[CONTRIBUTION FROM THE NORTHERN REGIONAL RESEARCH LABORATORY[1]]

Production of α-Ketoglutarate in Glucose Oxidation by *Pseudomonas fluorescens*[2]

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Fermentation of glucose in synthetic media by *Pseudomonas fluorescens* has led to yields of 0.50 to 0.55 mole of α-ketoglutaric acid per mole glucose consumed with indications that the maximum possible yield may be considerably higher. Gluconate and 2-ketogluconate accumulated as intermediates, suggesting that glucose was metabolized according to the hypothetical hexosemonophosphate shunt pathway. However, successive 1-carbon degradation up to the triose stage, as currently postulated for this pathway, is not consistent with the α-ketoglutarate yields obtained. A paper chromatogram procedure for following the course of fermentation is described.

Certain species of *Pseudomonas* bacteria have been shown to oxidize glucose to gluconate and 2-ketogluconate successively in essentially quantitative yield.[3] One species, *Ps. fluorescens* NRRL B-6, was found to produce α-ketoglutarate (about 0.2 mole per mole glucose oxidized) on continued oxidation.[4] Pyruvate accumulation was detected in the oxidation of gluconate by this organism, and evidence suggesting that pyruvate is converted to α-ketoglutarate was obtained.[5]

The observed sequence of oxidative steps would seem to indicate that glucose oxidation by this organism proceeds according to one version of the hypothetical "hexosemonophosphate shunt" (Warburg–Lipmann–Dickens) pathway, which is currently receiving considerable attention among biochemists as being a pathway alternate to aerobic glycolysis.[6] This version comprises oxidation of glucose-6-phosphate *via* 6-phosphogluconate and 6-phospho-2-ketogluconate to 5-phosphopentose. This reaction sequence constitutes stepwise 1-carbon degradation of the carbohydrate molecule, and it is frequently postulated that the sequence is repeated until one mole of triose phosphate plus three moles of carbon dioxide have been produced.[7] The triose phosphate would then be dissimilated by known reactions. *Ps. fluorescens* B-6, when grown on glucose or gluconate in synthetic media, accumulates 2-ketogluconate rapidly and almost stoichiometrically, whereas further oxidation leading to pyruvate and α-ketoglutarate accumulation is slow. An opportunity for studying broadly the nature of reactions in 2-ketogluconate oxidation is thus afforded, and information applicable to the obscure reactions of the hexosemonophosphate shunt pathway may be obtained.

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Presented before the Division of Agricultural and Food Chemistry at the 119th Meeting of the American Chemical Society, Cleveland, Ohio, April 8–13, 1951.

(3) L. B. Lockwood, B. Tabenkin and G. E. Ward, *J. Bact.*, **42**, 51 (1951).

(4) L. B. Lockwood and F. H. Stodola, *J. Biol. Chem.*, **164**, 81 (1946).

(5) H. J. Koepsell, *ibid.*, **186**, 743 (1950).

(6) V. R. Potter, *Adv. in Enzymol.*, **4**, 201 (1944); S. P. Colowick and N. O. Kaplan, *Ann. Rev. Biochem.*, **20**, 513 (1951).

(7) Another version of the hexosemonophosphate shunt pathway is emerging from the recent work of B. L. Horecker and P. Z. Smyrniotis, *J. Biol. Chem.*, **196**, 135 (1952), and others. In this version, as found in yeast, 6-phosphogluconate is simultaneously dehydrogenated and decarboxylated to yield 5-phosphoribulose. An important distinction between the two versions is that the pathway *via* the 2-ketogluconate ester involves a primary α-oxidation, whereas the pathway in yeast implies a β-oxidation.

The predicted theoretical yields of pyruvate and α-ketoglutarate conforming to intermediary production of one mole triose and three moles of carbon dioxide are 1.0 and 0.5 mole, respectively, per mole 2-ketogluconate consumed. A previous paper[5] has indicated that the sum of the yields of pyruvate and α-ketoglutarate obtained in 2-stage oxidative gluconate fermentation by *Ps. fluorescens* exceeded the predicted yields. This conclusion, however, depended on disregarding that stage of the fermentation in which no pyruvate or α-ketoglutarate accumulated. The evidence would be more conclusive if higher than predicted yields of either acid could be demonstrated in over-all fermentation. Simple conditions for obtaining α-ketoglutarate yields at or in excess of 0.5 mole (Table I) from glucose in fermentation of a synthetic medium (Table II) are described here. In addition to their value from a theoretical standpoint, the conditions provide a convenient and inexpensive source of α-ketoglutaric acid.

TABLE I

YIELDS OF α-KETOGLUTARATE AND PYRUVATE IN GLUCOSE OXIDATION BY *Ps. fluorescens*

Expt.	Fermentation time, hours	Initial glucose, μM./ml.	Final 2-ketogluconate, μM./ml.	Pyruvate Found μM./ml.	Pyruvate Molar yield	α-Ketoglutarate Found μM./ml.	α-Ketoglutarate Molar yield[a]
151	307	510	0	3	0.01	281	0.55
152	386	513	0	5	.01	252	.49
154	310	506	8	5	.01	260	.52
154	310	510	9	6	.01	259	.52
157	384	510	0	0	.00	241	.48
152	288	512	28	52	.11	227	.47
146	263	510	102	62	.15	242	.59
159	216	510	100	13	.03	223	.56

[a] A yield of 0.5 mole α-ketoglutaric acid per mole glucose corresponds to a yield of 40.6 g. per 100 g. of glucose.

TABLE II

SYNTHETIC MEDIUM FOR α-KETOGLUTARATE PRODUCTION

	μM./ml.	G./100 ml.
Glucose	500	9
$(NH_4)_2SO_4$[a]	12.5–25	0.16–0.32
KH_2PO_4	8	0.11
$MgSO_4$	2	.024
Fe[b]	0.02	1 p.p.m.
$CaCO_3$[c]	375	3.75

[a] Urea, sterilized separately, could be substituted for ammonium sulfate on an equimolar basis without affecting the results. [b] Supplied as ferrous ammonium sulfate. [c] Sterilized separately.

The upper group of data in Table I are analyses of fermentations in which oxidation beyond the 2-ketogluconate stage was complete, while the lower group represents analyses at the time of peak α-ketoglutarate yield but before 2-ketogluconate utilization was finished. No residual glucose was detectable on paper chromatograms despite heavy spotting at the time of analysis. It is seen that α-ketoglutarate yields were in the range of 0.47 to 0.59 mole per mole glucose consumed. These yields were verified by isolation of α-ketoglutaric acid from the fermentation liquor in one experiment.

The fermentations cited in Table I were conducted in erlenmeyer flasks stoppered with cotton plugs, and diffusion of gases (oxygen substrate and carbon dioxide product) precluded the obtaining of fermentation balances. Paper chromatography as described below was employed for following the course of fermentation and for noting the appearance of the various products. It was found that a considerable period of time, characterized by intense metabolic activity, expired before α-ketoglutarate accumulation began. Cell synthesis, as indicated by turbidity and by cell crop on differential centrifugation, commenced within 12 hours after inoculation and proceeded rapidly. By 40 hours there had been advanced conversion of glucose to gluconate and 2-ketogluconate. However, α-ketoglutarate and pyruvate were not detectable despite heavy spotting until between 40 and 64 hours. As in our experiments on gluconate oxidation,[5] these findings suggest that accumulation of α-ketoglutarate is the result of a partial breakdown of the normal mechanism for complete oxidation of glucose to carbon dioxide and water.

The chromatography procedure used also afforded an opportunity to test for the production of other organic acids or reducing sugars as major products of the fermentations. None was detected.

The yields of α-ketoglutarate obtained, together with the fact that α-ketoglutarate is a transient product which is not produced in the first stages of glucose oxidation and that pyruvate is converted to α-ketoglutarate, suggest that α-ketoglutarate arises by splitting of a 5- or 6-carbon molecule into several fragments of two or three carbons each, followed by condensation of the fragments, rather than stepwise degradation of 2-ketogluconate by the contemporary version of the hexosemonophosphate shunt pathway.

The conversion of 100 g. of glucose to about 41 g. of α-ketoglutaric acid, the inexpensiveness and simple composition of the media, and the ease with which α-ketoglutaric acid can be recovered will enhance the possibility of producing the substance on an industrial scale by fermentation methods.

Experimental

Fermentation Technique and Analysis.—The composition of the synthetic medium used is shown in Table II. The yield of α-ketoglutarate obtained was affected markedly by the amount of nitrogen supplied as previously experienced in gluconate oxidation.[5] At low nitrogen levels (less than 25 μM. per ml. calculated as 1 gram-atom per mole) fermentation was incomplete, while at high levels (above 50 μM. per ml.) fermentation was very much more rapid but α-ketoglutarate yields were low or non-existent. A nitrogen level of 25 μM. per ml. was used for most experiments. Substitution of urea for ammonium sulfate had no detectable effect on the results.

One-liter erlenmeyer flasks containing 200 ml. of medium inoculated with *Pseudomonas fluorescens* NRRL B-6 were incubated on a reciprocating shaker at 28°. Progress of the fermentations was followed by reducing-power analysis (detecting both glucose and 2-ketogluconate) according to Somogyi.[8] Pyruvate and α-ketoglutarate were estimated simultaneously by a modification of the method of Friedemann and Haugen.[9] Analytical values were corrected for evaporation during incubation.

Fermentation was intense initially, but reducing power changes became progressively slower after about 60 hours. Ten to fourteen days was usually required for complete reducing power disappearance. A silky precipitate of calcium α-ketoglutarate was found frequently after about the seventh day, and the medium became a thick slurry of crystals by the end of fermentation. Sometimes, however, the medium remained supersaturated and no precipitation occurred without seeding. Supersaturation or precipitation did not change the fermentation rate appreciably.

Isolation of α-Ketoglutaric Acid.—A portion (266 ml. from two flasks in expt. 154) of whole culture which contained 10.4 g. of α-ketoglutaric acid by the analytical method cited, was concentrated to a small volume, acidified with hydrochloric acid, and extracted repeatedly with ethyl acetate. Concentration of the extract gave a crystalline mass of crude α-ketoglutaric acid. Crystallization from ethyl acetate–petroleum ether gave the following fractions:

		C	H	Neut. equiv.	M.p.,[10] °C.
1	8.684 g.	41.0	4.16	74	112–114
2	0.777	41.2	4.14	76	107–111
3	0.289	40.6	3.97	73	114–115
	9.750 [Calcd.	41.1	4.14	73]	

From the mother liquor could be obtained 792 mg. of α-ketoglutaric acid semicarbazone (m.p. 197–198°) (equivalent to 570 mg. of α-ketoglutaric acid). Calcd. for $C_6H_9O_5N_3$: C, 35.5; H, 4.47; N, 20.7. Found: C, 35.6; H, 4.39; N, 20.7.

The total recovery of α-ketoglutaric acid, therefore, amounted to 10.32 g. or 99.2% of that found by analysis by the colorimetric method.

Detection of Fermentation Products by Paper Chromatography.—A number of procedures from the chemical literature were investigated for separating the fermentation products (gluconate, 2-ketogluconate, pyruvate and α-ketoglutarate) and other possible new products (*e.g.*, succinate, fumarate, citrate) on paper. The following procedure gave well defined separations. Pyruvic acid, which is moderately volatile, could be detected, and the individual acids of concern could be identified by the colors produced on color development.

A small amount (2 to 3 ml.) of centrifuged fermentation liquor was freed of cations by adding ion exchange resin until an oxalate test for calcium was negative. The liquor then was spotted on each of three sheets of filter paper. The papers were irrigated overnight by the descending technique with a solvent mixture consisting of 3 volumes of butanol, 2 volumes of pyridine and 1.5 volumes of water. This solvent formula was developed by Jeanes, Wise and Dimler[11] for the separation of mono- and disaccharides, and it served well in our separations of organic acids also. After drying, one of the three papers was sprayed with a 0.05% solution of brom phenol blue. Organic acids appeared as yellow spots on a blue background.

(8) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).

(9) T. E. Friedemann and G. E. Haugen, *ibid.*, **147**, 415 (1943). The single-solvent procedure, with ethyl acetate as solvent, was used. As applied to these experiments, it was found that greater accuracy and more precise differentiation between pyruvate and α-ketoglutarate could be obtained by reading the yellow color of the hydrazones, after extraction into sodium carbonate solution, at 380 mμ. NaOH to be 0.4 *N* in the solution was then added, and the resulting reddish-brown colors were read at 435 and 520 mμ. All readings were against appropriate reagent blanks. Results were calculated by simultaneous equations from pairs of the three readings.

(10) All melting points are capillary and corrected.

(11) A. Jeanes, C. S. Wise and R. J. Dimler, *Anal. Chem.*, **23**, 415 (1951).

The second paper was sprayed with a 0.5% solution of 3,5-dinitrosalicylic acid in 1 *N* sodium hydroxide. This reagent is frequently used to detect reducing sugars. In the present application, the paper while still moist was heated in an oven at 70°. α-Ketoglutaric and pyruvic acids appeared as brilliant orange spots after about 5 minutes. These spots were transient, and their location and relative intensities were noted. On continued heating, glucose and 2-ketogluconate appeared as permanent brown spots.

The third paper was sprayed with a 0.2% solution of orthophenylenediamine in ethyl alcohol, also containing 1% nitric acid. On heating in the 70° oven for about 30 minutes, keto substances on the paper appeared in characteristic colors. 2-Ketogluconic acid and its lactone were deep olive green. 5-Ketogluconic acid (present as a guide spot) was deep blue. Glucose was grayish-brown. α-Ketoglutaric acid was white on the steel-gray background. Pyruvic acid was rose-colored, and was easily detectable even at low concentrations. The R_f values of the various substances were approximately as follows, beginning at the starting line: 2-ketogluconolactone, 0.10; gluconic and 2-ketogluconic acids, 0.15; α-ketoglutaric acid, 0.25; pyruvic acid, 0.35; and glucose, 0.4. The R_f values of the organic acids, and especially of α-ketoglutaric acid, were considerably dependent on their concentration in the spot. The acids also tended to tail badly if overspotted. For these reasons, identification of the substances by their characteristic colors and their order in the line of spots was more satisfactory than identification by R_f value.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF CALIFORNIA]

Depsidones. II. Hydroxy and Methoxy Analogs[1]

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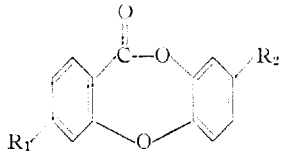
2-(2-Hydroxyphenoxy)-4-methoxybenzoic acid lactone (II) and 2-(2,4-dihydroxyphenoxy-)-4-methoxybenzoic acid lactone (III) have been prepared as further analogs of the depsidones. Methods have been developed for the formation of phenolic lactones, and a selective demethylation of phenolic ethers is described.

In a continuation of the study of the preparation of compounds related to the depsidones,[2] we have prepared hydroxy and methoxy derivatives of 2-(2-hydroxyphenoxy)-benzoic acid lactone (I). Tomita, Inubuse and Kusuda have suggested that I be called "depsidone."[3] We have chosen to use the systematic names throughout the present discussion, reserving "depsidone" as a generic name for the class.

Such substitution products (II, III) contain the important functional groups of the naturally occurring depsidones, some of which have shown bacteriostatic activity.[4]



I, R_1R_2 = H
II, R_1 = OCH_3, R_2 = H
III, R_1 = OCH_3, R_2 = OH

The preparation of 2-(2-hydroxyphenoxy)-4-methoxybenzoic acid lactone (II) proceeded from methyl 2-chloro-4-methoxybenzoate (IV) as outlined in Chart I. The condensation of IV with the sodium salt of guaiacol (V) proceeded smoothly to give methyl 2-(2-methoxyphenoxy)-4-methoxybenzoate (VI). Attempted demethylation of VI to 2-(2-hydroxyphenoxy)-4-hydroxybenzoic acid (VII) under the usual conditions with 48% hydrobromic acid in acetic acid was unsuccessful, resulting instead in 2,3′-dihydroxydiphenyl ether (VIII).

The ease of decarboxylation of *o*- or *p*-hydroxybenzoic acids is well known.[5] From consideration of the S_E2 mechanism proposed for similar decarboxylations,[6] it would appear probable that the methoxy acid (IX) is not undergoing decarboxylation, but more likely that decarboxylation is occurring subsequent to complete demethylation. It thus appeared attractive to attempt to achieve selective demethylation, and avoid loss of the carboxyl group to form 2-(2-hydroxyphenoxy)-4-methoxybenzoic acid (X). Such selectivity is suggested by the results of Ziegler, Weber and Gellert,[7] who observed the increased ease of cleavage of aromatic ethers, substituted *p*- with methoxyl (*o*–*p*-directing) groups.[8] It is further to be noted that the contributing canonical structures of VI suggest a lowered basicity for the 4 oxygen in VI, thereby inhibiting acid-catalyzed cleavage of the ether.

When demethylation was carried out with acetic acid saturated with anhydrous hydrogen bromide at 100° (steam-bath), there was isolated an acid, $C_{14}H_{12}O_5$, containing one methoxyl group. That the acid has the structure desired and expected (X) is supported by the fact that 2-phenoxy-4-methoxybenzoic acid is recovered unchanged under similar conditions and also by the further reactions of X.

Attempted cleavage of VI with acetyl iodide or with acetyl bromide[9] led to 3,5-dimethoxyxanthone.

Conversion of X to the lactone (II) was accomplished by the use of thionyl chloride in pyridine.[2] When II was treated with methanolic sodium

(1) From the thesis submitted by John W. Weldon to the Graduate Faculty of the University of California, 1951.

(2) D. S. Noyce and J. W. Weldon, THIS JOURNAL, **74**, 401 (1952).

(3) M. Tomita, Y. Inubuse and F. Kusuda, *J. Pharm. Soc. Japan*, **64**, 173 (1944); *C. A.*, **45**, 6173 (1951).

(4) A. Stoll, J. Renz and A. Brack, *Experientia*, **3**, 111 (1947); V. C. Barry, *Nature*, **158**, 131 (1946); S. Shibata, Y. Miura, H. Sugimura and Y. Toyoizumi, *J. Pharm. Soc. Japan*, **68**, 309 (1948).

(5) F. v. Hemmelmayr, *Sitz. Akad. Wiss. Wien*, **121**, 1359 (1912).

(6) R. R. Brown, D. Ll. Hammick and A. J. B. Scholefield, *J. Chem. Soc.*, 778 (1950).

(7) K. Ziegler, H. Weber and H. G. Gellert, *Ber.*, **75**, 1715 (1942).

(8) Compare G. B. Kolhatkar and V. V. Bapat, *J. Univ. Bombay*, **7**, 157 (1938); *C. A.*, **33**, 5268 (1939).

(9) P. G. Stevens, THIS JOURNAL, **62**, 1801 (1940).