Formation of Arabinose, Ribulose and Tartronic Acid from 2-Keto-D-gluconic Acid

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(Received 27 September 1954)

For some years evidence that 2-keto-D-gluconic acid is implicated in a number of biochemical systems, notably in *Acetobacter* species (Bernhauer & Görlich, 1935; Kulka & Walker, 1954) and in *Pseudomonas* species (Lockwood, Tabenkin & Ward, 1941; Norris & Campbell, 1949) has been accumulating. Studies by Warburg & Christian (1937) with red blood cells, by Lipmann (1936) with yeast macerate preparations and by Dickens (1938) with crude enzyme preparations from yeast, afforded substantial evidence for the existence of a pathway for the direct oxidation of glucose 6-phosphate by way of 6-phosphogluconic acid, 2-keto-6-phosphogluconic acid and pentose 5-phosphate. The work of Cohen & Scott (1951) with yeast preparations confirmed this scheme, since they detected the presence of 2-keto-6-phosphogluconate, ribose 5-phosphate and glyceraldehyde 3-phosphate in experiments on the oxidation of 6-phosphogluconate.

We have recently isolated 2-keto-D-gluconic acid and tartronic acid from a culture of *Acetobacter acetosum* on a glucose medium (Kulka, Hall & Walker, 1951). Tartronic acid had not been identified previously as a product of bacterial metabolism, although its presence in sugar-refinery liquor had been established by von Lippmann (1913). According to Rieben & Hastings (1946), tartronic acid is formed in various plants. In view of the isolation of this acid from bacterial cultures under conditions which might suggest that it was a decomposition product of 2-keto-D-gluconic acid, we have studied the decomposition of the latter under similar conditions. Our experiments have shown that tartronic acid was definitely formed and probably arabinose and ribulose.

EXPERIMENTAL

All melting points are uncorrected.

Formation of 2-keto-D-gluconic and tartronic acids in cultures of Acetobacter acetosum on a glucose medium

Acetobacter strain and cultural conditions. Strain N.C.I.B. 2224 was employed and the medium consisted of a solution of glucose (10%, w/v) in yeast-water with addi-

tion of CaCO_3 (precipitated chalk). The glucose was the B.P. ('Dextrosol') grade of Corn Products, Ltd. The yeast-water was prepared by boiling 300 g. of brewer's pressed yeast in 4 l. of water for 30 min., and filtering hot; the filtrate was clarified by repassing it through the extracted yeast which served as a filter pad. After addition of 400 g. of glucose the volume was made up to 4 l. with distilled water and the solution was distributed in equal portions among 10 Glaxo flasks of the type described by Clayton, Hunwicke, Hanes, Robinson & Andrews (1944). After sterilization (10 lb./sq.in. for 15 min.), 12 g. of sterile chalk were added aseptically to the contents of each flask and the pH value of the contents was then 7. After inoculation, the flasks were incubated at 30° for 4 weeks and then allowed to stand at room temperature for 5 months, when tests showed that viable bacteria were still present in the medium. Our strain of *A. acetosum*, when grown on glucose, first produces a high yield of gluconic acid. After 1 month at 30° the greater part of the latter is still present in the medium (Kulka & Walker, 1954), and it is not until 4-5 months have elapsed that the amount present is completely metabolized.

Isolation of tartronic acid. The contents of the flasks were combined and filtered. The clear filtrate was warmed to 40°, shaken with 40 g. of charcoal and, after 30 min., filtered through kaolin. The straw-coloured filtrate was concentrated under vacuum at 40° to a volume of 550 ml. and calcium salts were then precipitated with ethanol (96%, v/v). After 250 ml. of the latter had been added, the solid (*A*) which separated was removed by filtration and more ethanol (1500 ml.) was added to the filtrate, from which a syrup (*B*) separated. Fraction *A* (17 g.) was suspended in 400 ml. of boiling water and the solid remaining undissolved (13.4 g.) was treated with 20 ml. of HCl (20%, w/v) and the whole was shaken with charcoal and filtered. The filtrate was neutralized with dil. ammonia (1 vol. 0.88 ammonia/1 vol. water), and the calcium salt (or salts) which separated was filtered off, washed with water and dried over P_2O_5 in a vacuum (yield, 8.4 g.; found: Ca, 19.2%). A sample of this salt (3.8 g.) was warmed at 60° for 30 min. with an aqueous solution of 2.18 g. of oxalic acid dihydrate. Filtration of the suspension gave 2.92 g. of crude calcium oxalate monohydrate and a clear liquid which was concentrated at 40° under reduced pressure to a vol. of 2 ml. When kept *in vacuo* this concentrate crystallized in clusters of fine needles (2.22 g.). About 10 mg. were used for an ascending paper partition chromatogram which was run on Whatman no. 1 paper with the solvent system: *n*-butanol-formic acid-water (3:1:3, by vol.), according to the directions of Lugg & Overell (1948). On spraying with bromocresol green, one spot only was obtained and had an R_F value of 0.35, while authentic tartronic acid, which was run simultaneously on the same paper, gave a spot of R_F value 0.36. The bulk of the acidic product was recrystallized from absolute ether to

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constant m.p. (163° decomp.). The mixed m.p. with an authentic sample (m.p. 163°) of tartaric acid, prepared as described by Fenton (1898), was 163° . On titration, 53.0 mg. were equivalent to 8.64 ml. 0.102N-NaOH. Calc. for $C_4H_4O_6$: 8.68 ml. (Found: C, 30.1; H, 3.5. Calc.: C, 30.0; H, 3.4%.)

We have found (unpublished observation) that when a solution of diazotized *p*-nitraniline is added at 0° to an aqueous solution of authentic tartronic acid to which has been added an excess of sodium acetate, CO_2 is evolved and a reddish brown coupling product is precipitated. This behaviour serves to distinguish tartronic acid from the closely related mesoxalic acid, since the latter cannot couple with a diazonium acetate. When applied to the acid product of R_F value 0.35, this test was positive.

Isolation of 2-keto-D-gluconic acid. The syrup *B* was allowed to stand for several days under absolute ethanol at 0° when a calcium salt separated and was purified by redissolving in water and reprecipitating with 2 vol. of absolute ethanol. This salt (86 g.) had a strong reducing action on warm Fehling's solution and in several respects resembled the calcium salt of 2-keto-D-gluconic acid. Identity with the latter was established by preparation of a quinoxaline derivative (Ohle, 1934) by mixing a solution of 5 g. of the calcium salt in 20 ml. of water with 2.6 g. of *o*-phenylenediamine and 2 ml. of conc. HCl. The crystals (1.6 g.), which separated after 24 hr., were recrystallized from hot water to constant m.p. ($202\text{--}203^\circ$). The mixed m.p. with an authentic sample of 2-hydroxy-3-(*D*-arabotetrahydroxybutyl)quinoxaline was identical. (Found: C, 53.9; H, 5.4; N, 10.4. Calc. for $C_{12}H_{14}O_8N_2$: C, 54.1; H, 5.3; N, 10.5%.)

Formation of arabinose, ribulose and tartronic acid by the action of calcium hydroxide on 2-keto-D-gluconic acid

Following an unpublished observation by one of us (D.K.) that calcium salts are deposited when 2-keto-gluconate is heated with an aqueous solution of $Ca(OH)_2$ and that this deposit gives positive tests for tartronate, a solution of 2 g. of the calcium salt of 2-ketogluconic acid in 9 ml. of water was added to 180 ml. of a freshly prepared solution of

$Ca(OH)_2$ (0.11 g./100 ml.) and dispensed in 50 ml. quantities in Pyrex boiling tubes fitted with ground stoppers. The air above the liquid was displaced by N_2 and the tubes were then placed in a bath of boiling water for 20 min. A control tube containing an aqueous solution of the calcium salt of 2-ketogluconic acid but no $Ca(OH)_2$ was similarly treated; the tubes were then cooled and their contents were filtered. The control tube yielded 0.02 g. of precipitate but, from the other tubes, precipitates each amounting to approx. 0.11 g. were obtained. The latter consisted principally of $CaCO_3$ mixed with $Ca(OH)_2$ and with traces of organic matter. The filtrates from the deposits were deionized by passage first through Amberlite IR-120 (H) (Rohm & Haas, Co., Philadelphia, Pa., through British Drug Houses Ltd.) and then through De-Acidite E (Permutit Co. Ltd.). The solutions, in portions each of 10 ml., were then concentrated to very small volume at 40° *in vacuo* for examination by paper partition chromatography. Qualitative examination of these solutions revealed the presence of orcinol-reacting substances, presumably pentoses.

Chromatographic analysis of the products. Three solvent systems were employed on Whatman no. 1 paper and unknown spots produced by the deionized concentrate (*X*) were compared with spots made by authentic samples of *D*-arabinose, *D*-ribose and *L*-ribulose. The last-mentioned sugar was prepared from adonitol by the method of Reichstein (1934).

These chromatograms (Table 1) demonstrate the presence of arabinose and ribulose and the absence of ribose in the concentrates which were examined.

Isolation of tartronic acid. A solution of 4 g. of calcium 2-ketogluconate in 18 ml. of water was added to a stirred suspension of 1.5 g. $Ca(OH)_2$ in 360 ml. of water and the whole was maintained at 93° in an atmosphere of N_2 . After 20 min., the reaction mixture was cooled, allowed to stand overnight and filtered. The precipitate was dissolved in 20 ml. of 2N-HCl and the solution was neutralized with NH_4OH (1 vol. 0.88 ammonia/1 vol. water). The cream-coloured precipitate was washed successively with 10 ml. of water, 20 ml. of ethanol and 20 ml. of ether, when its dry weight was 0.71 g. It was dissolved in 6 ml. of HCl (20%, w/v) and the solution was extracted 10 times with 10 ml.

Table 1. *Chromatographic examination of the deionized concentrate X*

Reducing sugars were detected by the $AgNO_3$ reagent of Trevelyan & Harrison (1950), except in one case in which ketoses were revealed by use of the naphthoresorcin reagent of Partridge (1949). Solvent system 1 (Lugg & Overell, 1948) with ascending chromatography; solvent systems 2 and 3 (Jermyn & Isherwood, 1949) with descending technique.

	(1) <i>n</i> -Butanol-formic acid-water (3:1:3, by vol.)				(2) Ethyl acetate-pyridine-water (2:1:2, by vol.)		(3) Ethyl acetate-acetic acid-water (3:1:3, by vol.)	
	AgNO ₃ spray		Naphthoresorcin spray		AgNO ₃ spray		AgNO ₃ spray	
	<i>R_F</i> of reference compound	<i>R_F</i> of <i>X</i>	<i>R_F</i> of reference compound	<i>R_F</i> of <i>X</i>	Reference compound (cm.)	<i>X</i> (cm.)	Reference compound (cm.)	<i>X</i> (cm.)
D-Arabinose	0.14	0.15	0.11 (faint)	0.11 (faint)	40.2	41.2	—	19.7 24.5
D-Ribose	0.22	—	—	—	45.7	—	29.9	—
L-Ribulose	0.25	0.25 0.33 0.45	0.23	0.23	45.7	46.0	32.7	32.8

portions of ether. These ether extracts were combined and evaporated, yielding a solid residue which was acidic to litmus. Chromatography of the acid in the *n*-butanol-formic acid-water solvent and detection of the acid spot with bromocresol green spray gave an R_F value of 0.41. Authentic tartronic acid on similar treatment gave an identical R_F value. The greater part of the free acid obtained by the breakdown of 2-ketogluconic acid was then neutralized with NH_4OH and a silver salt was precipitated by addition of excess of AgNO_3 . The silver salt was dissolved in 10 ml. of 2N- HNO_3 and precipitated with NH_4OH . Repetition of this precipitation process followed by drying *in vacuo* at 76° yielded 0.18 g. of silver salt. (Found: Ag, 64.2. Calc. for $\text{C}_3\text{H}_2\text{O}_5\text{Ag}_2$: Ag, 64.6 %.)

DISCUSSION

Since the findings set out in this communication are concerned mainly with chemical phenomena and separate experiments with enzyme preparations have not been undertaken, it is not possible, on the basis of the results, to define with any degree of assurance, the pathways along which glucose metabolism proceeds in *A. acetosum*. The isolation of small quantities of calcium tartronate from among the products of reaction between calcium 2-ketogluconate and calcium hydroxide suggests that the tartronate isolated from the culture of *A. acetosum* may have arisen from 2-ketogluconate by a slow chemical process.

Oxidation of 2-keto-D-gluconic acid by permanganate has been described by Ohle & Berend (1927), who found that a controlled reaction in acidic conditions yielded D-arabonic acid, whereas under alkaline conditions they detected the formation of oxalic acid in 80 % of the yield which would be expected from a split of the keto acid to oxalic and erythronic acids. The last-mentioned acid was not isolated. The reaction in the present paper is clearly not of this type since oxygen was excluded from the system by a stream of nitrogen. Hence, oxidation probably occurred through intermolecular oxidoreduction. The chromatographic detection of arabinose and a ketopentose, identified as ribulose, as two major components of the mixture of products arising from interaction between calcium hydroxide and calcium 2-keto-D-gluconate, suggests that these two pentoses could be intermediates on the path from 2-ketogluconate to tartronate. Certain results obtained by Steinberg (1942) during a study of the assimilation of many different organic compounds by *Aspergillus niger* should be considered in connexion with the decomposition of 2-ketogluconate. Steinberg showed that 2-keto-D-gluconate was utilized more efficiently than D-gluconic acid by this mould and that a considerable increase in assimilability occurred when the test solution was heated before it was submitted to the action of the mould. After citing the report of Ohle & Berend (1927) on

Fig. 1

the detection of D-arabinose and D-arabonic acid in heated solutions of 2-keto-D-gluconic acid, Steinberg states that neither D-arabinose nor D-arabonic acid is utilized by *A. niger* for purposes of growth, and that the same is true of D-xylulose. Hence, it would appear that compounds other than these are formed in the thermal decomposition of 2-keto-D-gluconic acid, and it may well be that ribulose is a useful source of carbon for *A. niger*.

The scheme presented in Fig. 1 symbolizes our idea of the way in which calcium tartronate could arise from calcium 2-ketogluconate in presence of dilute alkali. A sequence of reactions leading to D-glyceraldehyde and the semialdehyde of tartronic acid followed by a Cannizzaro reaction between these two aldehydes would seem to constitute a reasonable explanation of the observed facts. D-Ribulose (I) may be presumed to arise by transformation of D-arabinose (II) or by decarboxylation of the hypothetical intermediate (III) which is 3-keto-D-gluconic acid. Our failure to detect ribose suggests that ribulose arises mainly by a mechanism other than isomerization of arabinose, since accumulation of ribose in appreciable quantity would be expected if this route was taken. In this connexion it is relevant to note that Gross & Lewis (1931) treated xylose with lime-water at 32° and detected the presence of D-xylulose and D-lyxose in equivalent quantities, at final equilibrium.

SUMMARY

1. The calcium salts of 2-keto-D-gluconic and tartronic acids were isolated from cultures of *Acetobacter acetosum* which had been grown in a medium containing glucose and calcium carbonate.

2. When an aqueous solution of calcium 2-keto-D-gluconate was heated at 100° for 20 min. with calcium hydroxide in an atmosphere of nitrogen,

calcium tartronate was formed and was isolated in small yield from the mixture of products. By paper chromatographic analysis it was demonstrated that arabinose and ribulose had been formed also in this reaction.

3. A scheme of reactions to account for these findings is presented.

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Some Minor Constituents of Liver Oils

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(Received 4 August 1954)

Minor constituents of liver oils, such as carotenoids, vitamins A and D and provitamins D, have attracted much study, but it is not unreasonable to assume that other, and possibly important, substances are present in small amount and have yet to be identified. The nature of such compounds might throw light on metabolic pathways of vitamins or sterols.

In order to isolate minor constituents, it may be

necessary to use large amounts of the starting material and to carry out lengthy manipulations with the attendant risk of producing artifacts, particularly from vitamin A. The greater the quantity of material used initially, the more serious is the risk of contamination; thus, liver tissues worked up in a commercial plant may yield lipid extracts slightly contaminated with impurities from solvents and these may accumulate in indi-