The Metabolism of Galactarate, D-Glucarate and Various Pentoses by Species of *Pseudomonas*

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1. When NAD^+ was present, cell extracts of *Pseudomonas* (A) grown with D-glucarate or galactarate converted 1 mol. of either substrate into 1 mol. each of 2-oxoglutarate and carbon dioxide; 70–80% of the gas originated from C-1 of the hexarate. 2. The enzyme system that liberated carbon dioxide from galactarate was inactive in air and was stabilized by galactarate or Fe^{2+} ions; the system that acted on D-glucarate was more stable and was stimulated by Mg^{2+} ions. 3. When NAD^+ was not added, 2-oxoglutarate semialdehyde accumulated from either substrate. This compound was isolated as its bis-2,4-dinitrophenylhydrazone, and several properties of the derivative were compared with those of the chemically synthesized material. Methods were developed for the determination of 2-oxoglutarate semialdehyde. 4. Synthetic 2-oxoglutarate semialdehyde was converted into 2-oxoglutarate by an enzyme that required NAD^+ ; the reaction rate with NADP^+ was about one-sixth of that with NAD^+ . 5. For extracts of *Pseudomonas* (A) grown with D-glucarate or galactarate, or for those of *Pseudomonas fragi* grown with L-arabinose or D-xylose, specific activities of 2-oxoglutarate semialdehyde-NAD oxidoreductase were much higher than for extracts of the organisms grown with (+)-tartrate and D-glucose respectively. 6. Extracts of *Pseudomonas fragi* grown with L-arabinose or D-xylose converted L-arabonate or D-xylonate into 2-oxoglutarate when NAD^+ was added to reaction mixtures and into 2-oxoglutarate semialdehyde when NAD^+ was omitted.

Galactaric acid (mucic acid) is a natural product that occurs in various fruits (Anet & Reynolds, 1954; Whiting & Coggins, 1960) and, like D-glucaric acid (D-glucosaccharic acid), serves as growth substrate for many organisms of the genera *Aerobacter* and *Escherichia* (Kay, 1926; Clapper & Poe, 1947). Blumenthal & Fish (1963) demonstrated that 1 mol. of D-glucarate is converted by *Escherichia coli* into 1 mol. each of pyruvate and glycerate, and have purified the aldolase that converts 3-deoxy-2-oxoglutarate into pyruvate and tartronate semialdehyde (Fish & Blumenthal, 1964). In the present work we have shown that a *Pseudomonas* does not metabolize D-glucarate by these reactions; instead, one carbon atom is removed as carbon dioxide and the rest of the molecule is converted into 2-oxoglutarate. An intermediate in this reaction sequence, and also in the formation of 2-oxoglutarate from galactarate, L-arabonate and D-xylonate by enzymes of *Pseudomonas*, appears to be 2-oxoglutarate semialdehyde (4-formyl-2-oxobutyric acid), which Singh & Adams (1964) have shown to be formed in the bacterial degradation of hydroxyproline. A brief account of some of

this work has already appeared (Dagley & Trudgill, 1964).

MATERIALS AND METHODS

Maintenance and growth of organisms. *Pseudomonas* (A) was one of four species isolated from poultry-house deep-litter (Bachrach, 1957), and was used in studies of the bacterial metabolism of glycine (Dagley, Trudgill & Callely, 1961) and tartrate (Dagley & Trudgill, 1963). The growth medium (carbon source, 1 g. of galactaric acid or D-glucaric acid/l., neutralized with NaOH), the conditions of growth of *Pseudomonas* (A), preparation of cell extracts and manometric methods were essentially as described by Dagley & Trudgill (1963). *Pseudomonas fragi* was a gift from Dr Ralph Weimberg and is listed B.25 in the collection of bacteria of the Northern Regional Research Laboratory, Peoria, Ill., U.S.A. The organism was grown with forced aeration in a medium that contained (per l. of 0.05 M- KH_2PO_4 - K_2HPO_4 buffer, pH 6.8): carbon source, 2.5 g.; $(\text{NH}_4)_2\text{SO}_4$, 1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g. Cells were harvested after growth for 15 hr. and cell extracts were prepared as for *Pseudomonas* (A).

Chromatography. One-dimensional paper chromatography of free carboxylic acids was used chiefly to examine preparations of ^{14}C -labelled hexaric acids. For descending chromatography on Whatman no. 1 papers solvent A

[butan-1-ol-pyridine-water (6:4:3, by vol.) (Hockenhull, 1953)] was used, and for the ascending method solvent B [phenol-formic acid-water (500:13:167, w/v/v) (Kornberg, 1958)] and solvent C [ethanol-formic acid-water (327:67:106, by vol.) (Page, 1961)] were used. These solvent systems separated hexaric acids from other compounds but did not distinguish between glucarate and galactarate; R_f values for the last-named compounds, with those of (+)-tartaric acid and tartronic acid for comparison, were as follows: glucarate and galactarate, 0.13 in solvent A, 0.15 in solvent B, 0.54 in solvent C; (+)-tartrate, 0.23 in solvent A, 0.26 in solvent B, 0.66 in solvent C; tartronate, 0.26 in solvent A, 0.32 in solvent B, 0.74 in solvent C. Amino acids were identified by two-dimensional chromatography on Whatman no. 1 papers by the ascending method, first in solvent B and then in butan-1-ol-propionic acid-water (178:125:90, by vol.) (Calvin & Benson, 1949). Radioactive compounds were located on chromatograms by radioautography and, after elution, they were identified by chromatography with authentic unlabelled compounds. When solvent C was used, 0.05% (w/v) of bromophenol blue and 0.06% (w/v) of sodium formate were added so that spots due to non-volatile carboxylic acids became visible when the paper was dried (Page, 1961); otherwise, acids were detected by spraying with a solution of aniline (1%, v/v) and xylose (1%, w/v) in 95% (v/v) methanol (Wood, 1958). A spray of 0.05% (w/v) 2',7'-dichlorofluorescein in ethanol was also used to locate various compounds that quenched or enhanced fluorescence when viewed in ultraviolet light. Chromatography of 2,4-dinitrophenylhydrazones of oxo acids was carried out either on paper (Dagley & Trudgill, 1963) or on thin layers of silica, by using as solvent light petroleum (b.p. 60–72°)-ethyl formate-propionic acid (650:350:77, by vol.) (Ronkainen, 1962).

Radioactive materials. D-[1- 14 C]Galactose was from The Radiochemical Centre, Amersham, Bucks.; D-[2- 14 C]galactose was from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; D-[1- 14 C]glucose and D-[6- 14 C]glucose were from the Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.; [14 C]glutamate was kindly given by Dr E. Bailey.

Preparation of [14 C]hexaric acids. Labelled hexaric acids were prepared by oxidizing the corresponding sugars with nitric acid (Mehlretter, 1963). Thus [14 C]galactarate was prepared by adding 2 m-moles of [14 C]galactose (30 μ c) slowly to a mixture of 0.64 ml. of 16N-HNO₃, 0.5 mg. of NaNO₂ and 0.04 ml. of water over a period of 20 min. The reaction mixture was stirred with a thermometer and held at 55–60° during the addition, and at 60° for 40 min. afterwards. The solution was cooled to 0°, and the slightly soluble galactaric acid was recovered by centrifuging, washed with 10 ml. of ice-cold water and two 10 ml. volumes of ethanol, and finally dried under vacuum. The yield was 50–60% of chromatographically pure [14 C]galactarate. [14 C]Glucarate was prepared from [14 C]glucose in a similar manner but was harvested as potassium hydrogen glucarate by bringing the solution to pH 9 with 50% (w/v) KOH at the end of the reaction and then readjusting to pH 3.4 with 16N-HNO₃; after crystallizing at 0° the glucarate was washed with cold aq. 25% (v/v) ethanol and dried under vacuum. The yield was 40–50% of chromatographically pure potassium hydrogen D-[14 C]glucarate.

Radioactivity measurements. Material labelled with 14 C

was counted in a Packard Tri-Carb liquid-scintillation spectrometer. The 14 CO₂ evolved in Warburg flasks was absorbed by hyamine hydroxide [(*p*-di-isobutylresorxyethoxyethyl)dimethylbenzylammonium hydroxide]. In each centre well was placed a strip of Whatman no. 1 paper (4 cm. $\times$ 2 cm.) that carried 30 μ l. of hyamine hydroxide (Passman, Radin & Cooper, 1956); after the reaction, papers were transferred to counting vials containing 75 mg. of 2,5-diphenyloxazole and 4.5 mg. of 2,2'-*p*-phenylenebis-5-phenyloxazole dissolved in 15 ml. of toluene. A counting efficiency of 36% was given when absorbed 14 CO₂, released from standard Na₂ 14 CO₃, was taken through this procedure. D-[14 C]Glucarate and [14 C]galactarate were assayed by applying a small volume (0.01–0.10 ml.) to strips of Whatman no. 1 paper (4 cm. $\times$ 2 cm.), drying in a cold-air stream and counting as described above. By reference to a sodium [14 C]benzoate standard a counting efficiency of 51% was obtained.

Preparation of 2-oxoglutarate semialdehyde. This compound was prepared by hydrolysis of methyl tetrahydro-2,5-dimethoxy-2-furoate, which was obtained by reduction of methyl 2,5-dihydro-2,5-dimethoxy-2-furoate; the last-named compound was prepared from methyl 2-furoate. The two intermediates in this sequence of reactions were isolated, purified by distillation under reduced pressure and examined by gas-liquid chromatography; also, their nuclear-magnetic-resonance spectra were recorded on a Varian A-60 spectrometer in deuteriochloroform, with tetramethylsilane as an internal reference. The conversion of methyl 2,5-dihydro-2,5-dimethoxy-2-furoate into methyl tetrahydro-2,5-dimethoxy-2-furoate was accompanied by the disappearance of a band at 3.67–4.06 τ due to vinyl protons and by the appearance of a band at 7.84–8.15 τ due to complex resonance of methylene group protons. These spectra showed the presence of three methoxy groups in both compounds, and also indicated a mixture of *cis* (37%) and *trans* (63%) isomers in each case. These isomers, which have not been previously reported, were also shown by gas-liquid chromatography with a Hy-Fi model 600C (Wilkins Instrument and Research Inc., Walnut Creek, Calif., U.S.A.). The instrument housed a column of stainless steel (5 ft. $\times$ 0.125 in.), packed with 5% silicone gum rubber (SE-30) on Chromosorb W sieved to 60–80 mesh (Johns-Manville, Celite Division, New York, N.Y., U.S.A.) and maintained at 126°; N₂ was used as carrier gas at a flow rate of 30 ml./min. Retention times were: methyl 2-furoate, 1.0 min.; methyl 2,5-dihydro-2,5-dimethoxy-2-furoate, 2.7 and 3.0 min.; methyl tetrahydro-2,5-dimethoxy-2-furoate, 2.7 and 2.3 min. Methyl 2,5-dihydro-2,5-dimethoxy-2-furoate was prepared from methyl 2-furoate by electrolysis in methanol (Clauson-Kaas & Limborg, 1952) and had b.p. 128–130°/25 mm. Hg. Our attempts to reduce this compound with Raney nickel and H₂ at 100 atm. (Clauson-Kaas & Limborg, 1952) were not successful, and we are indebted to Dr C. J. Sih for showing that it is readily reduced by Pd on carbon under H₂. Methyl tetrahydro-2,5-dimethoxy-2-furoate was prepared accordingly in batches of 10 g. by reducing methyl 2,5-dihydro-2,5-dimethoxy-2-furoate in a hydrogenator (Brown & Brown, 1962) with 5% Pd on carbon in H₂, generated at atmospheric pressure by the addition of NaBH₄ in ethanol to acetic acid. The consumption of H₂ was nearly theoretical (96%), and after distillation the product had b.p. 76–79°/2.3 mm. Hg. Small portions of methyl tetrahydro-2,5-dimethoxy-2-furoate

were hydrolysed to give 2-oxoglutarate semialdehyde (Murakami, Senoh & Hata, 1956) as required. The solution after hydrolysis was cooled, neutralized with 5N-NaOH, shaken with 4 vol. of diethyl ether to remove methanol and unchanged furoate, and then evaporated to a small volume under vacuum at 30°. A pale-yellow solid was recovered, dissolved in a suitable volume of water and stored at -15° until required. The yield was 65-70% of the methyl tetrahydro-2,5-dimethoxy-2-furoate taken. When thin-layer chromatograms of the solution were sprayed with ethanolic 2',7'-dichlorofluorescein, only one spot was detected under ultraviolet light. Attempts to crystallize 2-oxoglutarate semialdehyde either as the free acid or as the sodium or potassium salt were not successful.

Preparation of pentonic acids. D-Arabonic acid, L-arabonic acid and D-lyxonic acid were prepared from the pentoses by the method of Moore & Link (1940). Potassium D-ribonate and ammonium D-xylonate were kindly given by Dr Ralph Weimberg.

Other materials. Galactaric acid, D-glucaric acid and pentoses were from the California Corp. for Biochemical Research. Potassium hydrogen D-glucarate was from British Drug Houses Ltd., Poole, Dorset. Methyl 2-furoate was from Eastman Organic Chemicals, Rochester 3, N.Y., U.S.A. Thiamine pyrophosphate was from L. Light and Co. Ltd., Colnbrook, Bucks. NAD and NADP were from Sigma Chemical Co., St Louis, Mo., U.S.A.

Chemical analyses. Three methods were used to determine 2-oxoglutarate semialdehyde. The first was based on its conversion into a bis-2,4-dinitrophenylhydrazone that is taken down when centrifuged with kaolin, whereas the 2,4-dinitrophenylhydrazone of 2-oxoglutarate, when present at the concentrations encountered in this work, remains in the supernatant solution and may be determined by the method of Friedemann & Haugen (1943) when the kaolin has been removed. To 3 ml. of a solution containing 0.05 μ mole of 2-oxoglutarate semialdehyde was added 1 ml. of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2N-HCl, and after incubation for 1 hr. at 30° the precipitated bis-2,4-dinitrophenylhydrazone was centrifuged with 0.2 g. of kaolin, washed once with 3 ml. of 2N-HCl and twice with water, and drained. The precipitate was extracted twice with 5 ml. of 0.3% sodium ethylate solution at room temperature, the extracts were made up to 25 ml. with sodium ethylate solution, and the extinctions were read at 430 m μ in a spectrophotometer and referred to a curve prepared from standard solutions of 2-oxoglutarate semialdehyde similarly treated. This procedure suffers from the limitation that the colour developed begins to fade within 5-10 min. However,

the method proved valuable in those experiments that led to the discovery of 2-oxoglutarate semialdehyde-NAD oxidoreductase; later, this enzyme was used to determine 2-oxoglutarate semialdehyde by measurement of the increase in E_{340} due to reduction of NAD⁺ added in excess of the substrate. Synthetic 2-oxoglutarate semialdehyde was assayed by oxidizing with H₂O₂ to give an equimolar quantity of CO₂; the other product of the reaction is succinate semialdehyde (Singh & Adams, 1964). This assay cannot be used for enzymic reaction mixtures that may contain 2-oxoglutarate; its application to a solution that contained, per ml., sodium 2-oxoglutarate semialdehyde from hydrolysis of 10 μ moles of tetrahydro-2,5-dimethoxy-2-furoate was as follows. Warburg flasks at 30° contained 0.1-0.5 ml. of the solution, made up to a total volume of 3 ml. with sodium acetate buffer, pH 4.0 (150 μ moles), from which CO₂ was released by the addition of 0.1 ml. of aq. 3% (v/v) H₂O₂ from the side arms. The relationship between the amounts of solution and the CO₂ evolved was linear and indicated a conversion of 67% of the tetrahydro-2,5-dimethoxy-2-furoate into sodium 2-oxoglutarate semialdehyde. An assay with an extract of galactarate-grown *Pseudomonas* (A) gave a 61% conversion for the same sample. This assay was conducted at 27° in cuvettes that contained 2 μ moles of NAD⁺, 0.1 ml. of the solution of 2-oxoglutarate semialdehyde and 5.6 mg. of extract protein in 3 ml. of potassium phosphate buffer, pH 7.0 (39 μ moles); the amount of NADH formed was calculated from a value of 6.22×10^3 cm.² for the molar extinction coefficient at 340 m μ , and a small correction was applied for oxidation of NADH by the cell extract in the absence of substrate.

Infrared-absorption spectra. Infrared absorption was measured in compressed KBr disks on a Perkin-Elmer model 321 double-beam spectrophotometer.

Melting points. These were not corrected for the emergent stem of the thermometer.

RESULTS

Oxidation of substrates by whole cells of *Pseudomonas* (A). Galactarate and D-glucarate were both oxidized by suspensions of *Pseudomonas* (A), grown with either of these substrates as source of carbon, if cells were harvested within 3 hr. of the onset of the stationary phase. D-Arabonate, L-arabonate, D-xylonate and D-lyxonate were not oxidized at significant rates (Table 1). Although no carbon dioxide was evolved when these cells were

Table 1. *Rates of oxidation of various compounds by whole cells of Pseudomonas (A)*

The main compartments of Warburg flasks contained, in 3.0 ml., 40 μ moles of potassium phosphate buffer, pH 7.0, and washed cells (4 mg. dry wt.); centre wells contained 0.2 ml. of 20% (w/v) KOH. The gas phase was air. Each substrate (10 μ moles) was added from the side arms. Q_{O_2} values are expressed as μ l. of O₂ consumed/mg. dry wt. of cells/hr.; uptake of O₂ in the absence of substrate (Q_{O_2} 6) was subtracted.

Substrate	Q_{O_2} for substrate oxidized						
	Galactarate	D-Glucarate	DL-Glycerate	D-Arabonate	L-Arabonate	D-Xylonate	D-Lyxonate
Carbon source							
Galactarate	119	119	22	0	0	0	0
D-Glucarate	153	152	—	10	7	8	5

Table 2. Evolution of carbon dioxide from galactarate by *Pseudomonas* (A)

The main compartments of Warburg flasks contained, in 3.0 ml., 40 μ moles of potassium phosphate buffer, pH 7.0, and washed cells (12 mg. dry wt.) grown with galactarate. Centre wells contained 0.2 ml. of 20% (w/v) KOH, or 0.2 ml. of 5N-H₂SO₄, when uptake of O₂ or evolution of CO₂ was measured respectively. After equilibration at 30° reactions were started by the addition of 10 μ moles of disodium galactarate from the side arms. Gas exchange measured in the absence of substrate (*Q*₀, 4) was subtracted in each case.

Reaction time (min.)	Uptake of O ₂ (μ moles)	Evolution of CO ₂ (μ moles)	CO ₂ output / O ₂ uptake ratio
3	0.58	3.04	5.2
5	1.38	5.32	3.9
10	4.20	11.78	2.8
20	13.10	25.60	2.0
30	19.81	37.10	1.9
45	21.95	41.30	1.9

incubated with either galactarate or D-glucarate under nitrogen, a decarboxylation reaction during the early stages of aerobic metabolism was suggested by the observation that the ratio of carbon dioxide evolved to oxygen consumed was 2.7 times greater at 3 min. than at 30 min. during oxidation of galactarate (Table 2). When *Pseudomonas* (A) was grown with succinate, neither galactarate nor D-glucarate was oxidized.

Metabolism of galactarate and D-glucarate by cell extracts. Extracts of cells grown with D-glucarate or galactarate liberated 1.0 μ mole of carbon dioxide from 1.0 μ mole of either substrate when incubated until evolution of gas ceased (Fig. 3). The enzyme systems that attacked the two substrates differed in stability. In several experiments freshly prepared extracts showed specific activities of 1.5–2.0 μ moles of carbon dioxide/hr./mg. of protein towards galactarate and 5.0–8.0 μ moles of carbon dioxide/hr./mg. of protein towards D-glucarate. Ability to metabolize D-glucarate was maintained for several days, the extract being frozen and thawed between experiments; but that for galactarate was abolished by this treatment unless the cells were grown with galactarate as source of carbon. When an extract of D-glucarate-grown cells was held at 0° for 7 hr. its specific activity towards galactarate decreased more than tenfold; however, this loss did not occur if the cells were broken in a buffer containing either galactarate or Fe²⁺ ions (Table 3). When ability to metabolize galactarate was lost by allowing cell extracts to stand, additions of Fe²⁺ ions to the reaction mixture did not restore activity; but, when lost by passage

Table 3. Effects of ferrous sulphate and galactarate on enzymic activities of an extract of *Pseudomonas* (A) when stored at 0°

Pseudomonas (A) was grown with D-glucarate, and the washed suspension in potassium phosphate buffer, pH 7.0, was divided into four equal parts for ultrasonic disruption. Immediately before disruption the following additions were made to suspensions: (a) none; (b) 1 μ mole of disodium galactarate/ml.; (c) 1 μ mole of FeSO₄/ml.; (d) 1 μ mole of FeSO₄ plus 1 μ mole of galactarate/ml. After centrifuging, each cell-free extract contained 33–38 mg. of protein/ml. Main compartments of Warburg flasks contained, in 3.0 ml., 40 μ moles of potassium phosphate buffer, pH 7.0, and cell extract that had been stored at 0° for the times stated. Manometers were gassed with N₂ and equilibrated at 30°; reactions were started by addition of 5 μ moles of disodium galactarate or potassium sodium D-glucarate from the side arms and were stopped after 3 min. by tipping 50% (w/v) trichloroacetic acid from the centre well. Specific activities are expressed as μ moles of CO₂/hr./mg. of extract protein.

Specific activities of extract			
Substrate ...		Galactarate	D-Glucarate
Cell extract			
(a) Stored at 0° for 1 hr.		1.5	6.9
(b) {	Stored at 0° for 7 hr.	0.1	7.3
(c) {		1.6	—
(d) {		2.8	—
		2.6	8.4

through Sephadex G-50, activity could be restored if Fe²⁺ ions were added immediately after elution of the extract from the column. Little or no activity was restored by the addition of other metal ions and cofactors (Table 4.) When the nitrogen atmosphere over a reaction mixture was replaced by air, galactarate but not D-glucarate metabolism was inhibited: the specific activity of an extract towards galactarate was decreased from 1.8 to 0.2 μ mole of carbon dioxide/hr./mg. of protein by this change, whereas the respective values for D-glucarate were 5.2 and 4.9 μ moles of carbon dioxide/hr./mg. of protein.

Extracts of cells grown either with galactarate or with D-glucarate lost activity towards galactarate irreversibly when purification procedures, other than treatment with Sephadex G-50, were attempted. By contrast, D-glucarate was metabolized by cell extracts after they had been extensively dialysed: stimulation of activity by Mg²⁺ ions, but not by Fe²⁺ ions, could then be shown, and thiamine pyrophosphate was not effective (Table 5). Experiments with D-[1-¹⁴C]glucarate and D-[6-¹⁴C]glucarate showed that a cell extract catalysed the release of carbon dioxide from C-1 at about five times the rate of that from C-6. About 75% of the carbon dioxide from galactarate also originated

Table 4. *Effects of additions of metal ions and cofactors on the enzyme-catalysed evolution of carbon dioxide from galactarate*

An extract (3 ml.) of galactarate-grown *Pseudomonas* (A) containing 133 mg. of protein was passed through a Sephadex G-50 column by eluting with potassium phosphate buffer, pH 7.0 (2 g./l.), and 86 mg. of protein was recovered in 4.5 ml. For measurement of evolution of CO₂, Warburg cups contained, in 3.0 ml., 30 μ moles of potassium phosphate buffer, pH 7.0, and 8.5 mg. of cell-extract protein; metal ions were added as sulphate, except for Ca²⁺, which was added as nitrate. After gassing with N₂ and equilibrating at 30°, reactions were started by addition of 10 μ moles of disodium galactarate and were stopped by tipping 0.2 ml. of 5N-H₂SO₄ from the centre wells after 25 min. Before treatment with Sephadex, and on the addition of 5 μ moles of FeSO₄ after this treatment, the specific activities of the cell extract were 1.9 and 2.0 μ moles of CO₂/hr./mg. of protein respectively.

Cofactor added ...	None	Fe ²⁺ *	Fe ²⁺	Mg ²⁺	Mn ²⁺	Ca ²⁺	Zn ²⁺	Fe ³⁺	Co ²⁺	NADH	Cysteine	GSH
Cofactor (μ moles)	—	5	5	5	5	10	10	10	10	5	5	5
CO ₂ evolved (μ moles)	0.44	0.40	7.15	0.82	0.49	0.81	0.49	0.76	1.08	1.25	1.48	1.03

* Substrate absent.

Table 5. *Effects of additions of Mg²⁺ ions and thiamine pyrophosphate on the enzyme-catalysed evolution of carbon dioxide from D-glucarate*

An extract of *Pseudomonas* (A) grown with galactarate was dialysed with stirring for 20 hr. at 2° against a 0.3% (w/v) solution of EDTA in potassium phosphate buffer, pH 7.0 (2 g./l.), followed by dialysis for 24 hr. against a 0.9% (w/v) solution of KCl in the same buffer. For measurement of evolution of CO₂, Warburg flasks contained, in 3.0 ml., 30 μ moles of potassium phosphate buffer, pH 7.0, dialysed cell extract (7.6 mg. of protein) and addition of Mg²⁺ ions or thiamine pyrophosphate. Reactions were started by addition of 10 μ moles of substrate and stopped after 15 min. by tipping 0.2 ml. of 5N-H₂SO₄ from the centre wells.

MgSO ₄ added (μ moles)	Thiamine pyrophosphate added (μ moles)	D-Glucarate added (μ moles)	CO ₂ evolved (μ moles)
5	5	0	1.0
0	0	10	0.8
0	5	10	1.5
5	0	10	7.3
5	5	10	7.8

from C-1, whereas [2-¹⁴C]galactarate gave rise to little or no radioactive carbon dioxide (Table 6).

Conversion of galactarate and D-glucarate into 2-oxoglutarate semialdehyde by cell extracts. An orange-yellow precipitate was formed on incubation at 30° for 30 sec. when 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid was added to deproteinized solutions after carbon dioxide had been released from galactarate or D-glucarate by the action of cell extracts. A solution of this precipitate in 0.3% (w/v) sodium ethoxide gave a spectrum similar to that of the synthetic bis-2,4-dinitrophenylhydrazone of 2-oxoglutarate semialdehyde (Fig. 1), which had $\lambda_{\max}$ 433 and 517 m μ (ϵ 20400 and 39400 respectively). Both

the synthetic and enzymically formed 2-oxoglutarate semialdehyde reacted as aldehydes in solution, giving a strong 'silver-mirror' reaction with ammoniacal silver nitrate and an orange colour with o-dianisidine; equivalent concentrations of acetone gave only a faint pink colour with this reagent.

A sample of bis-2,4-dinitrophenylhydrazone was prepared from enzymically formed 2-oxoglutarate semialdehyde as follows. A large Warburg flask that contained, in a reaction volume of 60 ml., 3.75 m-moles of potassium phosphate buffer, pH 7.0, and cell extract (960 mg. of protein) was equilibrated at 30° and the reaction was started by addition of 5 m-moles of potassium sodium D-glucarate. After 75 min. the reaction was stopped by the addition of 5 ml. of 50% (w/v) trichloroacetic acid, the precipitate was removed by centrifuging at 0° and the clear solution was incubated at 30° for 60 min. with 250 ml. of 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid, when a copious orange-yellow precipitate was formed. By centrifuging successively at -5°, this precipitate was washed with 2N-hydrochloric acid, water and then methanol, and dried in air. The yield was 1.4 g. It was recrystallized twice from ethyl acetate, washed with anhydrous ether and dried at 100° under vacuum for 2 hr. (Found: C, 41.1; H, 3.2; N, 21.8. C₁₇H₁₄O₁₀N₈ requires C, 41.6; H, 2.9; N, 22.8%). A 2,4-dinitrophenylhydrazine derivative was similarly prepared from a sample of synthetic 2-oxoglutarate semialdehyde (Found: C, 41.6; H, 4.1; N, 21.8%). Both samples were dried further for 22 hr. at 50° in vacuum over phosphorus pentoxide, when the derivative of the product of enzymic reaction had m.p. 228–229° (decomp.) (Found: C, 40.6; H, 3.2; N, 22.1%) and that of the synthetic material had m.p. 219–220° (decomp.) (Found: C, 40.7; H, 3.2; N, 21.9%); the mixed m.p. was 223–226° (decomp.). For the synthetic derivative, Murakami *et al.* (1956) gave

Table 6. Release of carbon dioxide from radioactive galactarate and D-glucarate by extracts of *Pseudomonas (A)* grown with galactarate

The main compartments of Warburg flasks with two side arms contained, in 3.0 ml., 40 μ moles of potassium phosphate buffer, pH 7.0, and cell extract; centre wells contained 30 μ moles of hyamine hydroxide for absorption of radioactive CO₂. Flasks were gassed with N₂ and equilibrated at 30°. Reactions were started by the addition of substrate from one side arm and were stopped by the addition of 0.2 ml. of 5 N-H₂SO₄ from the other when the gas exchange in flasks containing no hyamine hydroxide showed that evolution of CO₂ had ceased. For reactions with D-glucarate, 14.3 mg. of extract protein was used; with galactarate, 28.6 mg. of extract protein.

Substrate			CO ₂	
	Amount (μ moles)	Radioactivity (disintegrations/min.)	Radioactivity (disintegrations/min.)	Percentage of total radioactivity
D-[1- ¹⁴ C]Glucarate	10	160 000	124 600	78
	20	320 000	264 700	83
D-[6- ¹⁴ C]Glucarate	10	189 600	26 540	14
	20	379 200	50 300	13
[1- ¹⁴ C]Galactarate	5	190 900	138 200	72
	10	381 800	295 300	77
[2- ¹⁴ C]Galactarate	5	109 169	39	—

Fig. 1. Absorption spectra of bis-2,4-dinitrophenylhydrazones in 0.3% (w/v) sodium ethoxide: —, derivative of synthetic sodium 2-oxoglutarate semialdehyde; ····, derivative of compound obtained by enzymic action on D-glucarate; ----, derivative of compound obtained by enzymic action on L-arabonate.

Fig. 2. Infrared-absorption spectra of the bis-2,4-dinitrophenylhydrazones of: A, compound obtained enzymically from D-glucarate; B, synthetic sodium 2-oxoglutarate semialdehyde; C, compound obtained enzymically from D-xylonate.

m.p. 205–206°; Singh & Adams (1964) gave m.p. 231–233° for the synthetic derivative and 227–229° for that prepared after enzymic degradation of L-hydroxyproline. The infrared-absorption spectra of the 2,4-dinitrophenylhydrazine derivatives of our synthetic and enzymically prepared material, examined before being finally dried over phosphorus pentoxide, showed that all absorption peaks were common to both substances (Fig. 2). They have one exceptional feature that has been pointed out by Murakami *et al.* (1956), namely that the band

at 5.9 μ due to carboxyl CO is relatively weak; the infrared-absorption spectrum of the 2,4-dinitrophenylhydrazine of 2-oxoglutaric acid shows a much stronger band at this wavelength. Chemically, the bis-2,4-dinitrophenylhydrazine of 2-oxoglutarate semialdehyde showed little evidence of acidic properties; like the derivative of tartronate semialdehyde (Dagley *et al.* 1961), it was not appreciably extracted from ethyl acetate into 10%

(w/v) sodium carbonate. However, the deposition of fine crystals in the aqueous phase at the interface between the two solutions showed that the compound had some tendency to enter the sodium carbonate solution; this was not observed when 10% (w/v) sodium chloride replaced the sodium carbonate.

Enzymic conversion of D-glucarate and 2-oxoglutarate semialdehyde into 2-oxoglutarate. Two series of Warburg flasks contained, in 3.0 ml., 40 μ moles of potassium phosphate buffer, pH 7.0, and cell extract (11 mg. of protein) of D-glucarate-grown *Pseudomonas* (A); in one series only, 10 μ moles of NAD⁺ were also added to each flask. After gassing with nitrogen and equilibrating at 30°, reactions were started by the addition of 10 μ moles of potassium sodium D-glucarate from each side arm and were stopped at intervals by tipping 0.2 ml.

Fig. 3. Compounds formed by the enzymic decomposition of 10 μ moles of D-glucarate by a cell extract of *Pseudomonas* (A). (a) No NAD⁺ added: ∇ , CO₂; Δ , 2-oxoglutarate semialdehyde; $\circ$, 2-oxoglutarate. (b) With 10 μ moles of NAD⁺ present: $\blacktriangle$, 2-oxoglutarate semialdehyde; $\bullet$, 2-oxoglutarate. Evolution of CO₂ was essentially unchanged by the addition of NAD⁺.

of 50% (w/v) trichloroacetic acid from centre wells to release carbon dioxide, which was measured. Precipitates were removed by centrifuging and the clear solutions were assayed for 2-oxoglutarate (Friedemann & Haugen, 1943) and for 2-oxoglutarate semialdehyde (by measuring E_{430} for the bis-2,4-dinitrophenylhydrazine dissolved in 0.3% sodium ethylate). After 20 min. there were evolved 9.5 μ moles of carbon dioxide/10 μ moles of D-glucarate, and at this time the concentration of 2-oxoglutarate semialdehyde reached a maximum in flasks that contained no added NAD⁺ (Fig. 3). In flasks that contained NAD⁺, 2-oxoglutarate semialdehyde was effectively absent and instead, over the first 30 min., 2-oxoglutarate was formed in amounts equimolar to the carbon dioxide evolved; a maximum yield of 8.6 μ moles of 2-oxoglutarate was given after 60 min. Since the concentrations of 2-oxoglutarate in these flasks decreased between 60 and 90 min., and 2-oxoglutarate semialdehyde was also slowly metabolized in those containing no NAD⁺, it was not possible in this particular experiment to study the stoichiometry of the conversion either of D-glucarate into 2-oxoglutarate semialdehyde or of 2-oxoglutarate semialdehyde into 2-oxoglutarate. However, suitable conditions for studying the oxidation of 2-oxoglutarate semialdehyde were arranged as follows. Two 100 ml. Erlenmeyer flasks each contained, in 10 ml., 150 μ moles of potassium phosphate buffer, pH 7.0, and an extract (5.7 mg. of protein) of *Pseudomonas* (A) grown with galactarate; to one flask 30 μ moles of NAD⁺ were also added. After equilibration at 30°, reactions were started by the addition of 17.5 μ moles of synthetic sodium 2-oxoglutarate semialdehyde to each flask. At intervals, 1.0 ml. samples were taken, 0.2 ml. of 50% (w/v) trichloroacetic acid was added to each one immediately and the precipitates were removed by centrifuging. The solutions were then assayed for 2-oxoglutarate and residual 2-oxoglutarate semialdehyde as described above. At 20 min., 17.1 μ moles of 2-oxoglutarate semialdehyde had disappeared from the reaction mixture containing NAD⁺ and the amount of 2-oxoglutarate determined was 17.9 μ moles. There was little change in the concentration of either compound in the flask that contained no NAD⁺ (Fig. 4). During the first 5 min. there was a linear rate of formation of 3.0 μ moles of 2-oxoglutarate/min.; when 30 μ moles of NADP⁺ were added instead of NAD⁺, the rate of formation was 0.5 μ mole of 2-oxoglutarate/min.

The identity of 2-oxoglutarate formed enzymically from 2-oxoglutarate semialdehyde was established by isolation of its 2,4-dinitrophenylhydrazone. A reaction mixture contained, in 10 ml., 150 μ moles of potassium phosphate buffer, pH 7.0, an extract of galactarate-grown *Pseudomonas* (A) and

Fig. 4. Conversion of synthetic sodium 2-oxoglutarate semialdehyde into 2-oxoglutarate by an extract of *Pseudomonas* (A) grown with galactarate. (a) No NAD^+ added: Δ , 2-oxoglutarate semialdehyde; $\circ$, 2-oxoglutarate. (b) With $30\mu\text{moles}$ of NAD^+ present: $\blacktriangle$, 2-oxoglutarate semialdehyde; $\bullet$, 2-oxoglutarate.

$140\mu\text{moles}$ of NAD^+ . After equilibration at 30° the reaction was started by the addition of $125\mu\text{moles}$ of synthetic sodium 2-oxoglutarate semialdehyde and stopped after 20 min. by the addition of 0.5ml. of 50% (w/v) trichloroacetic acid. The precipitate was removed by centrifuging, and, after passage through a 22μ Millipore filter, the filtrate was incubated at 30° for 30 min. with a solution of 30 mg. of 2,4-dinitrophenylhydrazine dissolved in 5 ml. of ethanol containing 0.1 ml. of concentrated sulphuric acid. The precipitate was extracted into ethyl acetate, re-extracted into 10% (w/v) sodium carbonate, and, after acidification of this solution, re-extracted into ethyl acetate. The solution was evaporated almost to dryness under vacuum at 30° and washed with cold ether to remove trichloroacetic acid; the 2,4-dinitrophenylhydrazine was then crystallized from ethyl acetate. When the crystals were compared with authentic 2-oxoglutarate 2,4-dinitrophenylhydrazine the two specimens showed: (1) identical R_f values on paper and thin-layer chromatograms; (2) identical spectra when dissolved in 10% (w/v) sodium carbonate-1.5N-sodium hydroxide (1:1, v/v); (3) infrared-absorption spectra with all absorption peaks common to both compounds after they had been dried at 100° for 2 hr. The identity of the derivative of 2-oxoglutarate formed by enzymic reaction was confirmed by its reduction to glutamic acid. About

Table 7. Relationship between concentration and activity for 2-oxoglutarate semialdehyde-NAD oxidoreductase present in a cell extract

Cuvettes contained, in 3.0 ml., $40\mu\text{moles}$ of potassium phosphate buffer, pH 7.0, $6\mu\text{moles}$ of NAD^+ and an extract (0.1–1.7 mg. of protein) of galactarate-grown *Pseudomonas* (A). Reactions at 27° were started by the addition of $7\mu\text{moles}$ of synthetic sodium 2-oxoglutarate semialdehyde and the increases in extinction at $340\text{m}\mu$ (ΔE_{340}) were recorded on a Cary spectrophotometer. Reaction rates were corrected for a decrease in extinction, due to oxidation of NADH, that occurred in the absence of substrate ($\Delta E_{340} = 0.044/\text{min.}/\text{mg.}$ of cell-extract protein; $0.3\mu\text{mole}$ of NADH/3 ml.).

Cell-extract protein (mg.)	Reaction rate ($\Delta E_{340}/\text{min.}$)	Ratio ($\Delta E_{340}/\text{min.}/\text{mg.}$)
0	0	—
0.113	0.097	0.860
0.340	0.29	0.853
0.567	0.50	0.888
1.134	1.02	0.900
1.701	1.60	0.941

$5\mu\text{moles}$ of the 2,4-dinitrophenylhydrazine were dissolved in 0.5 ml. of ethanol and diluted to 3 ml. with 2N-hydrochloric acid. The solution was placed in the water-cooled cell of an apparatus for electrolytic desalting (Stevens, Smith & Jepson, 1954; Smith, 1958), and a current of 1.0 A was passed until disappearance of the yellow colour showed that reduction of the 2,4-dinitrophenylhydrazine was complete. The solution was then carefully evaporated to about 0.1 ml. and the reduction product was identified as glutamic acid by co-chromatography with [^{14}C]glutamate.

A linear relation between concentration and activity was established for 2-oxoglutarate semialdehyde-NAD oxidoreductase by measuring the rate of increase in E_{340} due to reduction of NAD^+ by 2-oxoglutarate semialdehyde (Table 7). The specific activity of 2-oxoglutarate semialdehyde-NAD oxidoreductase present in extracts of *Pseudomonas* (A) grown with galactarate was more than 11 times the value for cells grown with (+)-tartrate (Table 8).

Metabolism of pentoses by Pseudomonas fragi. Weimberg (1961) showed that extracts of *Pseudomonas fragi* grown with L-arabinose, D-arabinose and D-xylose converted 1 mol. of each of the corresponding pentonic acids into 1 mol. of 2-oxoglutarate. We confirmed these observations for the same organism grown with L-arabinose and D-xylose and we found, as did Weimberg (1961), that extracts of cells grown with D-ribonate failed to convert D-ribonate into 2-oxoglutarate; growth with D-arabinose was sparse and we did not study

Table 8. *Specific activities of 2-oxoglutarate semialdehyde-NAD oxidoreductase present in extracts of Pseudomonas (A) and Pseudomonas fragi grown with various sources of carbon*

Cuvettes contained, in 3.0 ml., 40 μ moles of potassium phosphate buffer, pH 7.0, cell extract (0.1–7.0 mg. of protein) and 4 μ moles of NAD⁺. Reactions at 27° were started by the addition of 2 μ moles of sodium 2-oxoglutarate semialdehyde; the increases in extinction at 340 m μ were recorded on a Cary spectrophotometer and the corresponding amounts of NADH formed were calculated. Corrections were applied for oxidation of NADH that occurred in the absence of substrate, as in Table 7.

Organism	Growth substrate	Specific activity (μ moles of NADH/hr./mg. of protein)
<i>Pseudomonas (A)</i>	Galactarate	24.4
	(+)-Tartarate	1.2
<i>Pseudomonas fragi</i>	L-Arabinose	18.3
	D-Xylose	9.6
	D-Ribose	9.4
	D-Glucose	1.4

its metabolism further. The specific activities of 2-oxoglutarate semialdehyde-NAD oxidoreductase present in extracts of *Pseudomonas fragi* grown with L-arabinose, D-xylose and D-ribose were considerably greater than the values for cells grown with D-glucose (Table 8). When NAD⁺ was present, extracts of cells grown with the respective pentoses converted L-arabonate and D-xylonate into 2-oxoglutarate, but if NAD⁺ was not added a compound accumulated that gave a precipitate with 2,4-dinitrophenylhydrazine within 30 sec.; when L-arabonate was the substrate this was identified as 2-oxoglutarate semialdehyde from the absorption spectrum of the derivative in 0.3% (w/v) sodium ethoxide (Fig. 1). For cells grown with D-xylose, 180 μ moles of potassium D-xylonate were incubated at 30° for 60 min. with 33 mg. of cell-extract protein and a bis-2,4-dinitrophenylhydrazine was isolated by the methods described above; the yield was 58 mg., m.p. 221–223° (decomp.). The infrared-absorption spectrum of this derivative is shown in Fig. 2, and its spectrum in 0.3% sodium ethoxide was identical with that of the derivative isolated from the solution after treating L-arabonate with an extract of *Pseudomonas fragi* grown with L-arabinose.

DISCUSSION

Blumenthal & Fish (1963) showed that the first reaction in the metabolism of D-glucarate or galactarate by *E. coli* is dehydration to give a mixture of 3-deoxy-2-oxoglutarate and 4-deoxy-5-oxoglutarate, the second compound being the major product (Fish & Blumenthal, 1961). In *E. coli* this is

followed by reactions that do not occur in *Pseudomonas (A)* grown with D-glucarate or galactarate: in *E. coli* there is an aldolase fission to give pyruvate and tartronate semialdehyde, followed by reduction of the latter to glycerate (Fish & Blumenthal, 1964). Although tartronate semialdehyde reductase is strongly induced when *Pseudomonas (A)* metabolizes glycine (Dagley *et al.* 1961), it is effectively absent from cells grown with D-glucarate. Extracts of cells so grown converted the C₆ acids into a C₅ compound (2-oxoglutarate semialdehyde) and carbon dioxide under anaerobic conditions, an observation that indicates the occurrence of dehydrations of the type studied by Blumenthal & Fish (1963); two dehydrations are required for each substrate (Scheme 1). The first dehydration cannot be preceded by loss of carbon dioxide, since the products that would arise from decarboxylation of D-glucarate or galactarate, namely D-xylonate, D-lyxonate, D-arabonate and L-arabonate, were not attacked by cell extracts that convert the hexaric acids into 2-oxoglutarate semialdehyde. However, although the first reaction appears to be a dehydration, later reactions need not occur in the order suggested in Scheme 1; thus decarboxylation may precede the second dehydration, or the two dehydrations undergone by each of the substrates might, in principle, be catalysed simultaneously by the relevant enzyme. From the present work it is not possible to make a final choice between these alternatives; but the similarity between certain properties of our cell extracts and those of Blumenthal & Fish (1963) suggests that certain reactions are common to the metabolic pathways of *Pseudomonas (A)* and *E. coli*. Such common reactions, if they do exist, must be initial dehydrations, since in *E. coli* two C₃ compounds are formed by the next reaction in the sequence, whereas C₁ and C₅ compounds are given by *Pseudomonas (A)*. We found that the enzyme system for the metabolism of D-glucarate differed from that for galactarate in showing greater stability and in requiring Mg²⁺ ions for activity when dialysed; the system for galactarate was inactivated by the admission of air to the reaction, whereas that for D-glucarate was not; and the galactarate system was stabilized by Fe²⁺ ions, which had no effect on the D-glucarate system. Blumenthal & Campbell (1958) found that addition of Fe²⁺ ions to cell extracts stimulated the conversion of galactarate into pyruvate, whereas cell extracts that attacked D-glucarate showed a requirement for Mg²⁺ ions (Blumenthal, 1960). Accordingly in Scheme 1 the metabolism of D-glucarate is presented as differing from that of galactarate only by the existence of a different initial dehydratase, and for each hexaric acid the product is shown as 4-deoxy-5-oxoglutarate, which Fish & Blumenthal (1961) claim to be the

Scheme 1. Proposed reaction sequence for metabolism of D-glucarate and galactarate by *Pseudomonas* (A) and of pentoses by *Pseudomonas fragi*. The demonstration that 2-oxoglutarate is formed from certain pentoses and the elucidation of reactions by which the sugars are converted into the corresponding isomers of 4,5-dihydroxy-2-oxovalerate are the work of Weimberg & Doudoroff (1955) and Weimberg (1959, 1961).

main product of these dehydratases. Alternatively, removal of water from C-2 and C-3 of each substrate remains a possibility: this reaction would not give a metabolite common to both metabolic pathways, but the enzymes for the second dehydrations might exhibit low substrate specificities. However, since about three-quarters of the carbon dioxide arises from C-1 of each substrate (Table 6), one particular pathway is evidently preferred. The fact that a dehydratase can so distinguish between C-1 and C-6 of galactarate, which is a symmetrical and internally compensated molecule, can be interpreted on the three-point attachment concept of Ogston (1948).

Pseudomonas saccharophila converts L-arabinose into 2-oxoglutarate (Weimberg & Doudoroff, 1955); L-arabinolactone, L-arabonic acid and L-4,5-dihydroxy-2-oxovaleric acid (Weimberg, 1959) are reaction intermediates in this conversion. *Pseudomonas fragi* metabolizes D-ribose, D-xylose, D-arabinose and L-arabinose by similar reactions, and Weimberg (1961) obtained cell extracts that oxidized D-xylonate, D-arabonate and L-arabonate to 2-oxoglutarate. From this same organism grown with D-xylose and L-arabinose we prepared cell extracts that converted D-xylonate and L-arabonate respectively into 2-oxoglutarate semialdehyde in

the absence of NAD, and into 2-oxoglutarate when it was present. Further, when extracts of *Pseudomonas fragi* grown with D-xylose and L-arabinose were assayed for 2-oxoglutarate semialdehyde-NAD oxidoreductase their respective specific activities were about 13 and seven times as great as that measured for cells grown with D-glucose (Table 8). On this evidence, the metabolic pathways by which certain pentoses and hexaric acids are converted into 2-oxoglutarate are shown in Scheme 1 to merge at 2-oxoglutarate semialdehyde; this new metabolite is also a precursor of 2-oxoglutarate in the enzymic degradation of L-hydroxyproline by a *Pseudomonas* (Singh & Adams, 1964).

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REFERENCES

- Anet, E. F. J. & Reynolds, T. M. (1954). *Nature, Lond.*, **174**, 930.
- Bachrach, U. (1957). *J. gen. Microbiol.* **17**, 1.
- Blumenthal, H. J. (1960). *Bact. Proc.* p. 159.
- Blumenthal, H. J. & Campbell, A. (1958). *Bact. Proc.* p. 101.
- Blumenthal, H. J. & Fish, D. C. (1963). *Biochem. biophys. Res. Commun.* **11**, 239.

- Brown, C. A. & Brown, H. C. (1962). *J. Amer. chem. Soc.* **84**, 2829.
- Calvin, M. & Benson, A. A. (1949). *Science*, **109**, 140.
- Clapper, W. E. & Poe, C. F. (1947). *J. Bact.* **53**, 363.
- Clauson-Kaas, N. & Limborg, F. (1952). *Acta chem. scand.* **6**, 551.
- Dagley, S. & Trudgill, P. W. (1963). *Biochem. J.* **89**, 22.
- Dagley, S. & Trudgill, P. W. (1964). *Fed. Proc.* **23**, 225.
- Dagley, S., Trudgill, P. W. & Callely, A. G. (1961). *Biochem. J.* **81**, 623.
- Fish, D. C. & Blumenthal, H. J. (1961). *Bact. Proc.* p. 96.
- Fish, D. C. & Blumenthal, H. J. (1964). *Bact. Proc.* p. 112.
- Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Hockenhull, D. J. D. (1953). *Nature, Lond.*, **171**, 982.
- Kay, H. D. (1926). *Biochem. J.* **20**, 321.
- Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
- Mehlretter, C. L. (1963). In *Methods in Carbohydrate Chemistry*, vol. 2, p. 46. Ed. by Whistler, R. L. & Wolfrom, M. L. New York: Academic Press Inc.
- Moore, S. & Link, P. K. (1940). *J. biol. Chem.* **133**, 293.
- Murakami, M., Senoh, S. & Hata, Y. (1956). *Mem. Inst. sci. industr. Res. Osaka Univ.* **13**, 173.
- Ogston, A. G. (1948). *Nature, Lond.*, **162**, 963.
- Page, O. T. (1961). *Phytopathology*, **51**, 337.
- Passman, J. M., Radin, N. S. & Cooper, J. A. D. (1956). *Analyt. Chem.* **28**, 484.
- Ronkainen, P. (1962). *J. Chromat.* **11**, 228.
- Singh, R. M. M. & Adams, E. (1964). *Science*, **144**, 67.
- Smith, I. (1958). In *Chromatographic Techniques*, p. 37. Ed. by Smith, I. London: William Heinemann Ltd.
- Stevens, B. J., Smith, I. & Jepson, J. B. (1954). *Chem. & Ind.* p. 243.
- Weimberg, R. (1959). *J. biol. Chem.* **234**, 727.
- Weimberg, R. (1961). *J. biol. Chem.* **236**, 629.
- Weimberg, R. & Doudoroff, M. (1955). *J. biol. Chem.* **217**, 606.
- Whiting, G. C. & Coggins, R. A. (1960). *J. Sci. Fd Agric.* **11**, 337.
- Wood, T. (1958). In *Chromatographic Techniques*, p. 207. Ed. by Smith, I. London: William Heinemann Ltd.