than half of the original furfuryl alcohol distilled, leaving a black tarry residue from which no identifiable products could be extracted.

(b) (I) By **Ester Interchange**.—Employing the method of Calingaert, *et al.*,[7] furfuryl acetate, methyl oxalate and a trace of freshly-sublimed aluminum ethoxide were heated together at 50° for five hours, and distilled under reduced pressure. Products included small amounts of the original reactants, and a black tar from which no I could be extracted.

(c) **Difurfuryl Malonate, by the Diels Method**.—Carbon suboxide, prepared from malonic acid and phosphorus pentoxide, was treated with excess furfuryl alcohol. The clear solution was kept at −70° for 5 hours, gradually turning brown. On warming to room temperature the solution effervesced and turned black. The residue charred on heating, and distillation at 5 mm. produced no ester.

(d) **Difurfuryl Succinate from Acetyl Succinate Monofurfuryl Ester**.—A solution of furfuryl acid succinate in ether was treated with ketene at −10° to form acetyl succinate monofurfuryl ether. This was treated with furfuryl alcohol at the same temperature, but no difurfuryl succinate was obtained. Distillation at 5.8 mm. yielded a trace of furfuryl acetate and a black intractable tar.

Furfuryl Acid Succinate.—Using a procedure similar to that of Adams and Gauerke[11] for furfuryl acetate, 51 g. (0.52 mole) of furfuryl alcohol was heated with 50 g. (0.5 mole) of succinic anhydride and 40.5 g. of anhydrous sodium succinate. The product, a liquid which could not be distilled at 5 mm., was dissolved in aqueous sodium hydroxide, decolorized with charcoal, and precipitated with 10% hydrochloric acid. It was believed to be furfuryl acid succinate. No difurfuryl succinate was detected.

Methyl Furfuryl Oxalate.—Oxalyl chloride monomethyl ester was prepared by the method of von Frank and Caro.[12] The portion boiling at 118–120° (757 mm.) was added to sodium furfurylate, following the procedure employed for the preparation of I.

Oxidation of I.—A neutral solution of 15.8 g. (0.05 mole) of potassium permanganate in 200 ml. of water was added dropwise with stirring to a solution of 6.25 g. (0.025 mole) of I in 100 ml. of ether at 5°. Manganese dioxide was removed by filtration, and the ether and water layers were separated. Evaporation of the ether led to recovery of 2.8 g. of unreacted I. The aqueous layer was concentrated to 85 ml. on the steam-bath and treated with 10% lead acetate solution; 2.03 g. of lead oxalate precipitated.

The aqueous filtrate, after separation of the lead oxalate, was evaporated to dryness at 25°, yielding 1.48 g. of a crystalline compound which decomposed slowly when exposed to air. It gave a positive test for potassium with sodium cobaltinitrite, and when treated with phenylhydrazine formed a solid which decomposed without melting at 200–250°. Tentatively, it was regarded as the potassium salt of an aldehyde- or keto-acid. Attempts to secure the free acid by treatment with mineral acids led to decomposition.

Treatment of I in methanol with 4% hydrogen peroxide and a trace of ferrous sulfate for 24 hours at 0° resulted in complete destruction of the ester. No identifiable products could be isolated.

Methoxylation of I.—A mixture of 12.5 g. (0.05 mole) of I, 150 ml. of methanol and 20 g. of potassium acetate was cooled to −10°, and a cold solution of 8 g. (0.1 mole) of bromine in 50 ml. of methyl alcohol was added dropwise with stirring. Sodium bromide was removed by filtration, and the filtrate concentrated to 15 ml., precipitating 3.2 g. of methyl oxalate. The supernatant liquid, which presumably contained 2,5-dimethoxy-2,5-dihydrofurfuryl alcohol and perhaps some 2,5-dimethoxy-2,5-dihydrofurfuryl oxalate, was hydrolyzed and treated with 5 ml. (0.1 mole) of hydrazine hydrate, following the procedure of Clauson-Kaas and Limborg.[13] This gave 0.98 g. of 3-hydroxymethylpyridazine, identified by mixed melting point with a sample prepared from furfuryl acetate.

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Degradation of Isotopically-labeled Glucose *via* Periodate Oxidation of Gluconate[1]

BY FRANK EISENBERG, JR.[2]

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Radioactive tracer studies currently in progress in this Laboratory have required a method for assaying the radioactivity in the terminal carbon atoms of biosynthetic glucose. Gluconic acid suggested itself as a derivative of glucose suitable for degradation inasmuch as periodate oxidation of gluconate should yield both carbon-1 and carbon-6 separately from a single sample. To test this method, glucose, variously labeled with C^{14}, was oxidized to potassium gluconate in high yield and purity by the procedure of Moore and Link[3]; the gluconate was then oxidized with periodate with the expectation of obtaining CO_2 from C-1, HCOOH from C-2,3,4,5, and HCHO from C-6.

In the first attempts to evaluate this method periodic acid at *p*H 1 was selected as the oxidant. H_5IO_6 was added to a solution of potassium gluconate in the ratio of six moles of oxidant to one of gluconate, and the consumption of periodate was followed by the iodometric procedure of Jackson.[4] It can be seen from Fig. 1 that the oxidation proceeds rapidly until four moles are consumed and then slowly approaches the theoretical consumption of five moles after about 20 hours. The yield of CO_2, collected as $BaCO_3$ during the first two hours, was only 50% of theoretical, a fact which suggests that the bond between carbons 1 and 2 is the last to be split. When the method was tested with gluconate-1-C^{14}, the specific activity in $C^{14}O_2$ evolved during the same two hour period was only 80% of that in C-1 of the gluconate (Table I). Apparently CO_2 is coming from some other source as well as from C-1. In spite of the theoretical stoichiometry observed, the method is unsatisfactory for the allocation of isotope in labeled gluconate.

Some further experiments have been performed in an attempt to reveal the source of the extraneous CO_2 and possibly to suggest a mechanism to explain these results. Any mechanism must take into account the theoretical uptake of H_5IO_6; further oxidation of the fragments by H_5IO_6 is therefore ruled out unless the "theoretical" value observed is fortuitous. To test the possibility that HIO_3 (a product of the periodate oxidation) might oxidize the split products, a system was set up containing H_5IO_6, HIO_3, HCOOK and HCHO. No CO_2 was evolved over a period of three hours. Hence the preformed split products are not susceptible to further oxidation either by H_5IO_6 or HIO_3.

Gluconate-2-C^{14} and gluconate-6-C^{14} have both been subjected to periodate oxidation at *p*H 1 (Table I). Whereas no $C^{14}O_2$ was generated from gluconate-6-C^{14}, $C^{14}O_2$ was a product of oxidation of gluconate-2-C^{14} and accounted for some 2% of

(1) This investigation was supported in part by a research grant A-324, from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service

(2) National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

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TABLE I

DEGRADATION OF C14-LABELED POTASSIUM GLUCONATE

Oxidant	Comp. degraded	Sp. act. c.p.m./mAC (A)	Fragment isolated	Sp. act. c.p.m./mAC (B)	% mol. sp. act. Theoretical	(100B/6A) Found
H_5IO_6	Gluconate-1-C^{14}	1635	C-1	7735	100	80
pH 1	Gluconate-2-C^{14}	1157	C-1	120	0	2
	Gluconate-6-C^{14}	771	C-1	(0)[b]	0	0
$NaIO_4$	Gluconate-1-C^{14}	632	C-1	3730	100	99
pH 5.8			C-6	(0)	0	0
Phosphate buffer	Gluconate-2-C^{14}	1157	C-1	(0)	0	0
			C-2,3,4,5	1685	100	97[a]
			C-6	(0)	0	0
	Gluconate-6-C^{14}	771	C-1	(0)	0	0
			C-6	4385	100	95

[a] Calculated from (400B/6A). [b] Parentheses indicate activities of 1–2 counts above background.

the total CO_2 yield. Had C-2 been the sole source of the extraneous CO_2, the yield of $C^{14}O_2$ would have been 20%, as shown by the 20% dilution of $C^{14}O_2$ from gluconate-1-C^{14}. Since CO_2 can arise in periodate oxidations only from carboxyl groups, a mechanism may be postulated wherein C-2 of gluconate and at least one other carbon atom, but not C-6, are first oxidized to the carboxyl level. Possibly the fact that in strongly acidic solutions gluconic acid exists in part as a mixture of γ- and δ-lactones contributes to this anomalous behavior. No definitive explanation can be offered to account for these results.

A similar discrepancy has been observed by Abraham, *et al.*,[5] who oxidized calcium gluconate-1-C^{14} with H_2O_2 and Fe^{+++}, and could recover only 80% of the activity of C-1 in the CO_2 derived therefrom. Dimant, *et al.*,[6] have degraded gluconic and galactonic amides with hypobromite to obtain C-1 as CO_2. Their preference for this method over the simpler direct periodate oxidation of the acids was not explained, although they used periodate to obtain C-6 from the acids.

While these studies were in progress Bernstein[7] presented a bacterial method for the degradation of ribose. Among other chemical procedures designed to check the bacterial method, oxidation of ribose to ribonate followed by periodate oxidation in phosphate buffer at pH 5.8 was described. When these conditions were adopted by us for the analogous degradation of gluconate, the expected theoretical values were obtained from all species of labeled gluconate tested (Table I) and theoretical stoichiometry was achieved. It can be seen from Fig. 1 that the oxidation proceeds more rapidly with $NaIO_4$ buffered at pH 5.8 than at the strongly acid pH of H_5IO_6. Furthermore the reaction goes rapidly to completion and CO_2 yields of 90% or better were realized within an hour. That the reaction at pH 5.8 follows the usual behavior of periodate, oxidation is in harmony with the notion that the formation of lactones in some way disrupts the expected course of the oxidation.

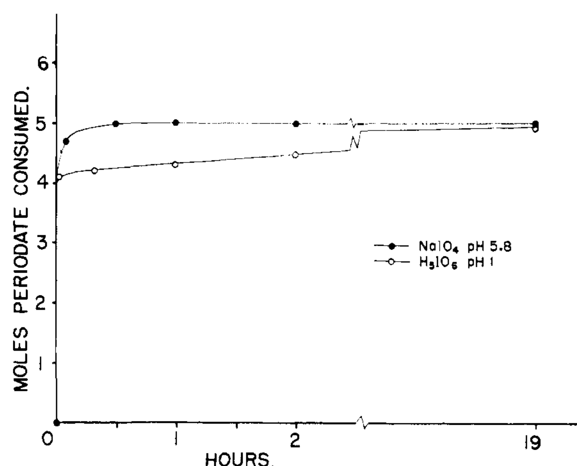

Fig. 1.—Periodate oxidation of potassium gluconate.

Experimental

Seventy mg. (0.3 mmole) of potassium gluconate (m.p. 180°) was dissolved in 12 ml. of 0.5 M sodium phosphate buffer at pH 5.8. The system was swept for five minutes with CO_2-free air to remove preformed CO_2 and 3.6 ml. of freshly prepared[8] 0.5 M $NaIO_4$ was added. Aeration was continued for 45 minutes, during which time the evolved CO_2 was trapped in half-saturated $Ba(OH)_2$ containing 2% $BaCl_2$. The resulting $BaCO_3$ was centrifuged, washed until neutral and held for plating and counting. At the end of an hour (*i.e.*, 15 minutes after the aeration was stopped) the mixture was chilled and the oxidation stopped by the addition of 1 ml. each of 6 N H_2SO_4 and 20% KI. Sufficient 2 M $NaAsO_2$ was then added to destroy the I_2. Four ml. of 3 N NaOH was added and the alkaline solution was distilled to near dryness several times after repeated additions of water. The distillate, containing formaldehyde, was collected in a receiver chilled in a salt–ice-bath. The partially frozen distillate was oxidized to formic acid by treatment with 20 ml. each of 1 N NaOH and 0.1 N I_2 for 1 hour at 0–5°, according to the method described by Sakami.[9] At the end of the oxidation period the solution was acidified and the I_2 destroyed with arsenite. The solution, now containing HCOOH, was neutralized, evaporated to 10 ml. and acidified with glacial acetic acid. A large wad of glass wool was added, and the mixture boiled under reflux with sweeping to remove preformed CO_2. To the boiling solution was added 30 ml. of $HgCl_2$ reagent.[9] By trapping the newly precipitated mercury salts the glass wool served to overcome the tendency of the suspension to bump. The evolved CO_2 was absorbed in $Ba(OH)_2$ as before, washed until neutral, plated and counted. This fraction proved to be derived virtually exclusively from C-6 of gluconate.

In testing the degradation of gluconate-2-C^{14} it was necessary to isolate HCOOH, presumed to originate from the C-2,3,4,5 fragment. To achieve this, the alkaline residue left from the distillation of formaldehyde (C-6) was acidified

(5) S. Abraham, I. L. Chaikoff and W. Z. Hassid, *J. Biol. Chem.*, **195**, 567 (1952).

(6) E. Dimant, V. R. Smith and H. A. Lardy, *ibid.*, **201**, 85 (1953).

(7) I. A. Bernstein, *ibid.*, **205**, 309 (1953).

(8) Solutions of $NaIO_4$ allowed to stand for more than a day evolve fumes of an oxidizing gas giving the reactions of ozone.

(9) W. Sakami, *J. Biol. Chem.*, **187**, 369 (1950).

and steam distilled. The distillate was then carried through the $HgCl_2$ oxidation as described above. As shown in Table I this HCOOH fraction contains all the carbon derived from C-2 of gluconate.

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Is Acetylcholinesterase a Metallo Enzyme?[1]

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The literature contains numerous examples of the activation of cholinesterases by divalent ions such as Ca^{++}, Mg^{++} and Mn^{++} (see among others).[3,4] The activity of dialyzed preparations is also increased by the presence of salts such as NaCl and KCl. This latter activation is a phenomenon associated with the ionic strength of the medium and is quite distinct from the activating effect of divalent ions which occurs at much lower concentrations and can always be superimposed upon the general salt effect. These observations have been obtained with more or less crude enzyme preparations. Despite the fact that the question of the essentiality of divalent ions in the catalytic process has never been studied in purified preparations, the belief persists that this enzyme is a "metallo enzyme."

Recently a paper[5] appeared which seems to indicate that Mg^{++} ion is essential for catalytic activity and a theory was advanced which incorporated Mg^{++} ion in the elementary processes. The basis of this view was an observed 96% inhibition at *p*H 7 of highly purified acetylcholinesterase preparation from *Electrophorus electricus* caused by the addition of 0.015 *M* citrate to an incubation medium containing 0.1 *M* NaCl, 0.010 *M* $MgCl_2$ and 3.3×10^{-3} *M* acetylcholine chloride. Eighty-six per cent. inhibition was obtained with 0.015 *M* borate. These findings are not in agreement with the experience of this Laboratory (unpublished data of D. Nachmansohn). It therefore appeared necessary to repeat these experiments and also to enquire more carefully into the question of whether some species of divalent ion was essential for activity. The results obtained were not in agreement with those of the previous investigators.[5] Accordingly an exchange of enzymes between the two laboratories was arranged and the experimental basis for the differences was resolved as described in a joint note which follows this paper. The procedure used for determining whether divalent cations are essential was to exclude their salts as completely as was conveniently feasible by using reagent grade analyzed sodium chloride, sodium hydroxide and the disodium salt of ethylenediaminetetraacetic acid[6] and highly purified water. The concentration of polyvalent cations was further reduced by adding the chelating agent ethylenediaminetetraacetic acid (EDTA). It was possible in all cases to calculate the maximum amount of any common polyvalent ion present. Since among the common ions which have an activating effect on this enzyme, Mg^{++} is one of the poorest bound by EDTA and is more or less the most plentiful as an impurity in the various reagents and will, therefore, be present as the free ion in the largest amount, we will present our results in terms of the concentration of this metallic ion.

Methods.—Enzymatic activity was studied by two experimental procedures.

(a) Continual automatic titration with 0.020 *N* NaOH using the Beckman Model K automatic titrator modified slightly so as to increase its rate of response and sensitivity and to decrease the amount delivered to about 3 λ per impulse. The *p*H is adjusted at intervals of less than one second and maintained constant to within less than 0.01 *p*H unit. The medium had a volume of 50–100 ml. and contained the salts indicated under results. The substrate concentration was 0.002 *M* acetylcholine bromide.

(b) Manometric assay[7] in 3.1 ml. of 0.16 *M* NaCl, 0.020 *M* $NaHCO_3$ and 0.1% gelatin medium gassed with 5% CO_2 in N_2 (*p*H 7.4). Additions are indicated with the results. The substrate concentration was 0.004 *M* acetylcholine bromide.

The enzyme preparation used in both procedures had originally been purified[8] to a specific activity (micromoles of acetylcholine hydrolyzed per hour per mg. of protein under optimum conditions) of 6.6×10^4 but in storage had fallen to 4.0×10^4. In addition a cruder preparation of specific activity 2.0×10^4 was used also in the manometric procedure.

The water used in the experiments had been distilled and passed through a resin demineralizer. The resulting water had a conductivity corresponding to 0.1 part per million of NaCl. If we assume 0.1 part per million of Mg^{++} in the water the concentration is about 5×10^{-9} *M*.

Acetylcholine bromide was analyzed for Mg^{++} by flame photometry and found to contain less than 0.05%.

Results

The results obtained by the method of continual titration are summarized in Tables I and II. We find in agreement with earlier observations that the activity is diminished in a medium of low ionic strength (ionic strength of substrate = 0.002 *M*) and can be increased to a maximum of about 20% in a medium of high ionic strength by the addition of 10^{-2} *M* or greater $MgCl_2$. That this Mg^{++} activation is not a general salt effect is shown by the fact that the rate is unaltered by the addition of the amount of NaCl which reproduces the ionic strength. At low ionic strength 10^{-4} *M* $MgCl_2$ is sufficient. The activating effect of Mg^{++} can be offset by the chelating agents citrate ion and EDTA.

Contrary to the results recently reported[5] and referred to above, citrate does not alter the basal activity of this enzyme. In the presence of added Mg^{++} and citrate the rate is somewhat higher than the basal rate and is equal to the rate corresponding to the concentration of free Mg^{++}.

Citrate ion is a relatively poor chelating agent for Mg^{++} ($pK = 3.2$[9]) compared to the tetravalent ion

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