A Study of the Glucose Oxidizing System of *Pseudomonas Aeruginosa*

by

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ABSTRACT

Cell free extracts of glucose grown cultures of P. aeruginosa were prepared by exposure to sonic vibration. These sonicates were capable of oxidizing glucose-6-phosphate, ribose-5-phosphate, glucose, gluconic acid and gluconolactone.

Treatment of the sonicate with $(NH_4)_2SO_4$ resulted in the formation of precipitateswhich possessed the ability to oxidize glucose, gluconolactone and gluconic acid. After $(NH_4)_2SO_4$ treatment neither the supernatant nor the precipitate could oxidize the phosphorylated compounds. Since 30.0% $(NH_4)_2SO_4$ or centrifugation for one half hour at 25,000 X g precipitated the enzymes they were considered to be insoluble.

Precipitation with different concentrations of $(NH_4)_2SO_4$ and $MnSO_4$ failed to separate the glucose and gluconate enzymes from one another.

Extraction with bile salts solubilized the gluconate enzyme, but precipitated the glucose enzyme along with a high proportion of the gluconate system. An increase in concentration of the bile salts destroyed the glucose enzyme while a reduction left a large percentage in solution.

The addition of glycine to the sonicate solubilized the enzymes but did not aid in their separation. When

added before the sonic treatment, the enzymes became labile to protein precipitants.

Ethyl alcohol, dioxane and acetone destroyed glucose oxidizing and gluconate oxidizing enzyme activity while ethyl ether destroyed only the glucose system.

The use of a growth substrate other than glucose resulted in the formation of a reduced gluconic acid system, but the results were not uniform in that the gluconate enzyme frequently was very active.

The glucose system was sensitive to KCN and NaN_3, and 8-hydroxy-quinoline but not to NaF. The 8-hydroxy-quinoline inhibition could be overcome by Mg^{++}.

Adenosinetriphosphate, flavine adenine dinucleotide, diphosphopyridinonucleotide, triphosphopyridinonucleotide, had no effect on the glucose oxidizing system.

Methylene blue, brilliant cresyl blue and pyocyanin had no ability to act as a hydrogen acceptor. However 2.6 dichlorobenzenoneindophenol stimulated the reaction. When this dye was added in the presence of $MgSO_4$ a 300.0% increase was noted.

The product of the reaction was determined by paper chromatography to be gluconic acid.

These data indicate that the glucose dehydrogenase differs from any previously described glucose dehydro-

genase and that some unknown hydrogen transport system apparently functions in the transport of electrons to the cytochromes. The data support earlier conclusions that the reaction does not involve phosphorylation.

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INTRODUCTION

Interest in the study of aerobic metabolism received an impetus when Dickens (1938 a, b) first demonstrated the metabolic scheme involving glucose-6-phosphate, 6-phosphogluconic and pentose phosphoric acids. This pathway was distinct from the glycolytic scheme and was the first positive evidence that an alternate pathway for carbohydrate metabolism existed. Prior to this finding, it was almost universally accepted that the oxidation and fermentation of glucose proceeded by a common pathway as far as the pyruvic acid. The oxidative steps in the degradation of glucose were pictured as taking place after the formation of pyruvate and involved the classical Kreb's tricarboxylic acid cycle. Under anaerobic conditions, pyruvate either acted as a hydrogen acceptor with the resultant formation of lactic acid, or was cleaved to compounds, such as acetaldehyde, which would act as hydrogen acceptors. However, fundamental studies on this problem were interrupted by the war and Dickens' initial observations were not confirmed or extended during the next ten years. In fact Colowick and Kaplan (1951) stated that "The universal existence of the Embden-Meyerhof pathway is so well accepted as to hardly require further documentation." Umbreit (1949) made a similar

statement, "The concept of a 'basic metabolic ground plan' has suffered no severe blows in the studies of the past year." At the time these statements were made considerable evidence was also available to indicate that glucose could be oxidized by a pathway which did not involve phosphorylated hexoses. However, these non-phosphorylated reactions were not considered to be important or even possible physiological reactions and so did not concern the majority of workers.

A scheme of glucose oxidation based on the initial observations of Dickens (1938,a,b) is now commonly accepted. This pathway, which is apparently very widely distributed, has been studied intensively by Horecker, Smyrniotis and Seegmiller (1951) using a purified yeast preparation. Glucose was shown to be oxidized by way of glucose-6-phosphate and 6-phosphogluconate. The latter compound was then oxidized with the formation of ribulose-5-phosphate (Cohen and Scott 1951, Horecker et al. 1951). In order to account for this reaction, a hypothetical intermediate was postulated (Horecker et al. 1951). It was shown that ribulose-5-phosphate was in equilibrium with ribose-5-phosphate.

Similarly, Seegmiller and Horecker (1952) obtained

an enzyme preparation from rabbit liver which catalysed the quantitative oxidation of 6-phosphogluconate to pentose phosphate and carbon dioxide. The presence of ribulose-5-phosphate and ribose-5-phosphate was demonstrated. An enzyme preparation from rabbit bone marrow catalysed the formation of glucose-6-phosphate from 6-phosphogluconate by way of a pentose phosphate intermediate, thereby demonstrating a reversal of the direct oxidative scheme.

While Horecker et al. have, at the present time, presented adequate proof of the direct oxidative scheme and have shown that it is present in a variety of tissue, a number of other workers have added to the completeness of the picture by studies on other organisms or tissues.

A variation of this oxidative scheme was discovered in Pseudomonas saccharophila by Entner and Doudoroff (1952). This organism oxidizes glucose by way of glucose-6-phosphate and 6-phosphogluconate, but the next step is the cleavage of the latter compound to yield 3-phosphoglyceraldehyde and pyruvate. The reaction may be quite widely distributed and could be important in the synthesis of hexoses.

Actually there was still earlier evidence of an alternate pathway of glucose oxidation, for in 1931, Harrison had isolated a glucose dehydrogenase from liver which could convert glucose to gluconic acid without the intermediate formation of any phosphorylated compounds. This reaction was not regarded seriously by biochemists for several reasons. The first was that, since phosphorylation was not demonstrated, the reaction was considered to be either an artifact or physiologically unimportant. The second was that, since the reaction was studied as an isolated system, it was not brought into a scheme of metabolism and the gluconic acid was pictured as an end product rather than an intermediate. Strecker and Korkes (1952) reported a further purification of this enzyme. This glucose dehydrogenase oxidized beta-glucose preferentially with delta-gluconolactone as the probable product. Diphosphopyridinonucleotide (DPN) was the prosthetic group of the system.

Keilin and Hartree (1949 a, b,), working with the enzyme Notatin, discovered by Müller (1928) in Aspergillus niger and Penicillium glaucum, established that flavine adenine dinucleotide (FAD) was the prosthetic group. The enzyme system was not affected by

cyanide and was glucose specific. Further work by Bentley and Neuberger (1949) established that Notatin was a dehydrogenase and that the product of glucose oxidation was delta gluconolactone.

Evidence of still another system of glucose oxidation began to accumulate with the demonstration by Barron and Friedemann (1941) that the oxidation of glucose by Pseudomonas aeruginosa was not affected by sodium fluoride and therefore did not involve phosphorylation. Lockwood et al. (1940) reported the formation of large quantities of gluconate and 2-ketogluconate during the growth of Pseudomonas fluorescens in a glucose medium. Lockwood and Stodola (1946) showed that with P. fluorescens NRRL B-6, alphaketoglutaric acid was obtained as a major product. With the same organism Lockwood and Nelson (1946) obtained a preparation which could oxidize pentoses to pentonic acids. However, these workers were interested only in the commercial production of the organic acids and did not interpret their data as indicating a new metabolic pathway.

Norris and Campbell (1949) found detectable amounts of both gluconic and 2-ketogluconic acids in sixteen and twenty four hour cultures of P. aeruginosa when

glucose was the growth substrate. Since strong systems for the oxidation of these compounds were also demonstrated over this period of time, it was concluded that the compounds must be continuously formed and oxidized and must therefore be part of the metabolic pathway for the complete oxidation of glucose.

Campbell and Norris (1951) concluded that the conventional glycolytic scheme was not present in P. aeruginosa. This conclusion was based on the insensitivity of the system to sodium fluoride, the lack of activity under anaerobic conditions, the absence of hexose phosphates, and the formation and utilization of gluconic and 2-ketogluconic acids. Using a dried cell preparation of P. aeruginosa, Stokes and Campbell (1951) found that glucose and gluconate were quantitatively converted to 2-ketogluconic acid indicating that at least in dried cells this was the only mechanism available for the oxidation of glucose. It was also confirmed that gluconic acid was an intermediate in the formation of 2-ketogluconate.

Warburton, Eagles and Campbell (1951) determined pyruvic acid in glucose cultures of P. aeruginosa at sixteen, twenty-eight and forty hours and at the same time were able to demonstrate a strong system for its

oxidation. This led them to conclude that pyruvic acid was a normal intermediate in glucose oxidation. Campbell and Stokes (1951), working with the same organism, found that while resting cells had no ability to oxidize citrate, cis aconitate, isocitrate, alpha-ketoglutarate, succinate or fumurate, dried cells could oxidize the previously listed compounds as well as malate, acetate, oxalacetate and pyruvate. They concluded that the organism possessed a conventional tricarboxylic acid cycle.

Klein and Doudoroff (1950) in their studies of Pseudomonas putrefaciens found that the wild type had little or no ability to attack glucose and did not possess hexokinase and could utilize glucose quite readily. This agrees quite well with the data reported by Wood and Schwerdt (1953, 1954), obtained with a cell free sonic extract of P. fluorescens. The latter workers were able to demonstrate that the sonic extract possessed the ability to oxidize glucose-6-phosphate, 6-phosphogluconate, ribose-5-phosphate, glucose and gluconic acid. Using ammonium sulphate fractionation they were able to separate the glucose-gluconic oxidizing system from the soluble enzyme system which oxidized the phosphorylated compounds.

The glucose-gluconic system was not a flavoprotein oxidase and did not involve a DPN or TPN specific glucose

dehydrogenase. Cytochrome carriers apparently were involved and so this glucose dehydrogenase differs from Notatin or from that of Harrison.

The soluble enzyme system for the phosphorylated compounds appears to be quite closely related to the one reported by Entner and Doudoroff (1952) for P. saccharophila. Wood and Schwerdt could not show the existence of hexokinase and so the presence of glucose-6-phosphate oxidizing system is difficult to explain. However these data agree with the findings of Klein and Doudoroff.

The literature reviewed offers abundant proof for the existence of a direct pathway for the oxidation of glucose. Moreover, the Embden-Meyerhof scheme seems to play a minor role in aerobic systems.

Work with resting and dried cells has shown numerous routes and while these studies have been rewarding, the actual mechanism of action will not be definitely shown or characterized until purified enzyme systems are available.

Very little work has been done on the glucose oxidizing enzymes of bacteria. Since the glucose oxidizing system of P. aeruginosa has been shown to be similar in many respects to the glucose dehydrogenase

of Harrison (1931) and Müller's Notatin (1928), it was thought that the study of this enzyme would be profitable.

Although oxidative enzymes in general are insoluble, attempts were made to isolate the enzyme responsible for the initial oxidation of glucose. The task immediately at hand was the isolation, purification and characterization of the non-phosphorylative glucose oxidizing enzyme system of Pseudomonas aeruginosa ATCC 9027.

METHODS

Bacteriological

Pseudomas aeruginosa ATCC 9027 was used throughout these studies. The organism was maintained on stock culture media consisting of tryptone 1.0%, dipotassium hydrogen phosphate 0.3%, glucose 0.1%, glycerol 0.3%, yeast extract 0.1%, agar 0.5%, gelatin 2.0%, adjusted to pH 7.2. The stock cultures were stored at approximately 10°C.

The medium used for obtaining a good yield of cells was that of Campbell et al. (1949 b). In general the medium was dispensed in 200 ml. quantities in 500 ml. capacity Florence flasks. After inoculation these were shaken on a horizontal rotary shaker for approximately 18 hours.

The inoculum was prepared from a stock culture by transferring at least three times, at 24 hour intervals, on glucose agar slants. The resulting growth was washed off with sterile water and added to a Florence flask containing the previously described liquid medium. After 18 to 24 hours incubation this flask was used as the source of the 1.0% inoculum for the large volume of medium.

The cells were harvested by use of a Serval Angle centrifuge at 5,000 revolutions per minute with a Serval SS-1 centrifuge at 14,000 revolutions per minute, or with a Sharples continuous centrifuge at approximately 25,000 revolutions per minute. After harvesting, the cells were washed once with distilled water and then resuspended in distilled water at a concentration of 200 to 400 milligrams per millilitre.

This cell suspension was subjected to sonic oscillation for fifteen to twenty minutes in a Raytheon 10 Kilocycle Oscillator. The resulting mixture, called a sonicate, was then centrifuged for ten minutes at 14,000 revolutions per minute and resulted in the separation of a slight amount of sedimentable material, which was discarded. The centrifuged sonicate, which was a reddish colour, exhibited a marked Tyndall effect.

Chemical

Metabolic gas exchanges were measured in the Warburg respirometer according to the standard procedures of Umbreit et al. (1949).

Gluconic acid was detected by paper chromatography with an ethanol-methanol-water (45:45:10) solvent system

and 0.1N $AgNO_3$ in 5N NH_4OH spray as described by Norris and Campbell (1949 a). The reaction was carried out in a 125 ml. capacity Warburg reaction vessel containing 50 micromoles of glucose (2 ml.), 15 ml. of a pH 7.3 M/15 phosphate buffer, 10 ml. of a sonicate from asparagine grown cells (showing no gluconic acid oxidation), 1 ml. of $8X10^{-3}$ M $MgSO_4$, 2 ml. of sodium 2, 6, dichlorobenzenoneindophenol (0.75 mg. per ml.) and 10 ml. of distilled water. The rate of oxidation was followed with a conventional Warburg system containing one tenth of the previous constituents. When the reaction was almost complete the large cup was cleared of protein by the use of dilute sulphuric acid which lowered the pH to 2.0. After centrifugation, the pH was raised to 7.4 and the reaction mixture was concentrated to four ml. in vacuo at 25°C. This solution was then used for chromatographic purposes.

Protein was determined in the following manner: 5 ml. of 10.0% trichloro acetic acid were added to 0.5 ml. of the protein solution, the resulting precipitate was separated by centrifugation at 5,000 revolutions per minute. The precipitate was dissolved in freshly prepared 3.0% NaOH and 0.6 ml. of a 20.0% $CuSO_4 \cdot 5H_2O$ solution was added for the biuret colour reaction. The solution

was rapidly brought to a final volume of 25 ml. with 3.0% NaOH and shaken vigourously for one minute; allowed to stand for ten minutes, and centrifuged again at 5,000 revolutions per minute for 10 minutes. The supernatant was then read at 560 millimicrons in a Beckman Model B. Spectrophotometer. This procedure is a modification of the method of Robinson and Hogden (1940) and uses casein as a standard.

A saturated solution of $(NH_4)_2\ SO_4$ was prepared by adding 70.6 gm. to 100 ml. of distilled water. All the fractions reported are given as the per cent of saturation. The fractions were obtained by adding $(NH_4)_2SO_4$ slowly with stirring to the protein solution. After five minutes the solution was centrifuged at 10,000 revolutions per minute for ten minutes. The temperature was kept below 10°C to lessen the danger of denaturation. After separation, the fractions were dialysed, with stirring, against ice cold distilled water for one hour, using a cellophane sac.

The alumina c-alpha was prepared according to the method of Hawk, Oser and Summerson (1949). The alumina was used in amounts equal to the solids in solution in the sonicate, i.e., if the sonicate had 200 mg. of cell material per ml. then 200 mg. of Alumina were added per

ml. of solution.

The alumina was shaken on a rotary shaker with the preparation and then centrifuged to remove it from solution. Elution was carried out by shaking the eluting agent with the alumina and again removing the alumina by centrifugation.

The IRC-50 resin was conditioned by allowing it to remain for 12 hours in 10.0% HCl, washing free of acid with distilled water and then suspending in pH 7.2 M/15 phosphate buffer. The solution was adjusted to pH 7.2 with NaOH until the pH remained constant, and was then left for 12 hours at less than 10°C. The pH was tested after 12 hours and the resin was then washed with a large excess of pH 7.2 M/15 phosphate buffer. The pH was tested again and if the resin was at the desired pH it was ready for use. This procedure is a modification of the one used by Hirs, Moore and Stein (1953).

The solvents ethyl alcohol, ethyl ether, and acetone were cooled to -18°C before use. The dioxane was used at room temperature. The solvents were added slowly with stirring to a sonicate whose temperature was below 10°C. The precipitates were removed by centrifugation at 10,000 revolutions per minute for 10 minutes. The precipitates were resuspended to their original volume in distilled

water or buffer pH 7.3. The traces of solvent were removed from the precipitates by dialysis as before or by aeration. The solvents were removed from the supernatant by separating the layers, by dialysis or by aeration.

The sonicate was made 0.0235M with regard to $MnSO_4$, according to the method of Kuby and Lardy (1953) and was allowed to stand at approximately 10°C for 38 hours. The mixture containing the precipitate was divided into four parts and centrifuged at 14,000 revolutions per minute. The four samples were centrifuged for 5, 10, 20 and 30 minutes respectively. Another experiment was undertaken in which the concentration of $MnSO_4$ was added to two separate volumes of the sonicate bringing the concentration of $MnSO_4$ to one half and one tenth of the 0.0235M originally used. These two samples were then centrifuged at 14,000 revolutions per minute for five minutes. The $MnSO_4$ was removed by dialysis and the various fractions were tested for activity.

The protamine sulphate solution was prepared according to the method of Lindstrom (1953), in which the protamine sulphate is dissolved in pH 5.0 buffer with a final concentration of 20 mg. per ml. The cold protamine sulphate was added slowly beneath the surface of a stirred

protein solution. The resulting precipitate was removed by centrifugation. The protamine sulphate presumably removes nucleoprotein from solution on a mole for mole basis. Therefore there should be no protamine sulphate left in solution.

The bile salts, sodium taurocholate and sodium glycocholate, were added directly to a sonicate and homogenized in a Van Potter homogenizer. The homogenates were centrifuged for t en minutes at 14,000 revolutions per minute and then dialysed against distilled water. This method is a modification of the procedure followed by Williams and Sreenivasan (1953).

The inhibitors, whose final concentration in the reaction mixtures is reported here, were added directly to the Warburg cup. Substrate was tipped in within twenty minutes of the addition of the inhibitor. The centre wells of the cups used for cyanide inhibition contained 0.2 ml. of 4N NaCN in 10.0% KOH (Eisenberg (1953)).

Final Concentration of Inhibitors

Sodium fluoride	2.5×10^{-2} M
Sodium azide	3×10^{-3} M
8-hydroxy quinoline	1×10^{-4} M
Sodium iodoacetate	1×10^{-2} M
2.4 dinitrophenol	2×10^{-4} M
sodium arsenite	1×10^{-2} M
Potassium cyanide	1.5×10^{-4} M
Pyocyanin	3×10^{-4} M

The co-enzymes were prepared according to the Merck Index (1952) and added directly to the Warburg cup. Their final concentrations in the reaction mixtures are reported here.

Final Amounts of Co-enzymes

Riboflavin phosphate		5.0 micromoles
Flavine adenine dinucleotide	(FAD)	5.0 micromoles
Triphosphopyridinonucleotide	(TPN)	5.0 micromoles
Diphosphopyridinonucleotide	(DPN)	5.0 micromoles
Adenosinetriphosphate	(ATP)	100 micrograms
Cytochrome - C		520 micrograms
Magnesium sulphate	($MgSO_4$)	2.5×10^{-3} M

The hydrogen ion acceptors were added in the same manner as the inhibitors. The final concentration in the reaction mixture is reported here.

Final Concentration of Hydrogen Acceptors

Methylene blue	1×10^{-4} M
Brilliant cresyl blue	1×10^{-4} M
2.6 dichlorobenzenoneindophenol	1×10^{-3} M
Potassium ferricyanide	1×10^{-3} M
Pyocyanin	1×10^{-4} M

EXPERIMENTAL

I Isolation and Purification of the Enzyme System

The isolation and the purification of the glucose oxidizing system were to be the first steps in the procedure. Therefore a method of rupturing the cell membranes with the release of the system in an intact form was required. Cell free extracts were obtained by the exposure of a cell suspension containing approximately 31 mg. of protein per ml. to sonic vibration generated by a 10 Kilocycle 60 cycle Raytheon oscillator. The exposure time which gave the greatest liberation of active enzyme was fifteen minutes.

Physiological saline, M/30 phosphate buffers pH6 and pH7, Tris buffer pH7.5 and pH8.0, and distilled water were tested in an effort to determine the optimum medium for suspending the cells for exposure to sonic vibration. Distilled water proved to be the best medium.

Dialysis of the sonicate against distilled water reduced the endogenous respiration by seventy-five per cent with only a slight decrease in the rate of glucose oxidation.

Ammonium Sulphate Fractionation

The sonicate prepared from glucose grown cells was active against glucose, gluconic acid, delta-gluconolactone, and to a lesser degree against glucose-6-phosphate and ribose-5-phosphate. When $(NH_4)_2SO_4$ (30.0% of saturation) was added, the precipitate so formed was capable of oxidizing glucose, gluconic acid and delta-gluconolactone. The glucose-6-phosphate, ribose-5-phosphate and endogenous systems remained in solution.

An attempt was then made to separate the glucose enzyme from the gluconic acid enzyme by a more refined fractionation of the system with $(NH_4)_2SO_4$. Fractions of the sonicate were obtained by adding 2.0% of saturation $(NH_4)_2SO_4$ and increasing it by 2.0% increments. The resulting precipitates showed equal activity with regard to glucose and gluconic acid and so no separation was achieved.

The glucose and gluconic acid enzyme system was concluded to be insoluble as determined by its precipitation by $(NH_4)_2SO_4$ (30.0% of saturation). Since the two enzymes were inseparable with regard to $(NH_4)_2S\Phi_4$ treatment it was decided to attempt a separation by the use of adsorbents.

Alumina C-Alpha

Alumina C-alpha was used in the hope that it would either adsorb the enzyme selectively or take out the other constituents of the preparation preferentially. However, after treatment the suspension was found to have retained reduced but equal activity toward glucose and gluconic acid. Elution of the adsorbent released the endogenous activity and the portion of the glucose and gluconate oxidizing system which had been adsorbed.

Since the endogenous system was adsorbed, it was thought that it might have interfered with the adsorption of the enzymes in question. Therefore an ammonium sulphate (25.0% of saturation) fraction of a sonicate was used for the adsorption procedure. The results remained the same in that the glucose and gluconate enzymes were still adsorbed and eluted in the same fractions, as shown by Table I.

TABLE I

THE RESPIRATORY ACTIVITY OF SONICATE FRACTIONS TREATED WITH ALUMINA-C-ALPHA

FRACTION	OXYGEN UPTAKE IN 1.5 HOURS		
	Endogenous micro-litres	Glucose + micro-litres	Gluconic acid + microlitres
Original Sonicate	72	101	39
Fraction not Adsorbed on Alumina	12	82	31
Fraction Eluted from Alumina	65	94	38
25% $(NH_4)_2 SO_4$ Fraction	0	98	50
Fraction not Adsorbed on Alumina	0	46	27
Fraction Eluted from Alumina	0	83	41

+ The values reported in these columns refer to net oxygen uptake.

The Warburg cups contained 1.5 ml. pH 7.4 M/15 TRIS buffer; 1.0 ml. enzyme preparation; 0.2 ml. substrate containing 5 micromoles of substrate; 0.3 ml. distilled water; 0.15 ml. of 20.0% KOH in centre well. Final volume in the main chamber equalled 3.0 ml.

The samples treated with $(NH_4)_2SO_4$ were dialysed for 1.0 hours with stirring against distilled water.

IRC-50 Resin

The resin was prepared as described earlier and packed in a column 10 inches high and 2 centimetres in diameter. The sonicate was passed through this column at a slow rate and tested for activity after each passage.

After the first passage the endogenous activity was reduced by half, but the glucose and gluconic acid activity remained the same. The second, third and fourth passages reduced the glucose-gluconate system but evidently eluted the endogenous system. The elution with 10 ml. of M/30 phosphate buffer pH 7.2 gave a high endogenous with slight glucose and gluconate activity after the first washing. The next four eluents gave a low endogenous and only slightly higher glucose and gluconate oxidation rates. The sixth eluent which was M/5 phosphate pH 7.2 gave no activity. The resin did not give any separation except with the endogenous system which can be readily separated from the glucose-gluconate system by $(NH_4)_2SO_4$ fractionation. The data are summarized in Table II.

TABLE II

ACTIVITY OF SONICATE TREATED WITH I.R. C-50

STEP	OXYGEN UPTAKE IN 1.5 HOURS		
	Endogenous micro-litres	Glucose + micro-litres	Gluconic Acid + microlitres
Original Sonicate	84	90	43
After 1st Passage through Column	45	86	52
After 2nd Passage Through Column	61	45	27
After 3rd Passage Through Column	58	33	14
After 4th Passage Through Column	69	32	20

Elution with M/30 PO_4 Buffer (10ml. fractions)	Endogenous	Glucose+	Gluconic Acid +
Fraction 1	41	24	13
Fraction 2	14	16	7
Fraction 3	8	8	4
Fraction 4	6	6	6
Fraction 5	7	5	2
Elution with M/5 PO_4 Buffer (10 ml.)	0	0	0

+ The values reported in these columns refer to net oxygen uptake

The Warburg cups contained 1.5 ml. pH 7.2 M/15 phosphate buffer; 1.0 ml. enzyme preparation; 0.2 ml.

substrate (5.0 micromoles); 0.3 ml. distilled water; 0.15 ml. of 20.0% KOH in the centre well.

Precipitation by Solvents

When a sonicate was treated with 2.0% acetone, ethyl alcohol or dioxane, the oxidative ability of both the precipitate and the supernatant toward glucose and gluconic acid was destroyed. Ethyl ether in the same concentration proved to be an exception. The activity of the supernatant was destroyed but the precipitate had the ability to oxidize gluconic acid alone. This loss of ability was not due to a separation of the protein part of the enzyme from its co-factors since a recombination of the precipitate with the supernatant did not result in a recovery of activity. (Table III).

TABLE III

EFFECT OF SOLVENT PRECIPITATION ON THE RESPIRATORY ACTIVITY OF SONICATE

Solvent Added (2% by volume)		Oxygen Uptake		
		Endogenous micro-litres	Glucose micro-litres	Gluconic Acid microlitres
Acetone	A	0	0	0
	B	0	0	0
Ethyl Alcohol	A	0	0	0
	B	0	0	0
Dioxane	A	0	0	0
	B	0	0	0
Ethyl Ether	A	0	0	0
	B	0	0	50

A = Supernatant
B = Ppt. resuspended in original volume

Ppt. and Supernatant Recombined	Endogenous	Glucose	Gluconic Acid
Acetone	0	0	0
Ethyl Alcohol	0	0	0
Dioxane	0	0	0
Ethyl Ether	0	0	45

The Warburg cups contained 1.5 ml. pH 7.3 M/15 phosphate buffer; 1 ml. enzyme preparation; 0.2 ml. substrate (5.0 micromoles); 0.3 ml. distilled water; 0.15 ml. of 20.0% KOH in centre well.

Precipitation by Manganous Sulphate

Manganous sulphate treatment of a protein solution has been shown to remove nucleoproteins. An attempt was made to remove interfering nucleoproteins from solution in order to aid in dissolving the glucose enzyme. It was thought that the removal of all extraneous material would greatly aid in the separation of a pure enzyme. The sonicate was treated with $MnSO_4$ as described previously. Upon resuspension the precipitate proved to have the greatest amount of the glucose-gluconate oxidizing enzymes. It was found that varying concentrations of the $MnSO_4$, and varying times of centrifugation removed corresponding amounts of both enzymes but did not effect a separation as can be seen from Table IV.

TABLE IV

EFFECT OF MANGANOUS SULPHATE ON RESPIRATORY ACTIVITY OF SONICATE

0.0235M. $MnSO_4$

Time of Centrifugation (14,000 R.P.M.)	Net Oxygen Uptake			
	Glucose microlitres		Gluconic Acid microlitres	
	A	B	A	B
5 minutes	35	112	15	56
10 minutes	15	112	0	56
20 minutes	10	112	0	56
30 minutes	0	112	0	56

A = Supernatant
B = Ppt. Resuspended in original volume

5 Min. Centrifugation (14,000 RPM)

Concentration of $MnSO_4$	Glucose microlitres		Gluconic Acid microlitres	
	A	B	A	B
0.0235M.	35	112	15	56
0.01175M.	45	112	20	56
0.00235M.	55	112	25	56

A = Supernatant
B = Ppt. Resuspended in original volume

The Warburg cups contained 1.5 mls. pH 7.3 m/15 phosphate buffer; 1.0 ml. enzyme preparation; 0.2 ml. substrate (5.0 micromoles); 0.3 ml. distilled water; 0.15 ml. of 20.0% KOH. All enzyme fractions were dialysed as before.

Bile Salt Extraction

When glycocholate was added to a sonicate in the proportion of 0.5 gm. per 10.0 ml. of the sonicate, the resulting supernatant oxidized only gluconic acid in the presence of 2.6 dichlorobenzenoneindophenol as the hydrogen acceptor. The precipitate oxidized gluconic acid more rapidly than it did glucose and the addition of the dye was again necessary for the oxidation. Combination of the dialysed precipitate and the dialysed supernatant did not restore the activity on glucose to its original rate.

Taurocholate used in the same concentration as the glycocholate gave similar results. The precipitate contained both enzymes and the supernatant only the gluconic enzyme. An increase in the concentration of bile salts caused a destruction of both enzymes. The data are summarized in Table V.

TABLE V

EXTRACTION OF THE SONICATE WITH BILE SALTS

Bile Salt Used as Extractant	Oxygen Uptake	
	Glucose micro-litres	Gluconic Acid micro-litres
1. Glycocholate		
A = Supernatant	0	0
A + 2, 6, dichlorobenzenoneindophenol	0	56
Precipitate	35	50
2. Taurocholate		
A = Supernatant	0	0
A + 2, 6, dichlorobenzenoneindophenol	0	56
Precipitate	35	50

The Warburg cups contained 1.5 ml. pH 7.3 M/15 phosphate buffer; 1.0 ml. of enzyme preparation; 0.2 ml. substrate (5.0 micromoles); 0.3 ml. distilled water except when the 2.6 dichlorobenzenoneindophenol was added, in 0.2 ml. volumes in which case the water was reduced to 0.1 ml; 0.15 ml. of 20.0% KOH in the centre well. All enzyme fractions were dialysed as before.

Use of Carbon Source Unrelated to Glucose

Since the glucose oxidizing enzyme has been reported

to be constitutive while the gluconic acid enzyme was adaptive it should be possible to grow the organism on a medium which would not stimulate the formation of the gluconic acid oxidizing enzyme system (Entner and Stanier (1951)).

In some cases, when grown in 0.3% asparagine alone, 0.2% asparagine plus 0.2% glycerol, and 0.2% asparagine plus 0.2% succinate, intact cells proved to be adaptive to all substrates except glucose. Acetate grown cells had a constitutive though slightly reduced system for the oxidation of gluconic acid. (See Fig. I.)

The sonicates of the cells grown on the various carbon sources had only a very weak system for the oxidation of gluconic acid. However the systems were not dependable, in that the gluconic acid enzyme would frequently appear quite strongly, though not to the same degree as in the glucose grown cellls. This would appear to be a promising approach however and should be investigated in greater detail.

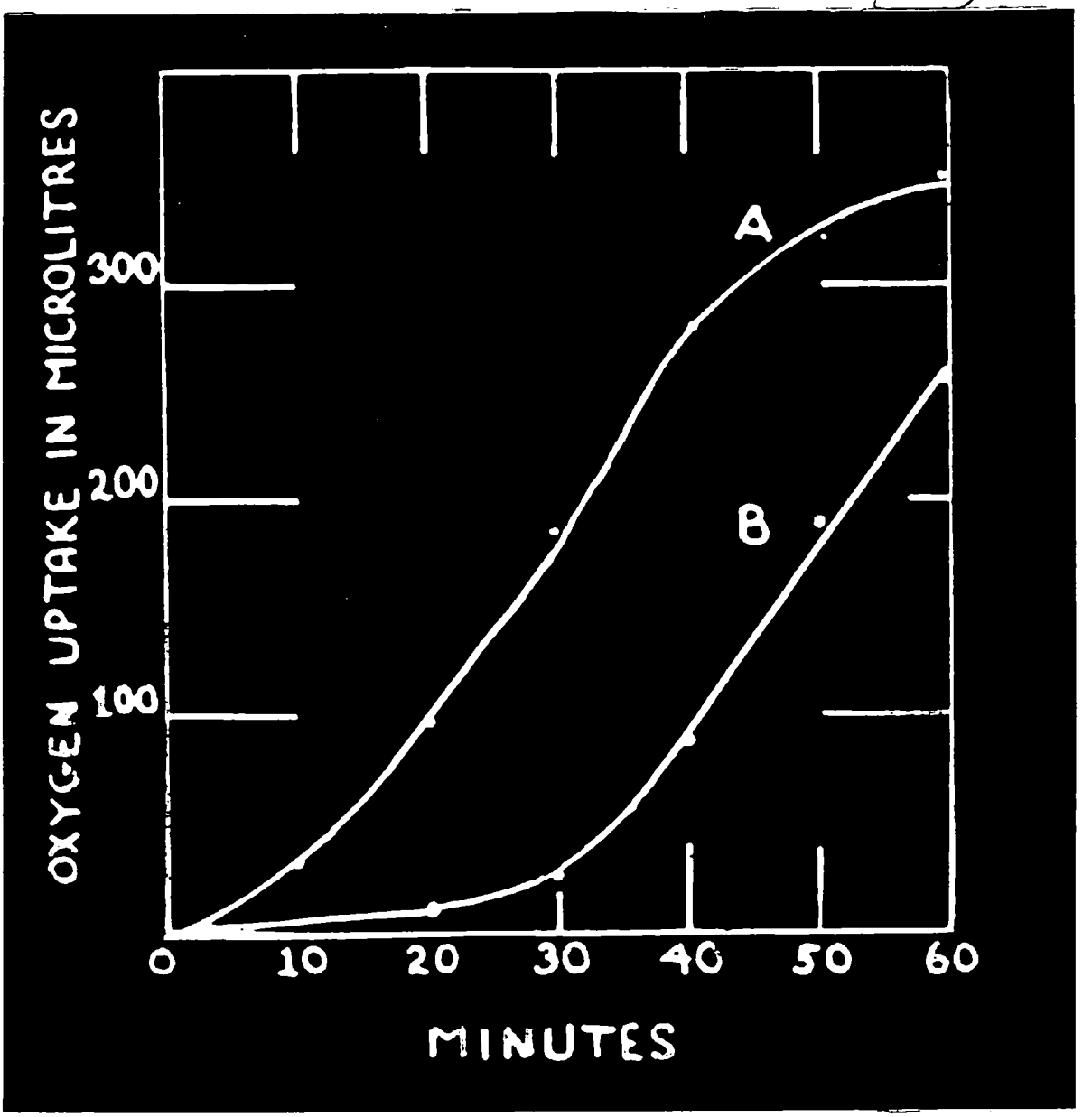

FIG. I. The constitutive nature of the glucose enzyme (A) and the adaptive nature of the gluconic enzyme (B).

These curves demonstrate typical glucose and gluconate oxidation by whole cells when asparagine, asparagine + succinate or asparagine + glycerol was used as the carbon source for growth. Sonicates of these cells possessed normal activity towards glucose but negligible activity towards gluconate.

Net oxygen uptake is shown, using 5 micromoles of substrate.

Glycine and Aniline Treatment

The method of Crewther et al. (1953) was followed in which glycine was added to a sonicate in order to solubilize the enzymes. According to these authors glycine and aniline increase the solubility of proteins so that they can withstand higher concentrations of protein precipitants.

It was found that aniline, added to a final concentration of 0.01M., increased the solubility but inhibited the enzyme action.

When glycine was added to the sonicate at a final concentration of 0.1 M. it did not inhibit the enzyme action, and moreover greatly increased the solubility. Precipitation with $(NH_4)_2$ SO_4 and protamine sulphate did not separate the glucose enzyme from the gluconic acid enzyme however.

Sonic vibration in the presence of 0.1M. glycine (final concentration) resulted in an active preparation. However $(NH_4)_2SO_4$ and protamine sulphate deactivated both the precipitate and the supernatant.

TABLE VI

THE EFFECT OF GLYCINE AND ANILINE ON THE SONICATE

Step		Oxygen Uptake in 1.5 Hours	
		Glucose microlitres	Gluconic Acid microlitres
Original Sonicate		112	56
Made 0.1M with Glycine		112	56
Made 0.1M with Aniline		80	35
Original Sonicate	A	15	7
	B	112	56
0.1M with glycine	A	112	56
	B	10	0
0.1M with aniline	A	75	35
	B	10	0
30.0% $(NH_4)_2SO_4$ Fraction of			
Original Sonicate		112	56
0.1M.Glycine Sonicate		112	56
Supernatant of 30.0% $(NH_4)_2SO_4$ Fraction of			
Original Sonicate		0	0
0.1M.Glycine Sonicate		45	25
Supernatant of 6.0% Protamine Sulphate			
Original Sonicate		70	30
0.1M.Glycine Sonicate		85	40
Sonicate prepared in Presence of 0.1M.Glycine		112	56
20.0% $(NH_4)_2SO_4$ Fraction		15	0
Supernatant of 20.0% $(NH_4)_2SO_4$ Fraction		0	0
Supernatant of 6.0% Protamine Sulphate		0	0

A = Supernatant
B = Precipitate

The Warburg cups contained 1.5 ml. pH 7.3/M/15 phosphate

buffer; 1.0 ml. enzyme preparation; 0.2 ml. substrate (5.0 micromoles); 0.3 ml. distilled water; 0.15 ml. of 20.0% KOH in the centre well.

All $(NH_4)_2SO_4$ treated samples were dialysed as before.

II Properties of the Enzyme System

The attempts to isolate and purify the enzyme had met with very little success, therefore it was decided to study the enzyme system as it existed in the original dialyzed sonicate without the benefit of any purification procedure.

Inhibition Studies

The effect of sodium iodoacetate, sodium fluoride, sodium arsenite, 2.4 dinitrophenol, sodium azide, potassium cyanide and pyocyanin, on a sonicate of asparagine-succinate grown cells was studied.

As shown in Table VII only sodium azide and potassium cyanide showed marked inhibition. These two inhibitors depressed the respiration of the glucose oxidizing enzyme almost completely.

When 8-hydroxy-quinoline was used on a 25% $(NH_4)_2SO_4$ fraction of an asparagine grown sonicate, the inhibition could be overcome by the addition of magnesium.

TABLE VII

THE EFFECT OF VARIOUS INHIBITORS ON GLUCOSE OXIDATION

INHIBITOR	% INHIBITION
Sodium Iodoacetate	0
Sodium Arsenite	0
Sodium Fluoride	0
Sodium Azide	60
Potassium Cyanide	100
2.4 Dinitrophenol	0
Pyocyanin	0
8 OH Quinoline	80
8 OH Quinoline + 2.5×10^{-3} M. $MgSO_4$	0

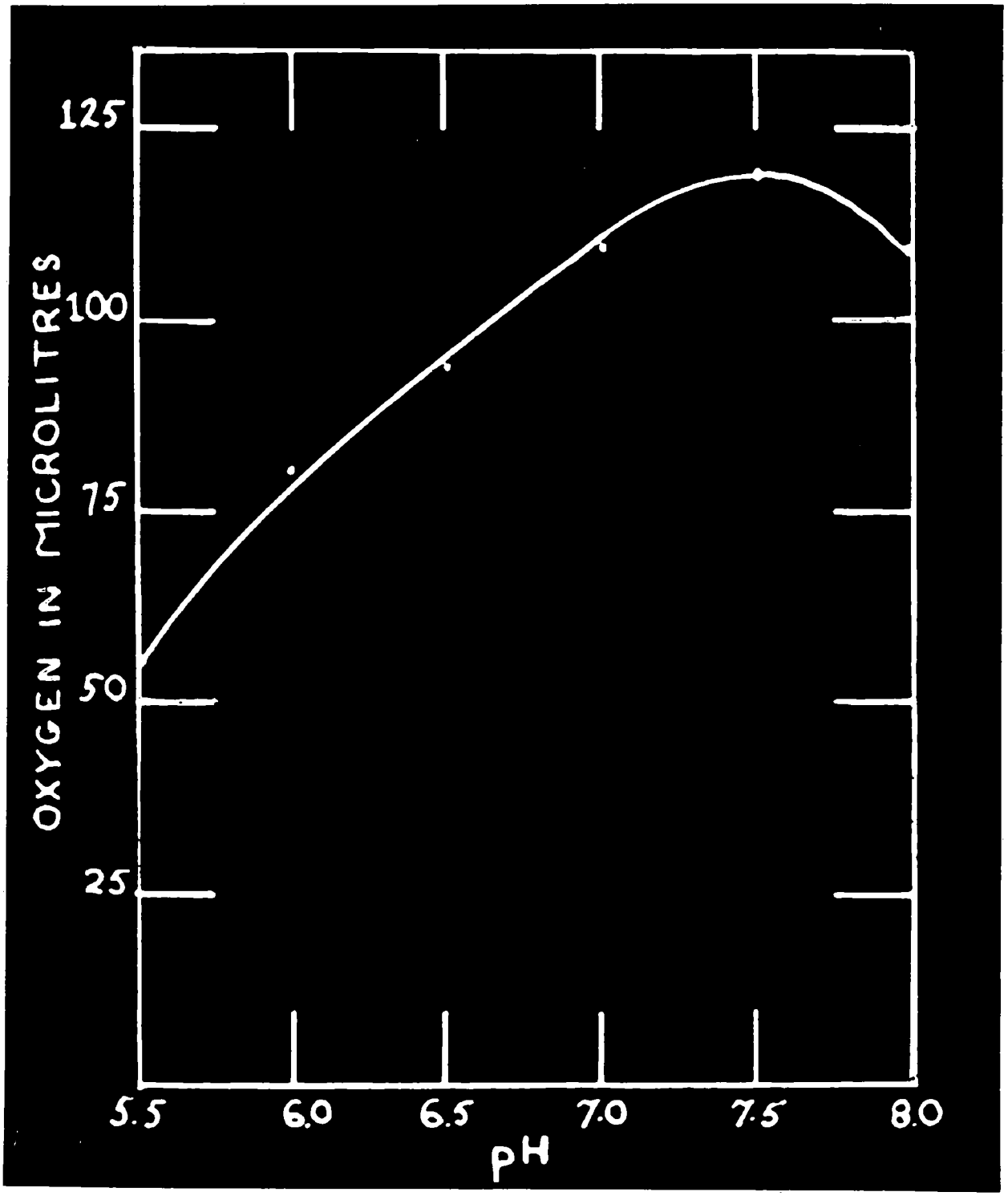

FIG. II pH Curve

The Warburg cups contained 1 ml. enzyme preparation, 0.2 ml. glucose (5.0 micromoles), 0.3 ml. distilled water and 1.5 ml. M/15 phosphate buffer.

The pH was raised in increments of 0.5 pH units between pH 5.5 and pH 7.0, and 0.2 pH units between pH 7.0 and pH 8.0

Net oxygen uptake in 1.5 hours.

Hydrogen Ion Acceptors

Brilliant cresyl blue, methylene blue, 2.6 dichlorobenzenoneindophenol, ferricyanide and pyocyanin were tested as hydrogen ion acceptors. The ferricyanide proved to be inhibitory, while only the 2.6 dichlorobenzoneindophenol was stimulatory as shown in Table VIII.

TABLE VIII

THE EFFECT OF HYDROGEN ACCEPTORS ON GLUCOSE OXIDATION

HYDROGEN ACCEPTOR	EFFECT
Brilliant Cresylblue	None
Methylene Blue	None
2.6 Dichlorobenzenoneindophenol	100% Stimulation
Ferricyanide	40% Inhibition
Pyocyanin	None

Coenzymes

ATP, DPN, TPN, FAD, riboflavin phosphate and cytochrome-C had no effect on the glucose oxidizing system.

Magnesium sulphate in a final concentration of 2.5×10^{-3} stimulated the oxidation of glucose by over thirty per cent when used alone while when used in

conjunction with 2.6 dichlorobenzenoneindophenol the system was stimulated by approximately 300.0% (Table IX).

TABLE IX

THE EFFECT OF VARIOUS COENZYMES ON GLUCOSE OXIDATION

COENZYME	EFFECT
ATP	None
FAD	None
DPN	None
TPN	None
Riboflavin Phosphate	None
Cytochrome C	None
$MgSO_4$	33% Stimulation
2.6 Dichlorobenzenoneindophenol	100% Stimulation
$MgSO_4$ + 2.6 Dichlorobenzenone-indophenol	286% Stimulation

The Reaction Product

Gluconic acid was determined as the product of the reaction by paper chromatography with the previously described ethanol-methanol-water solvent system.

An asparagine grown sonicate which showed only glucose oxidation was used. The reaction was allowed to continue

until it was almost complete. The contents of the large Warburg cup, after being treated as described in the methods, was chromatographed. Two spots were observed which corresponded to glucose and gluconic acid standards. The glucose spot had an Rf of 0.55 and the gluconic acid spot had an Rf of 0.40.

DISCUSSION

Several related non-phosphorylative glucose oxidizing systems have previously been described in the literature. It should be noted however, that although the reaction which they catalyse is similar, the nature of the enzyme systems is quite different. Since the enzymes are normally species specific their differences in chemical composition will be manifested by variations in such physical constants as solubility, stability and pH optimum. This means that the isolation and purification of each enzyme system has to be established independently. It is for this reason that a detailed study of the glucose oxidizing system of P. aeruginosa was essential.

Manometric experiments have revealed that sonic extracts were able to oxidize glucose, gluconate, glucose-6-phosphate, ribose-5-phosphate and delta-gluconolactone. Centrifugation or $(NH_4)_2SO_4$ fractionation precipitated the glucose, gluconate, and delta-gluconolactone oxidizing systems in an active form. However, the system for oxidizing the phosphorylated compounds was lost after treatment with $(NH_4)_2SO_4$.

Attempts to separate the glucose enzyme from the gluconic acid enzyme were not successful. The fact

that both enzymes were thrown down by half hour centrifugation at 25,000 g or by 30.0% $(NH_4)_2SO_4$ showed that they were insoluble. An attempt to fractionate by the addition of different percentages of $(NH_4)_2SO_4$ resulted in a series of precipitates which had equal amounts of the two enzymes.

The adsorption procedures using IRC-50 and Alumina-c-alpha also resulted in fractions containing equivalent amounts of the two enzymes.

Treatment by $MnSO_4$ was ineffective in that it removed corresponding amounts of the two enzymes from the solution. The use of various solvents for fractionation denatured the enzymes. However ethyl ether was an exception in that it did not destroy the gluconic acid enzyme.

Extraction of the sonicate with bile salts resulted in a solution showing strong activity toward gluconic acid when 2.6 dichlorobenzenoneindophenol was present. The precipitate possessed a strong system for the oxidation of both glucose and gluconic acid. Reduction of the concentration of bile salt left the glucose enzyme in solution while an increase in concentration destroyed the glucose activity in both fractions.

When added before the sonic treatment, glycine aided in solubilizing both the glucose and gluconic acid enzymes

but did not aid in their separation. Moreover the solubilized glucose and gluconic acid enzymes became labile to such precipitants as protamine sulphate and $(NH_4)_2SO_4$.

Growth on a carbon source other than glucose may almost entirely eliminate the gluconic acid enzyme. However, this method does not give uniform results. The glucose enzyme is always constitutive but the gluconic acid enzyme frequently appears very strongly, although not to the same degree as when glucose is the carbon source.

Coulthard et al. (1942) were able to purify Notatin by acetone extraction of a tannic acid precipitate of the mould culture filtrate. Strecker and Korkes (1952) were able to remove extraneous material from an aqueous extract of liver homogenate with $(NH_4)_2SO_4$. Both liver glucose dehydrogenase and Notatin catalyse the oxidation of glucose to gluconic acid. These two enzymes, apparently possessing the same function as the glucose oxidizing enzyme of P. aeruginosa and therefore isodynamic are adequate proof of the differences in the physical constants of three enzymes classed as being isodynamic.

The glucose oxidizing enzyme of P. aeruginosa is inhibited by 1.5×10^{-4} potassium cyanide and 1×10^{-3}M

sodium azide thereby suggesting the function of cytochrome carriers, and distinguishing it from the flavoprotein glucose oxidase. The lack of inhibition by sodium fluoride and 2.4 dinitrophenol would indicate the lack of phosphorylation or the absence of the glycolytic scheme. Since DPN, TPN, FAD, ATP, cytochrome-c or riboflavin phosphate caused no stimulation these factors were regarded as not being coenzymes of this system. This is in contrast to the liver dehydrogenase which requires either DPN or TPN and Notatin which requires FAD as the prosthetic groups.

2.6 dichlorobenzenoneindophenol stimulated the glucose oxidation while methylene blue, pyocyanin and brilliant cresyl blue did not affect the rate of oxidation. This may indicate a role of cytochrome b or a similar component in the oxidation (Wood and Schwerdt (1953)).

A requirement for magnesium was shown when the inhibition by $1X10^{-4}M$ 8-hydroxyquinoline was overcome by addition of $2.5 \times 10^{-3}M$ $MgSO_4$.

The reaction product of glucose oxidation was found to be gluconic acid when determined under conditions which would split a lactone to the acid. Strecker and Korkes (1952) found that the product of the reaction catalysed by the liver dehydrogenase of Harrison (1931)

was gluconolactone. Bentley and Neuberger (1949) showed that the enzymatic product of Notatin was delta-gluconolactone. The reaction product of glucose oxidation by P. aeruginosa could well be gluconolactone, since the sonicate can oxidize gluconolactone as readily as gluconic acid. Brodie and Lipmann(1954) have reported that an enzyme from the particulate fraction of Azotobacter vinelandii oxidized glucose and quantitatively accumulated delta gluconolactone. A second enzyme from the supernatant of A. vinelandii and baker's yeast rapidly hydrolysed the lactone to the corresponding acid. This mechanism activated by Mg^{++} is similar to the glucose oxidizing system of P. aeruginosa.

From these results it may be concluded that the glucose oxidizing enzyme of P. aeruginosa is insoluble and is cyanide and azide sensitive. It requires Mg^{++} as a co-factor and can use 2.6 dichlorobenzenoneindophenol as a hydrogen acceptor. Its mechanism of action is probably the removal of hydrogen from glucose to form gluconolactone which may then undergo enzymatic or non-enzymatic hydrolysis to gluconic acid.

SUMMARY

1. A cell free extract of P. aeruginosa was obtained by exposure to sonic vibration. This sonicate possessed two systems, one directly oxidizing glucose, gluconate and gluconolactone, the other an independent system oxidizing glucose-6-phosphate and ribose-5-phosphate.

2. The glucose, gluconate and gluconolactone oxidizing system could be separated from the soluble proteins by centrifugation or $(NH_4)_2SO_4$ precipitation but the enzymes could not be separated from each other.

3. pH 7.5 was found to be the optimum pH for the glucose system.

4. The enzymes were precipitated by $MnSO_4$ in amounts proportional to the concentration of the salt. The relative amounts of each of the enzymes in the precipitate remained constant, with no separation of enzymes being achieved in the process.

5. The resin IRC-50 adsorbed the enzymes equally. It was not possible to elute them separately.

6. Alumina-c-alpha absorbed the glucose and gluconate enzymes completely and it was not possible to elute them separately.

7. Ethyl alcohol, dioxane and acetone destroyed glucose and gluconate enzymes. Ethyl ether destroyed only the glucose enzyme.
8. The addition of glycine to the sonicate solubilized the enzymes but did not aid in their separation. Addition of glycine before sonic treatment caused the enzymes to become labile to protein precipitants.
9. Extraction of the sonicate with bile salts left a precipitate with equal activity toward glucose and gluconic acid. The supernatant possessed a portion of the gluconic acid enzyme in a soluble form. An increase in the bile salt concentration destroyed the glucose activity.
10. Growth on a carbon source other than glucose resulted in the formation of a reduced gluconic acid system. However, the results were not uniform in that the gluconate enzyme frequently was very active.
11. Methylene blue, pyocyanin, and brilliant cresyl blue had no ability to act as hydrogen acceptors.
12. 2.6 dichlorobenzenoneindophenol acted as a hydrogen acceptor.
13. Ferricyanide inhibited glucose oxidation.
14. A.T.P., F.A.D., D.P.N., T.P.N., cytochrome-c and

riboflavin phosphate had no effect on glucose oxidation.

15. KCN, NaN_3 and 8-hydroxy quinoline inhibited glucose oxidation.
16. $MgSO_4$ overcame the inhibition caused by 8-hydroxyquinoline.
17. $MgSO_4$ stimulated glucose oxidation.
18. Sodium fluoride, 2.4 dinitrophenol, sodium iodoacetate, and sodium arsenite caused no inhibition.

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