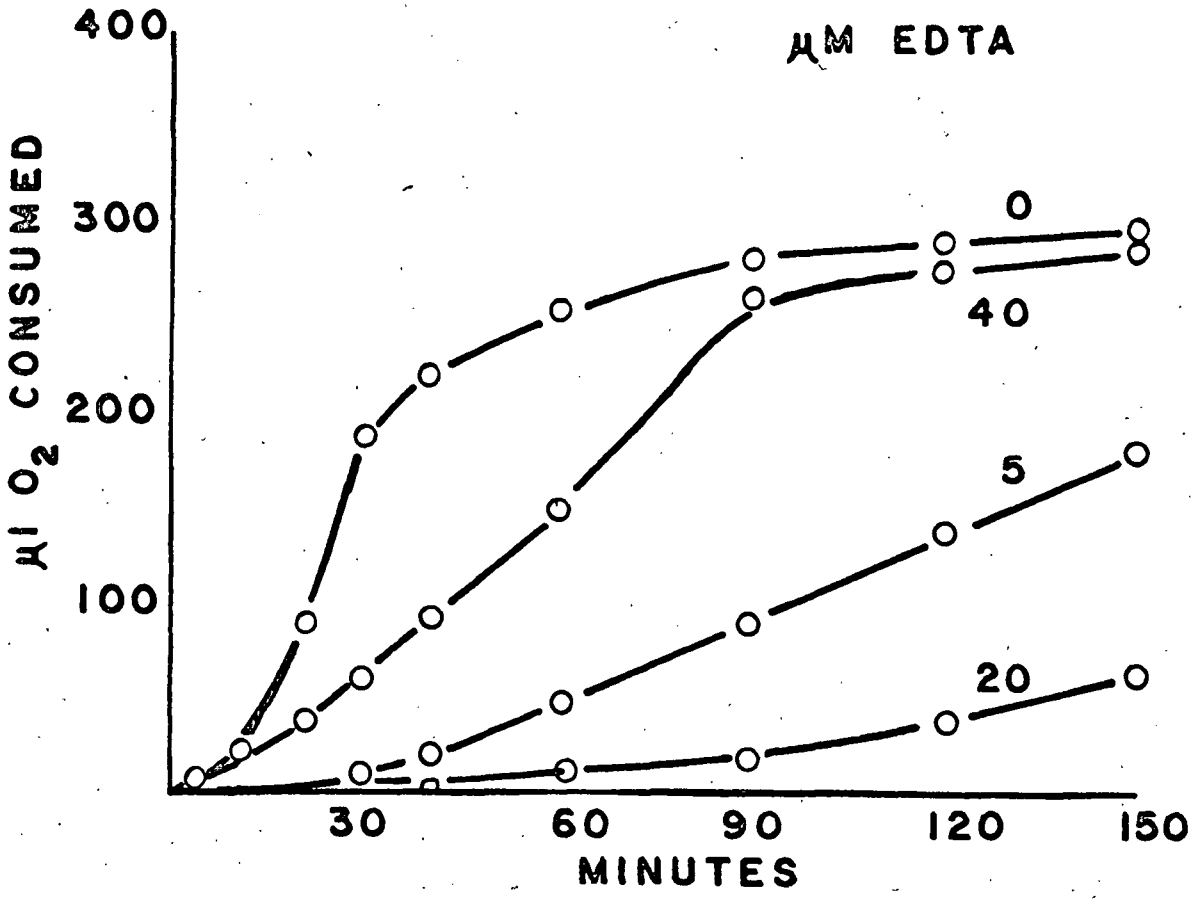

Fig. 11. Oxygen uptake by washed cell suspensions in the presence of various concentrations of EDTA and 5μM 2-KG. Endogenous values have been subtracted.

The identification of accumulating metabolic intermediates was attempted using large Warburg cups, 50μM 2-KG, and concentrations of the various inhibitors which would reduce oxygen consumption to less than 100μl. Cups were removed at various time intervals and chromatography was performed as previously described for the uninhibited reaction mixtures. Pyocyanine, a physiological hydrogen acceptor synthesized by this organism (Campbell, MacQuillan, Eagles and Smith, 1957), was also used as a metabolic inhibitor (Table 9). With the four inhibitors employed only pyruvate, ∝-ketoglutarate, and the starting compound were isolated. In each case, with the exception of the iodoacetamide inhibited reactions, pyruvate was present, as expected, and 2-KG was detected in all systems. It appeared likely then, that although the dissimilatory reactions for 2-KG utilization were functioning, the observed inhibition was a general retardation of the rate of 2-KG breakdown or perhaps inhibition of the initial step. Either explanation would result in much of the 2-KG remaining essentially unreacted. Since no other intermediary compounds were detected by the methods used it was felt that compounds may be present only in minute amounts.

3. Inhibitors and 2-KG-U-C^{14}

Various inhibited reactions using 2-KG-U-C^{14} as the substrate were run. Paper chromatography of the

Table 8(a). Pyruvate accumulation by washed cell suspensions in the presence of metabolic inhibitors

Inhibitor	Substrate	
	Glucose	2-KG
	μM pyruvate	
arsenite 3μM*	7.32	5.63
iodoacetamide 10μM	2.40	0.00
selenite 20μM	3.66	1.50

*Concentration of inhibitor per 3 ml reaction mixture.

Table 9. Products of 2-KG dissimilation by washed cell suspensions in the presence of various metabolic inhibitors

Inhibitor	Time minutes	Paper chromatography Supernatant fluid	Hydrazone	Paper Electro-phoresis
Arsenite	20	2-KG	pyruvate	2-KG or gluconic
	60	2-KG	pyruvate, α-keto-glutarate	"
Iodoacetamide	20	2-KG	-	"
	60	2-KG	-	
Pyocyanine	20	2-KG	pyruvate	"
	60	2-KG	pyruvate	"
Selenite	20	2-KG	pyruvate	"
	60	2-KG	pyruvate	"

hydrazones and of the unreacted supernatants was performed. From Table 10 it can be seen that no new intermediates were detected by this approach. From the amount of radioactivity incorporated into the cells it was observed that in most cases assimilation had been interrupted almost completely by the concentrations of inhibitors used.

The inhibitory effect of iodoacetamide was unusual due to the fact that no product of accumulation was detected yet 50μL of oxygen was still consumed. The radioactivity loss from the supernatant fluid was accounted for as $C^{14}O_2$. It is perhaps unlikely that all of the utilized substrate was oxidized completely to $C^{14}O_2$ and water, unless perhaps a primary decarboxylation took place and the resulting pentose was completely oxidized to $C^{14}O_2$ via recycling through the pentose-phosphate shunt. To gain further insight to this peculiarity, iodoacetamide reaction mixtures were repeated but 2-KG-1-C^{14}, 2-KC-6-C^{14}, and 2-KG-U-C^{14} were used as substrates. The reactions were allowed to proceed for one hour at which time the cells were fractionated and the CO_2 analyzed for radioactivity (Table 11). If the assumption that triose-phosphate dehydrogenase is greatly inhibited by iodoacetamide is correct, then these data suggest that a 3:3 split of a six carbon compound occurs. If the Entner-Doudoroff pathway was operative, then one would expect pyruvate-1-C^{14} from 2-KG-1-C^{14}, and the radioactivity would largely be

Table 10. Products of 2-KG-U-C^{14} dissimilation by washed cell suspensions in the presence of various metabolic inhibitors

Inhibitor	μM/cup	Paper chromatography supernatant	hydrazones	% C^{14} cells
Arsenite	6	2-KG	pyruvate	2.10
Bromo-pyruvate	80	"	"	0.19
Iodoaceta-mide	10	"	-	0.13
Sodium fluoride	750	"	-	2.26
Pyocyanine	3.6	"	pyruvate	0.15
Selenite	5	"	"	4.54
Anaerobic		"	" (Trace)	0.82

Table 11. $C^{14}O_2$ release from variously labelled 2-KG by washed cell suspensions inhibited with 10μM of iodoacetamide

Substrate	$C^{14}O_2$ * %
2-KG-1-C^{14}	18.0
2-KG-6-C^{14}	11.6
2-KG-U-C^{14}	4.8

* Expressed as a percentage of the total radioactivity in the Warburg vessel.

lost by decarboxylation on conversion to acetyl-CoA. However, 2-KG-6-C^{14} would not be converted to pyruvate due to the assumed inhibition of 3-phosphoglyceraldehyde dehydrogenase and would be expected to accumulate. Since no triose-phosphates were detected in the presence of iodoacetamide it is suggested that a condensation of two triose-phosphates, a reversal of the aldolase reaction, a dephosphorylation of fructose-1,6-diphosphate to fructose-6-phosphate and hence to G-6-P, an oxidation to 6-PG, and dissimilation primarily via the Entner-Doudoroff pathway, would result in a lesser $C^{14}O_2$ yield from 2-KG-6-C^{14} relative to 2-KG-1-C^{14}. This could be primarily due to the retardation of this complex reaction sequence by the inhibitor and possibly by a minor participation of the pentose phosphate pathway resulting in a much slower release of $C^{14}O_2$ which could result from a decarboxylation of 6-PG to ribulose-5-phosphate.

4. Identification of intermediates of 2-KG dissimilation by "carrier" experimentation

The failure to accumulate or detect key intermediates in any investigation of this nature may be due to one or both of the following: (a) that the intermediates are transient and never accumulate in detectable amounts even under inhibited conditions; (b) that the intermediates are always bound to the requisite enzymes concerned with substrate dissimilation. One available

method to circumvent these problems is to flood an enzyme system with an unlabelled possible intermediate during the course of degradation of a labelled substrate. If the cell is freely permeable to the possible intermediate, it will, theoretically, equilibrate with the radioactive intermediate thereby creating a "carrier" pool of the intermediate which then would be radioactive and more easily detectable.

a) non-phosphorylated "carriers"

Dickens and Glock (1951), explained the presence of pentoses and pentose-phosphates in animal tissues by a postulated decarboxylation of 2-K-6-PG. Fewester (1957), demonstrated that 5-KG underwent decarboxylation to give xylulose in extracts of A. suboxydans, and Datta et al. (1958), purified a decarboxylase from A. melanogenum which catalyzed the formation of a pentose from 2,5-diketogluconic acid. Hough and Jones (1938), proposed that 2-KG underwent an aldolase-type split to give β-OH-pyruvate and glyceraldehyde with glycolaldehyde, a product of the decarboxylation of β-OH-pyruvate, condensing with glyceraldehyde to give ribose. Stafford (1954), proposed that 2-ketohexonic acids were cleaved by an aldolase-like enzyme to give OH-pyruvic acid and glyoxylic acid in plants. They demonstrated a specific glyceric dehydrogenase which would reduce hydroxypyruvic acid to glyceric acid. De Ley (1954), suggested an aldolase split

of 2-K-6-PG into triose-phosphate and hydroxy-pyruvate or, as an alternative, the transformation of 2-K-6-PG to a dienol, and this into 3-keto-6-phosphogluconate, which was decarboxylated with the formation of ribulose-5-phosphate which, in turn, was transformed to triose-phosphate in A. cloacae.

Several of these compounds were selected as possible intermediates of 2-KG dissimilation by P. aeruginosa and were utilized as "carriers" during the oxidation of radioactive 2-KG. These reactions were carried out in double-sidearm Warburg cups. The labelled 2-KG was oxidized by the washed cell suspension for 12 minutes and then the "carrier" was added from a second sidearm. Oxidation was allowed to continue for an additional 12 minutes, with the exception of the gluconate carrier reaction which was terminated at 5 minutes. The reactions were stopped by pipetting the cup contents into cold centrifuge tubes followed by centrifugation. The carriers were isolated by electrophoresis and chromatography as their 2,4-dinitrophenylhydrazone derivative or as the free compound. From the results of these experiments (Table 12), it was concluded that neither ribose, which could have formed from a direct decarboxylation of 2-KG, hydroxypyruvic acid, which could have formed from a 3:3 aldolase-type split of 2-KG, nor glyoxylate, which could have formed from a 2:4 split of 2-KG or 2-K-6-PG, were intermediates involved in 2-KG dissimilation unless they are very

Table 12. Compounds isolated during 2-KG-U-C^{14} dissimilation by washed cell suspensions in the presence of "carrier" substrates

Carrier	Paper chromatography				Electrophoresis	C^{14}
	Supernatant	C^{14}	Hydrazones	C^{14}		
Ribose	2-KG	+			2-KG or gluconic	+
	Ribose	-			ribose	-
	unknown (1)	+			unknown (1)	+
Glyceralde-hyde	2-KG	+			2-KG or gluconic	+
	Glyceraldehyde	-			glyceraldehyde	-
	unknown (1)	+			unknown (1)	+
Gluconic acid	2-KG	+			2-KG or gluconic	+
	Gluconic	-				
Hydroxypyru-vate	2-KG	+	2-KG	+	2-KG or gluconic	+
			OH-Py[2]	-		
			α-KG[1]	+		
	unknown (1)	+	-	-	unknown (1)	+
Glyoxylate	2-KG	+	2-KG	+	2-KG or gluconic	+
			glyoxylate	-		
			pyruvate	+		
			α-KG[1]	+		
	unknown (1)	+			unknown (1)	+
Glyceralde-hyde + 5μM Iodo-acetamide	2-KG	+			2-KG or gluconic	+

1 α-KG- α-ketoglutarate.

2 OH-Py- hydroxypyruvate

tightly bound to the enzymes involved in their formation and subsequent utilization. Since carrier gluconate did not become radioactive it is most probable that gluconic dehydrogenase is not freely reversible. It was also thought possible that gluconate-C^{14} could have been formed from 6-PG-C^{14} by the action of a specific phosphatase following the conversion of 2-K-6-PG to 6-PG. Similarly, glyceraldehyde could have arisen not only from an aldolase-like reaction involving non-phosphorylated 2-KG but perhaps by the action of a phosphatase on glyceraldehyde-3-phosphate a product of the Entner-Doudoroff pathway. Glyceraldehyde did not become radioactive even in the presence of 5μM of iodoacetamide.

In each of the radioactive reaction mixtures, with the exception of the gluconate carrier and iodoacetamide inhibited reactions, an unknown compound accumulated in the supernatant fluid. It was of interest that the greatest accumulation was observed in the hydroxypyruvic acid carrier experiment. Whether there exists a correlation between the apparent repression of pyruvate formation and the formation of the unknown radioactive compound is unknown. It was suspected from the position of the compound on paper chromatograms and on elution from an anion exchange resin that this labelled compound was an amino acid. Subsequent paper chromatography and reactions with ninhydrin showed that this radioactive compound was, in fact, three amino acids, two of which were identified

as aspartic acid and glutamic acid and the third resisted identification. Since pyruvate-C^{14}, α-ketoglutarate-C^{14}, and glutamate-C^{14} have been identified in this investigation then oxidative assimilation with 2-KG-U-C^{14} likely follows a similar mechanism as with glucose-U-C^{14}. Aspartate could have arisen by a direct amination or transamination of oxaloacetic acid. Phenylalanine and methionine were also shown to be present in the hydroxypyruvate "carrier" reaction mixture but in much smaller quantities. It is significant that serine, which would arise from transamination involving hydroxypyruvate, was not found in the reaction mixture.

b) phosphorylated carriers

Certain classes of chemical compounds are known not to penetrate bacterial cells under normal conditions. Nucleotides and hexose phosphates provide classic examples of such compounds. (Kepes and Cohen, 1962). However, Roberts, Abelson, Cowie, Bolton, and Britten (1957), have shown that at lowered temperatures (20 C) glucose-1-phosphate and fructose-1:6-diphosphate as well as many amino acids were taken up by washed cell suspensions of E. coli. It was also observed that some diffusion of intracellular "pool" materials out of the cell occurred at lower temperatures. Lowered temperatures therefore seemed amenable to carrier experiments involving possible phosphorylated intermediates in 2-KG metabolism. A

preliminary investigation into the applicability of this approach was conducted using the oxidation of glucose-6-phosphate and 6-phosphogluconate by washed cell suspensions as a test system. It was found that at 20 C there was significant oxygen consumption, although at a slow rate, by washed cell suspensions indicating that both phosphorylated compounds were able to enter the cell.

Reaction mixtures containing 5μM glucose-6-phosphate or 5μM 6-PG, 10μM ATP, 10μM $MgCl_2$, and 0.10 M Tris (pH 7.4) were allowed to incubate at 20 C for 100 minutes prior to the addition of labelled substrate and oxygen consumption was followed to ensure that the phosphorylated compounds were diffusing into the cell. Five μM of glucose-U-C^{14} were added and the reaction was allowed to proceed for an additional 20 minutes and was terminated by the addition of PCA. The two supernatant fluids after centrifugation were neutralized, and applied to a Dowex-1-formate column and eluted with formic acid and ammonium formate. The products of the 6-PG "carrier" reaction with glucose-C^{14} as substrate are shown in the elution profile of Figure 12. Peaks 1, 2 and 3 were identified as glucose, glutamic acid and 2-KG respectively. Peaks 4 and 5 were identified as glucose-6-phosphate and 6-PG respectively, by paper chromatography before and after phosphate hydrolysis with bacterial alkaline phosphatase (Table 13). Since this procedure was successful with glucose-U-C^{14} oxidation it was decided to apply it to 2-KG-U-C^{14} oxidation.

Table 13. Identification of phosphorylated intermediates of glucose-U-C^{14} oxidation by paper chromatography

Compounds	Rg.*	After treatment with bacterial alkaline phosphatase Rg.	Inference
Glucose	1.00	-	
Gluconate	0.31	-	
2-KG	0.52	-	
Glucose-6-phosphate	0.12	1.00	
6-PG	0.06	0.31	
Peak I	1.00	-	glucose
Peak II	0.41	-	glutamic acid**
Peak III	0.50	-	2-KG
Peak IV	0.12	1.00	G-6-P
Peak V	0.06	0.32	6-P.G

*Rg - Relative to glucose (Solvent, Ethylacetate/Pyridine/Boric acid) and developed with benzidine-periodate.

** - Checked with Butanol/Acetic acid and with ninhydrin.

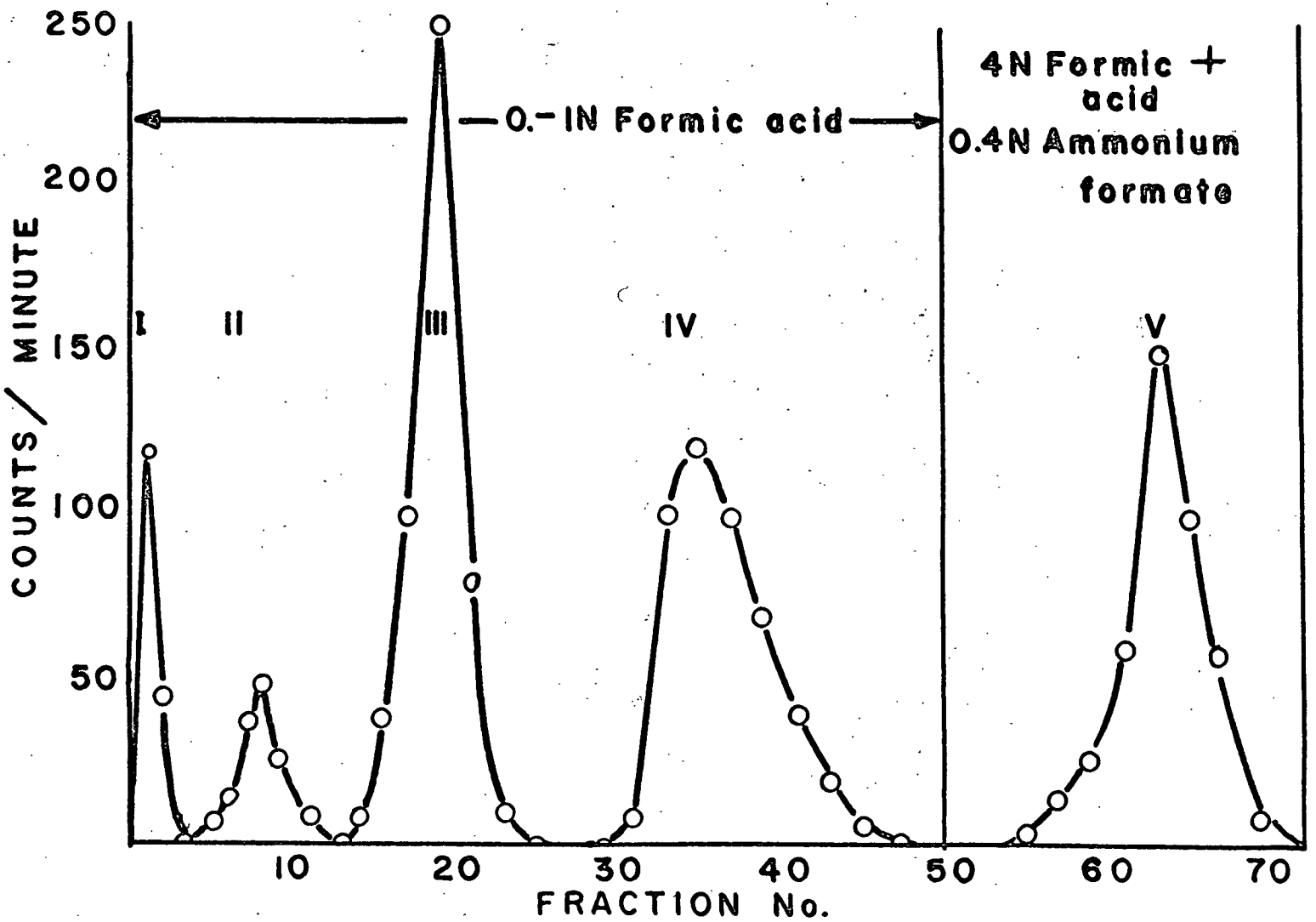

Fig. 12. Elution sequence from a Dowex 1-formate anion exchange column of compounds from glucose-U-C^{14} oxidation by P. aeruginosa

Identical reaction mixtures were prepared substituting 2-KG-U-C^{14} for glucose-U-C^{14} and 6-PG, 2-K-6-PG, and 2-K-3-D-6-PG were used as carriers. Figure 13 depicts the relative rates of diffusion of the various carriers as measured by oxygen consumption. The supernatant fluids were treated as previously described and a representative elution sequence is demonstrated (Fig. 14). The intermediates contained in the formic acid-ammonium formate eluate were identified by paper chromatography (Table 14A). In both the 2-K-6-PG and 6-PG carrier experiments, 2-K-6-PG and 3-phosphoglyceric acid were isolated as radioactive compounds. Unfortunately, 6-PG was not radioactive, even though reisolated. The identification of 2-KG is considered to be an artifact of the purification of these compounds resulting from an acid hydrolysis of 2-K-6-PG to 2-KG and phosphate. This is feasible considering the position of elution from the formate column (Fig. 14), and the highly acid conditions encountered. Only 2-K-3-D-6-PG was isolated as a labelled product of the "cold" 2-K-3-D-6-PG carrier experiment. These data demonstrate conclusively that 2-K-6-PG, 2-K-3-D-6-PG, and 3-phosphoglyceric acid are intermediate compounds during the oxidation of 2-KG by glucose grown cells of P. aeruginosa and this represents the first evidence for a constitutive 2-KG-kinase in whole cells of any Pseudomonas species and also demonstrates the participation of the Entner-Doudoroff pathway during the oxidation of 2-KG.

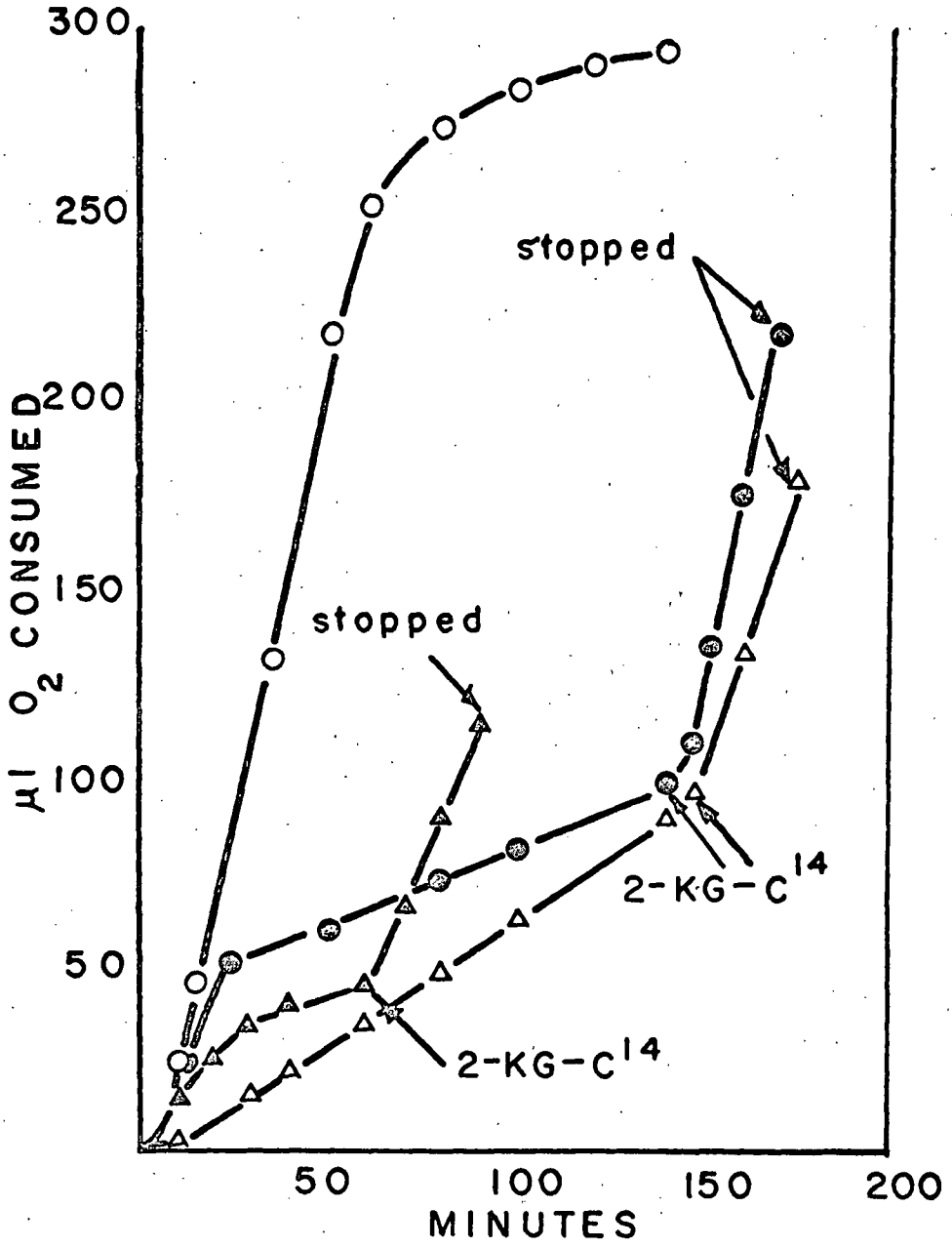

Fig. 13. Oxygen uptake at 20°C by washed-cell suspensions of P. aeruginosa with 2-KG(O—O), 2-KGPG (● — ●), 6PG(▲—▲) and 2-K-3-D-6-PG (△ — △). 2-KG-C^{14} was added and reactions were stopped where indicated.

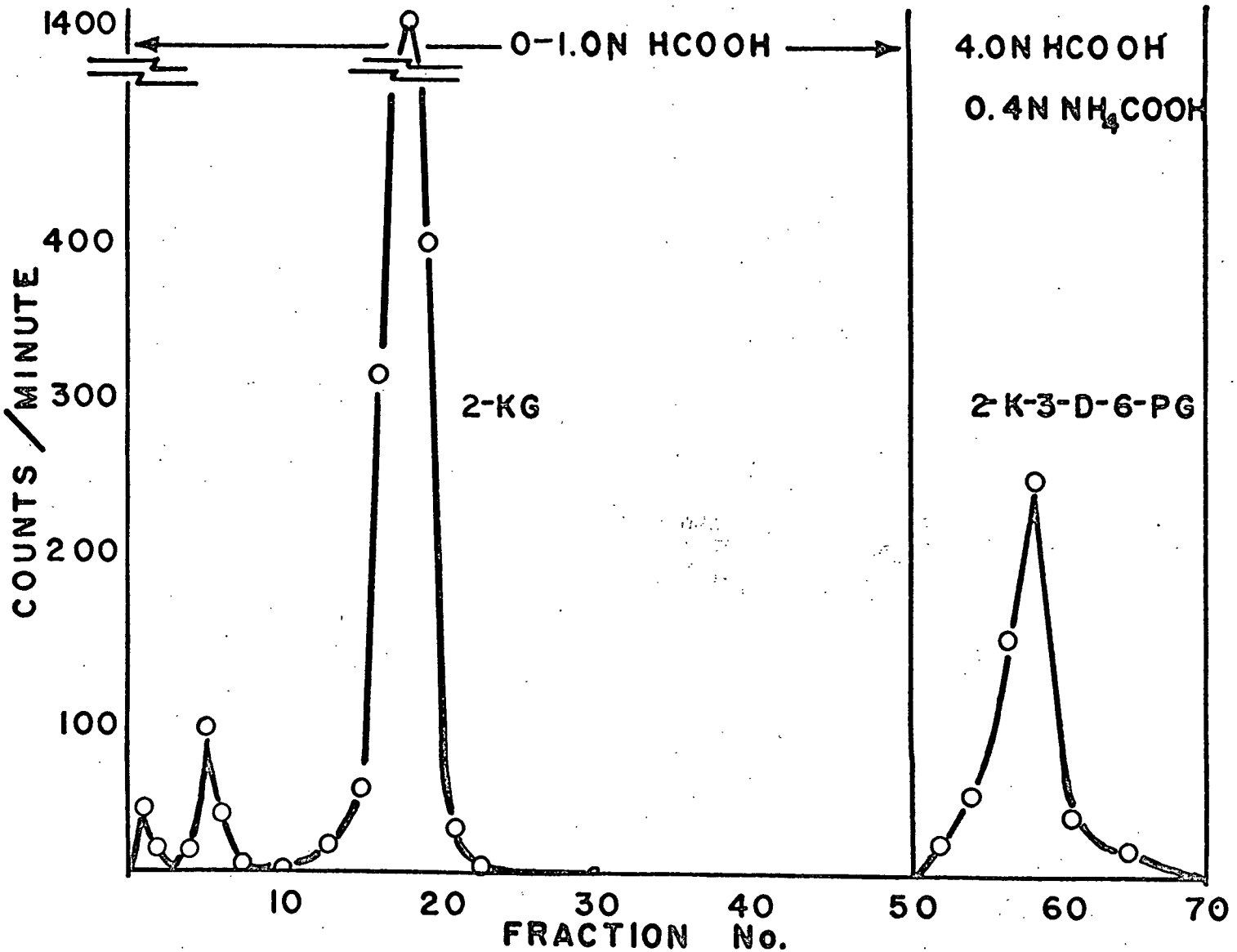

Fig. 14. Elution sequence of C^{14} compounds from a Dowex-1-formate anion exchange column after 2-KG-C^{14}, oxidation in the presence of 2-K-3-D-6-PG.

5. Demonstration of 2-KG metabolism in cell-free extracts

a) Enzyme assays

Although a 2-KG-kinase has been reported in a variety of bacteria it has, in almost all cases, been an induced kinase. Ghiretti and Guzman-Barron (1954), demonstrated a kinase for 2-KG using a cell-free extract of C. creatinovorans but no product isolation was attempted and the kinase activity was not constantly reproducible. However, Narrod and Wood (1955), demonstrated a relatively weak 2-KG kinase in glucose grown cells of P. fluorescens, partially purified the enzyme, and isolated and identified 2-K-6-PG as the end product of the enzymic transphosphorylation with ATP.

During the course of this investigation attempts were made to demonstrate a 2-KG kinase as a constitutive enzyme in glucose grown cells. Sonic extracts, Hughes' pressates, and French cell pressates were seemingly void of this enzyme. Cell breakage was carried out in bovine-serum albumin, glutathione, cysteine, 2-mercaptoethanol, or glycylglycine in an effort to stabilize the 2-KG kinase--all without apparent success. Neither ATP, CTP, GTP, nor ITP gave any evidence of activity as phosphate donors and attempts to demonstrate 2-KG-kinase activity in pressates from 20 hour, 0.2% 2-KG grown cells were also unsuccessful.

Ciferri et al. (1959) showed that the 2-KG-kinase activity of L. mesenteroides was poor when cells were harvested from the stationary phase of growth and that the purified enzyme was rapidly inactivated above 30 C.

In an attempt to demonstrate kinase activity in log phase cells, the cells were grown on 0.5% 2-KG or on 0.5% glucose as carbon sources and harvested at 20 hours. The cells were broken in $\frac{M}{5}$ Tris pH 7.4 and 0.025 M glycylglycine by means of a French pressure cell. The 2-K-6-P-G reductase, 6-PG dehydrase and 2-K-3-D-6-PG aldolase, as well as 2-KG kinase activities were measured with both the glucose grown and 2-KG grown cells. Figures 15 and 16 demonstrate the various enzyme activities found in 2-KG grown cells and the relative enzyme activities of 2-KG or glucose grown cells are shown in Table 15. The activities of the enzymes measured were, with the exception of the kinase, twice as high when the cells were grown on 2-KG rather than glucose. The high relative value for the 2-KG-kinase from 2-KG grown cells was obviously due to the low--almost immeasurable--activity from cells grown on glucose. The 2-KG induced kinase was the least active of all the enzymes measured with an activity of approximately one-tenth the activity of the relatively weak 6-PG-dehydrase.

As a confirmation of these enzymic reactions attempts were made to isolate the end-products of the various

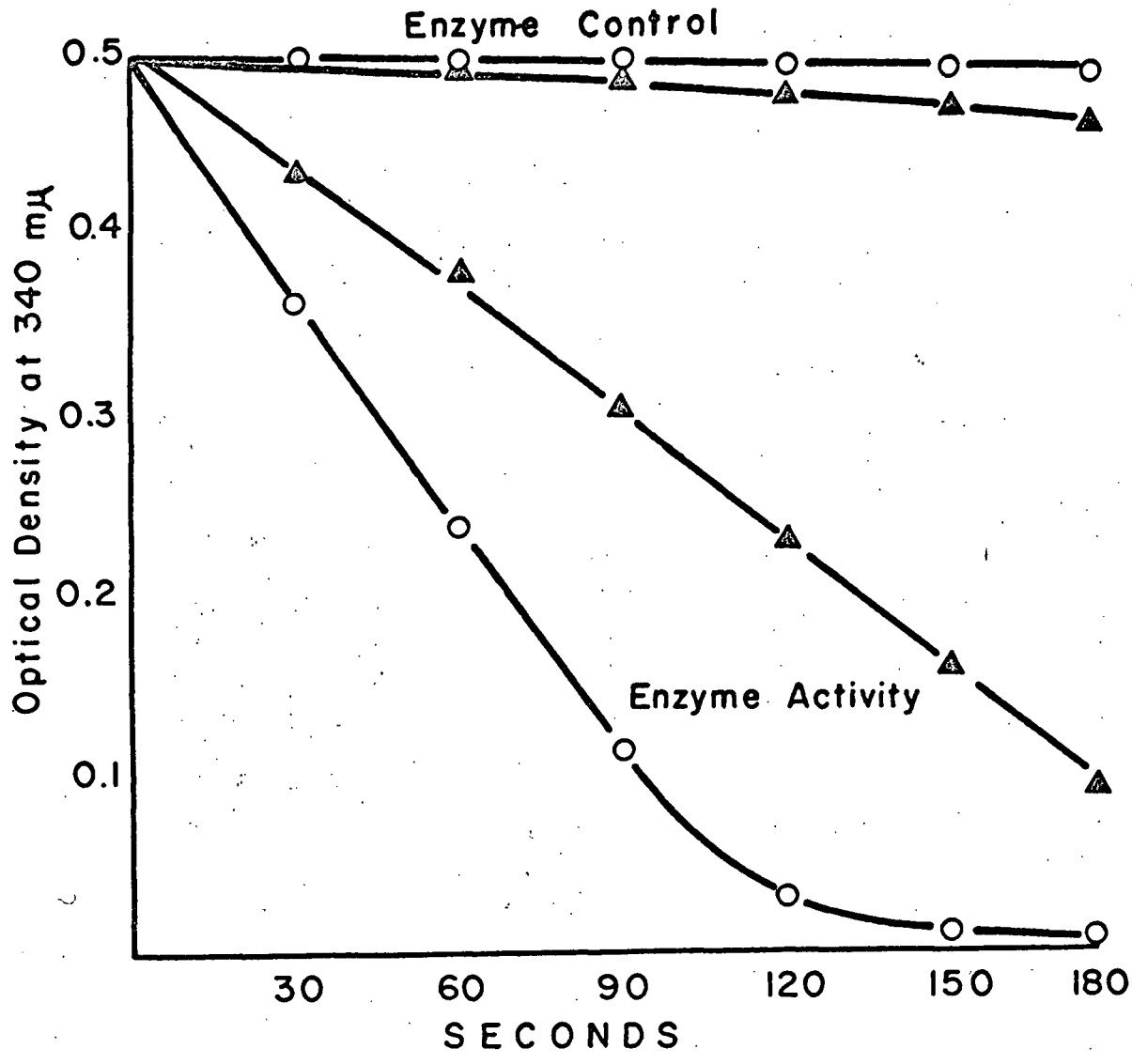

Fig. 15. Demonstration of 2-ketogluconokinase activity (▲-▲) and 2-ketogluconoreductase activity (o-o) in cell-free extracts of 2-KG grown cells

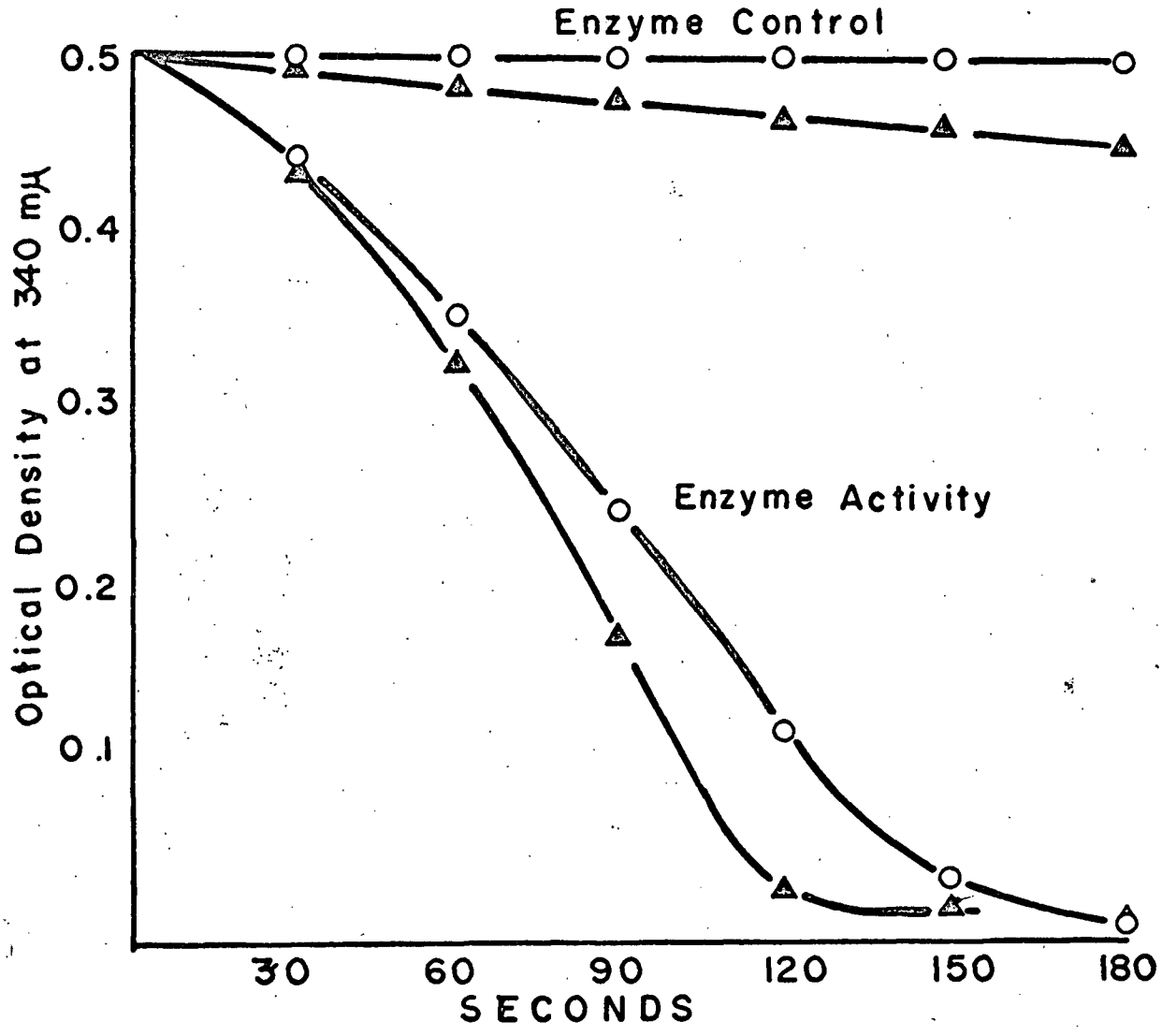

Fig. 16. Demonstration of 6-PG dehydrase (▲-▲) in cell-free extracts of glucose grown cells and 2-K-3-D-6-PG aldolase (o-o) in cell-free extracts of 2-KG grown cells

Table 14. Identification of phosphorylated intermediates of 2-KG-U-C^{14} metabolism

standards	solvent[1] 1 R_{gl}[2]	solvent[3] 2 R_{gl}	solvent 2 after phosphatase R_{gl}	compound
2-KG	0.94	0.58	-	-
gluconate	0.94	0.36	-	-
glycerate	1.00	1.00	-	-
2-K-6-PG	0.72	0.18	-	-
6-PG	0.46	0.10	-	-
2-K-3-D-6-PG	0.80	0.30	-	-
3-PG[4]	0.85	0.20	-	-
reaction mixture				
A - whole cell reactions				
2-KG-C^{14} +	0.94	0.58		2-KG-C^{14}
2-K-6-PG	0.72	0.18-	0.58	2-K-6-PG-C^{14}
	0.86	0.20	1.00[5]	3-PG-C^{14}
2-KG-C^{14} +	0.94	0.58		2-KG-C^{14}
6-PG	0.72	0.18-	0.58	2-K-6-PG-C^{14}
	0.86	0.20	1.00[6]	3-PG-C^{14}
2-KG-C^{14} +	0.94	0.58		2-KG-C^{14}
2-K-3-D-6-PG	0.80	0.30	-	2-K-3-D-6-PG-C^{14}
B - cell-free extract reactions				
2-KG-C^{14}, ATP,	0.94	0.58	0.58	2-K-6-PG-C^{14}
Mg^{++}	0.72	0.18	0.58	
2-KG-C^{14}, ATP,	0.94	0.58	0.58	2-K-6-PG-C^{14}
Mg^{++}, $NADPH_2$	0.72	0.18	0.58	

1. acetone/pyridine/water
2. R_{gl}--relative to glycerate
3. ethylacetate/pyridine/boric acid
4. 3-PG--3-phosphoglyceric acid

5,6. Only a trace of radioactive glyceric acid was found here, but not enough to account for the disappearance of the R_{gl} 0.18-0.20 compounds.

reactions. Reaction mixtures were prepared in a manner identical to those of the kinase assay with the exception that three times the amount of cell-free extract was used and that one reaction was without $NADPH_2$. Warburg flasks were used for the incubations which were carried out at 30 C for one-hour at which time the reactions were terminated with 1.4 N PCA, centrifuged, and the clear supernatant fluid neutralized with IN KOH. These supernatants were analyzed in a manner analogous to the phosphorylated carrier experiments. From Table 14B it can be seen that 2-K-6-PG was the identified end product of the transphosphorylation with ATP and that 6-PG was not present, but rather 2-K-6-PG was again isolated. The active Entner-Doudoroff enzymes probably degraded the 6-PG as fast as it was formed. The above observations then establish the existence of a dissimilatory pathway for 2-KG metabolism in P. aeruginosa. A similar pathway has been demonstrated in P. fluorescens (Narrod and Wood, 1955; Frampton and Wood, 1961), but it should be noted that cell-free extracts of P. fluorescens contain relatively active enzymes for 2-KG utilization since cells grown on glucose or 2-KG exhibit demonstrable 2-KG-kinase activity and the cell-free preparations degrade 2-KG quantitatively to pyruvate--in the presence of arsenite. Cell-free extracts of P. aeruginosa do not exhibit this apparent activity. It is incomprehensible why the presumably initial step involved in 2-KG utilization should be relatively inactive in cell-free extracts and

Table 15. Comparative enzyme activities of glucose and 2-KG grown cells of *P. aeruginosa*[*]

Enzyme	Substrate		Ratio of activity
	Glucose	2-KG	2-KG/glucose
2-KG-kinase	0.13	1.81	12.95
2-K-6-PG-reductase	45.00	84.40	1.87
6-PG dehydrase	8.70	17.50	2.01
2-K-3-D-6-PG aldolase	30.30	60.30	1.99

[*]Enzyme activities are expressed as change in O. D. per minute per mg protein x 100

also in whole cells. Whole cells do not immediately metabolize 2-KG as they do glucose and gluconate (Figure 1), but rather a distinct 5-10 minute lag occurs prior to rapid oxidation. A constitutive enzyme exists for the oxidation of 2-KG since this apparent lag period remained unchanged when cells were treated with chloramphenicol. It has been observed that 2-KG induces a brief degradation of ribonucleic acid (RNA)--presumably ribosomal--during this lag period, whereas substrates such as glucose and α-ketoglutarate do not (Gronlund and Campbell, 1965). It is possible that a necessary cofactor for 2-KG degradation is derived either directly or indirectly from the degradation products of R.N.A. It was reasoned that if this postulate is valid then cells extremely deficient in ribosomal complement would be unable to utilize 2-KG as an energy source. Phosphate starved cells of P. aeruginosa were demonstrated to be deficient in ribosomal material relative to non-starved cells (Hou, 1965). Preliminary experiments with phosphate-starved cells disclosed that these cells were in fact unable to utilize 2-KG as observed by oxygen consumption (Table 16). Since the first step involved in 2-KG utilization by this organism is a phosphorylation it could be argued that the limiting requirement was phosphate. However, the addition of inorganic phosphate had relatively little stimulatory effect (Table 16), but the addition of a nucleoside pool had a

Table 16. The stimulation of 2-KG oxidation in phosphate starved cell suspensions of P. aeruginosa by inorganic phosphate.

Compound added	Oxygen consumption at 150 minutes μl
2-KG	70
2-KG + Pi*	90

*Pi - inorganic phosphate

Table 17. The stimulation of 2-KG oxidation in phosphate starved cells of P. aeruginosa by a nucleoside pool

Compound added	Oxygen consumption at 150 minutes μl
nucleoside pool*	162
" " + 2-KG	346
" " + Pi	197
" " + 2-KG + Pi	467

*½ μM of each of 4 nucleosides (adenosine, cytidine, guanosine, and uridine)

marked stimulation on oxygen uptake (Table 17). Subsequent studies using one μM of individual nucleosides demonstrated that all the nucleosides were stimulatory but not to the same degree (Table 18). Guanosine was found to be the best stimulator, cytidine was slightly better than uridine, and adenosine was the least stimulatory. The free base guanine was found to have a stimulatory effect similar to guanosine (Table 19) and thereby eliminated the possible importance of the ribose moiety. Since P. aeruginosa has been shown to liberate ammonia during the degradation of purine and pyrimidine compounds (Campbell, Gronlund, and Duncan, 1963), and since guanine and guanosine both had essentially the same stimulatory power on 2-KG utilization it was decided to test inorganic ammonia as a possible stimulatory agent. It can be seen from Table 19 that ammonia also had a marked stimulatory effect. This latter observation initiated two queries: was the decreased rate of oxygen uptake with phosphate-starved cells the result of the accumulation of a toxic intermediate; or was ammonia required for the formation of some cofactor mandatory for the phosphorylation of 2-KG? The former postulation was feasible in light of the work of Tomlinson (1964), who demonstrated that starvation decreased oxidative assimilation and permitted greater accumulation of keto-acid intermediates in P. aeruginosa. However, in this investigation the analysis of reaction supernatants gave no indication of any keto-acid accumulation and

manometric data (Table 20) demonstrated that α-ketoglutarate was oxidized to the same degree whether 2-KG was present in the reaction or not. The latter postulation that ammonia was necessary for perhaps some cofactor synthesis is feasible, but is open to criticism in view of the limited data available at this time. A new type of enzymatic phosphorylation was discovered by Smith (Fujimoto and Smith, 1960; Smith, 1961). It was found that E. coli extracts catalyzed direct phosphorylation of glucose with potassium phosphoramidate, with N-phosphorylglycine or, more slowly, with monophosphorylhistidine. This then would suffice as one possible explanation for an ammonia requirement for 2-KG phosphorylation.

Table 18. The stimulation of 2-KG oxidation in phosphate starved cells of P. aeruginosa by purine and pyrimidine ribosides

Compound added	Oxygen consumption at 150 minutes μl above 2-KG + Pi control
2-KG + Pi + adenosine	215
2-KG + Pi + cytidine	350
2-KG + Pi + guanosine	400
2-KG + Pi + uridine	315

Table 19. The stimulation of 2-KG oxidation in phosphate starved cell suspensions of P. aeruginosa

Compound added	Oxygen consumption at 140 minutes μl above 2-KG + Pi control
guanine	95
guanosine	80
NH_3	70

Table 20. The effect of α-ketoglutarate on 2-KG oxidation in phosphate starved cell suspensions of P. aeruginosa

Compound added	Oxygen consumption at 180 minutes μl
α-ketoglutarate + Pi	91
2-KG + Pi	226
α-ketoglutarate + 2-KG + Pi	271

GENERAL DISCUSSION

The demonstration of sequential enzymes concerned in substrate dissimilation alone is not necessarily valid evidence for the existence of a particular functioning metabolic pathway. The isolation of the requisite intermediates however does qualify the existence of the pathway in cell-extracts but the quantitative estimation of the participation of such a pathway cannot be applied to whole cells where the cellular integrity has not been drastically altered. The ability of a cell to use a particular pathway is no assurance that the organism readily utilizes that route during growth but will, unquestionably, vary according to the availability of alternate pathways and also according to the needs of the organism as governed by both growth conditions and developmental stage. Similarly, the use of metabolic inhibitors is a useful tool for the elucidation of catabolic pathways but is limited due to a general lack of specificity exhibited by most inhibitors. Indeed, in this investigation the various inhibitors used were too restrictive in affecting the initial stages of 2-KG utilization. It again must be emphasized that extremely unphysiological conditions are imposed upon the cells by the accumulation of metabolites and conclusions of pathway participation under these conditions are nebulous. Entner and Doudoroff (1952), concluded that P. saccharophila metabolized glucose and gluconate via one pathway to pyruvic acid, but the cells were poisoned with

arsenite, thereby leaving such a conclusion open to criticism. However, their conclusion was substantiated in non-inhibited cells by Stern, Wang, and Gilmour (1959), using radiorespirometry. Frampton and Wood (1961), showed that 2-KG was quantitatively dissimilated via the Entner-Doudoroff pathway to pyruvate under conditions of arsenite inhibition in extracts of P. fluorescens, but no data is available as to the relative participation of this pathway in a non-poisoned cell, although Stern, Wang, and Gilmour (1959), have demonstrated that Pseudomonas reptilovora, a closely related organism, dissimilates 28% of the available glucose by the pentose-phosphate route. In this investigation, P. aeruginosa was shown to degrade 2-KG quantitatively to pyruvate under conditions of arsenite or bromopyruvate poisoning, but without isotope distribution data it cannot be concluded that all the pyruvate was a product of only the Entner-Doudoroff pathway. Stern, Wang, and Gilmour calculated that the pentose-phosphate pathway functions to the same extent in P. aeruginosa as in P. reptilovora.

The use of lowered temperatures as an aid to permeability in whole cells is an exciting concept. Whether active transport mechanisms are readily inactivated or whether physical cellular barriers are broken down, thus allowing a greater freedom of diffusion, may be the reason for increased permeability is unknown at present, but the

application of this technique to the elucidation of pathways of carbohydrate dissimilation is of value as demonstrated during the course of this investigation. Thus, in this study, incubations of whole cells with the phosphorylated compounds, glucose-6-phosphate, 6-PG, 2-K-6-PG, or 2-K-3-D-6-PG at 20C permitted oxidation of these compounds and when 2-KG-C^{14} and the latter two compounds were used as "carrier" substrates and from the appropriate labelling which subsequently ensued, evidence was obtained for a dissimilatory pathway of 2-KG degradation in intact cells. This technique and the results which were obtained from its application permitted a full investigation of the enzymes functioning in 2-KG dissimilation.

The failure to accumulate α-ketoglutaric acid during the active dissimilation of 2-KG by P. aeruginosa is, on first consideration, a detriment to the economy of the cell as it has obviously lost a means of conserving the carbon compound which acts as the point of entry into protein and nucleic acid synthesis when ammonia becomes available. However, MacKelvie (1965), demonstrated that cultures grown in a nitrogen limiting medium with glucose as the sole carbon source accumulate far greater quantities of 2-KG relative to pyruvate or α-ketoglutarate and since these cells are analogous, at least in ribosomal complement, to phosphate-starved cells it becomes obvious that 2-KG accumulation is a more efficient means of conserving carbon compounds than is

α-ketoglutarate because: α-ketoglutarate is almost a universal metabolite among microorganisms but 2-KG is not; 2-KG can support more cellular synthesis than α-ketoglutarate; and from indirect data presented in this report 2-KG may in fact be incorporated directly into cellular constituents.

The mechanism of ammonia stimulation of 2-KG utilization is as yet a mystery but it is probable that ammonia is functioning either as a control mechanism or that it assumes a more direct role in the degradation of 2-KG and functions as a precursor to the synthesis of a necessary cofactor. A phosphoramidate (Fujimoto and Smith, 1960; Smith, 1961) may be such a cofactor.

The unusual lability of 2-KG kinase in cell-free extracts of P. aeruginosa relative to the other enzymes functioning in 2-KG degradation is puzzling since none of the common agents used to stabilize the enzyme were effective with the exception of glycylglycine. Glycylglycine may be serving as the substrate for a protease which, when liberated during cell breakage, attacks 2-KG kinase. Another possibility is that glycylglycine is serving as a substrate the utilization of which may produce a supply of ammonia and stimulate 2-KG degradation. However, the latter postulation is perhaps the most unlikely since cell breakage should result in the availability of a variety of nitrogenous compounds which would also serve as sources of ammonia.

SUMMARY

The non-phosphorylated oxidation of glucose through gluconolactone, gluconic acid and 2-KG, as well as the role of 2-KG as a transient intermediate during glucose catabolism by P. aeruginosa have been confirmed.

It was found that glucose grown cells metabolized 2-KG via 2-K-6-PG and 2-K-3-D-6-PG intermediates thereby establishing the existence of a kinase for 2-KG and the participation of the Entner-Doudoroff pathway during 2-KG degradation.

Two-keto-6-phosphogluconoreductase, 6-phosphogluconate dehydrase, and 2-keto-3-deoxy-6-phosphogluconate aldolase were confirmed as constitutive enzymes of glucose grown cells and these three enzymes as well as a 2-ketoglucono-kinase were demonstrated spectrophotometrically in 2-KG grown cells. Although 2-KG kinase could not be demonstrated spectrophotometrically in glucose grown cells the product of 2-KG transphosphorylation with ATP in the presence of magnesium with cell-free extracts was shown to be 2-K-6-PG.

The role of 2-KG in oxidative assimilation was demonstrated to be considerably different from glucose especially with respect to the quantitation and speed of assimilation.

Evidence was accrued to establish 2-KG as a key carbon conserving intermediate in the oxidation of glucose under conditions of limiting nitrogen.

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