[CONTRIBUTION FROM THE KENT CHEMICAL LABORATORY, UNIVERSITY OF CHICAGO]

Trihydroxy-*iso*butyric Acid and Some of its Derivatives[1]

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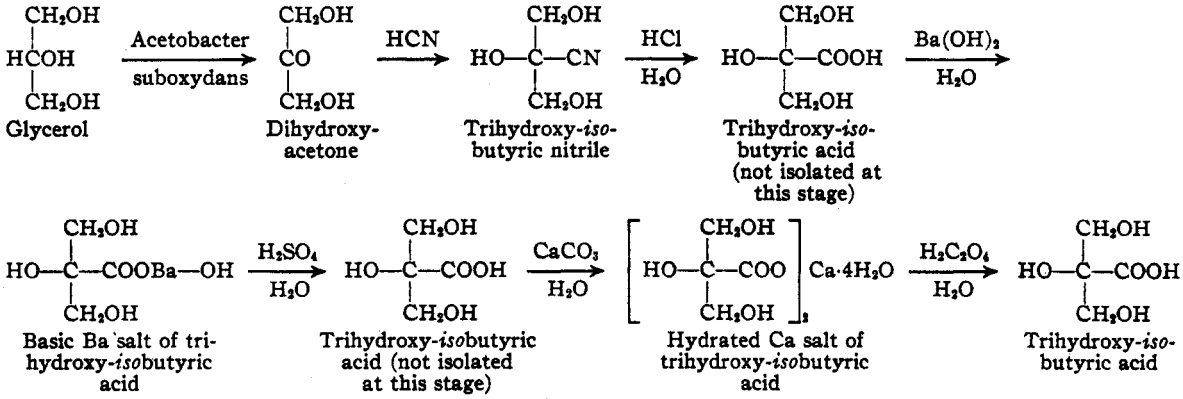
Work on the preparation of compounds considered necessary for a study of the rearrangement of tetroses in alkaline solution has been proceeding in this Laboratory for some time. The treatment of hexoses with strong alkali leads to the production of a complicated mixture containing saccharinic acids,[3] the analysis of which presents great experimental difficulties. The number of saccharinic acids possible[4] from a tetrose is much smaller than that from a hexose. It was, therefore, decided that the mechanism of the transformation of monosaccharides into saccharinic acids might be more easily discovered by working with the tetroses than with the hexoses. The preparation of the tetroses and their corresponding saccharinic acids was therefore undertaken. When this preparative work has been completed and the properties of the theoretically possible saccharinic acids established, the task of determining which acids are formed in an alkaline rearrangement of a tetrose should be comparatively easy. Finally, these facts should pave the way for an understanding of the mechanism of the rearrangement.

The aldo-tetroses could conceivably lead to the production of eleven C_4-saccharinic acids. Ten of these have been prepared in this Laboratory.[5] The one which has so far not been prepared in quantity is 2,2′-dihydroxy-*iso*butyric acid.[5b]

This paper reports the preparation of trihydroxy-*iso*butyric acid and some of its derivatives. The work was done for three reasons: (1) the acid might prove of service in the quantity preparation of 2,2′-dihydroxy-*iso*butyric acid; (2) while not a saccharinic acid, it might prove to be a product of the alkaline decomposition of tetroses in which case means for its identification and isolation would be necessary; and (3) it might be possible to transform the acid into trihydroxy-*iso*butyric aldehyde, the alkaline decomposition products of which, because of its close relationship to tetroses, might contribute information important in the understanding of the saccharinic rearrangement.

Very few references to trihydroxy-*iso*butyric acid are found in the literature. Of two investigations, one by Fischer and Tafel,[6] the other by A. Widiger,[7] the former yielded the basic method by which the acid was finally isolated in the work reported below. In their attempt to prove the presence of glyceric aldehyde in a mixture obtained by the reaction between lead glycerate and bromine, Fischer and Tafel added hydrogen cyanide to the reaction mixture, hydrolyzed the products, and found trihydroxy-*iso*butyric acid to be the chief product. Widiger utilized 2,2′-dichloro-1-hydroxy-*iso*butyric acid[5b] to prepare the acid. He hydrolyzed this compound, precipitated the acid as the basic barium salt, transformed the latter into the calcium salt, and liberated the acid in 50% yield by means of oxalic acid.

To produce the acid in the most direct manner, the authors chose dihydroxyacetone as the source material. By treatment with hydrogen cyanide, the nitrile was prepared which, by acid hydrolysis, was transformed into the acid. The preparation of dihydroxyacetone in maximum yield presented some difficulties. The method of preparation finally used was the bacteriological oxidation of glycerol by means of *Acetobacter suboxydans*. The series of steps employed as shown.



(1) This paper is a condensation of a dissertation presented by Harold M. Coleman in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Chicago.

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(3) J. U. Nef, *Ann.*, **357**, 214 (1907); **376**, 1 (1910); **403**, 204 (1913).

(4) For definition of "saccharinic acid," see Glattfeld and Sherman, THIS JOURNAL, **47**, 1742 (1925).

(5) Glattfeld and co-workers; see, for instance, (a) *ibid.*, **42**, 2314 (1920); (b) **60**, 415 (1938).

(6) E. Fischer and Tafel, *Ber.*, **21**, 2634 (1888); **22**, 106 (1889).

(7) A. Widiger, M.S. Dissertation, University of Chicago, 1936.

Previous authors, van Deen,[8a] Grimaux,[8b] Fischer and Tafel,[8c] Piloty and Ruff,[9a] Den Otter,[9b] and Dimroth and Schweizer,[9c] using chemical methods of producing dihydroxyacetone or its derivatives, report low yields. The work of Bertrand,[10a] Brown,[10b] and Emmerling,[10c] using *Bacterium xylinum*, that of Waterman,[11a] Hermann and Neuschul,[11b] Sazerac,[11c] Bernhauer and Gorlich,[12a] and Fernbach,[12b] using different strains of the acetic acid group of bacteria; and that of Kluyver and DeLeew,[13a] Virtanen and Nordlund,[13b] Sjollema and Seekles,[14a] Virtanen and Bärlund,[14b] Furth and Marion,[14c] and visser't Hooft,[15] using *Acetobacter suboxydans;* contributed useful information. While dihydroxyacetone was produced from glycerol in most of these investigations, the technique of its isolation and purification still remained unsatisfactory. Finally, the work of Underkofler and Fulmer,[16] using a procedure developed by Neuberg and Hofmann[17] provided the conditions for maximum yield. Their procedure, with some modification, was used in this research.

Experimental Part

Experiments on the Rate and Extent of Conversion of Glycerol into Dihydroxy-acetone by Acetobacter suboxydans.—The progress of the conversion of glycerol to dihydroxyacetone was followed by an adaptation of the results of work by Rappaport and Reifer.[18]

Approximately 55 g. of glycerol[19] was weighed accurately, washed with water into a 1 l. volumetric flask, 5 g. of yeast extract[20] and 3 g. of potassium dihydrogen phosphate added, the contents diluted to the mark, and transferred to the fermentation flask.[21] Three 25-ml. samples were pipetted out and titrated with 0.5 *N* sodium hydroxide to the blue tint of brom cresol purple. It was found necessary to add 14.8 ml. of the 0.5 *N* sodium hydroxide solution to the remaining 925 ml. of fermentation solution to bring the *p*H to 6.0–6.8.[22] This fermentation solution (from the weight of glycerol added) was calculated as 2.25 *N* with respect to glycerol as a reducing agent toward potassium periodate in acid solution.[23]

The adjusted solution was then sterilized for twenty minutes at a pressure of 2 atmospheres and a temperature of 120°, permitted to cool, and inoculated aseptically through the inoculation-tube entrance of the fermentation flask with several drops of a four-day old activated culture[24] of *Acetobacter suboxydans*.[25,26] After inoculation, the solution was shaken vigorously and the time recorded. Portions of the fluid were then withdrawn through the stopcock into a small flask in order to wash both the short portion of the tube beyond the stopcock and the flask itself with fermentation solution. Finally, sufficient solution was withdrawn

(8) (a) J. van Deen, *Jahresberichte für Chemie*, 501 (1863); Tydschrift voor Geneeskunde Jahregang, 4 and 5; (b) Grimaux, *Compt. rend.*, **104**, 1276 (1887); (c) E. Fischer and Tafel, *Ber.*, **20**, 1088 (1887), 3384 (1887).

(9) (a) Piloty and Ruff, *ibid.*, **30**, 1656 (1897); **30**, 3161 (1897); (b) H. P. Den Otter, *Rec. trav. chim.*, **56**, 474 (1937); (c) O. Dimroth and R. Schweizer, *Ber.*, **56**, 1375 (1923).

(10) (a) G. Bertrand, *Compt. rend.*, **122**, 900 (1896); **126**, 984 (1898); *Bull. soc. chim.*, [3] **15**, 627 (1896); **19**, 502 (1898); (b) A. Brown, *J. Chem. Soc.*, [3] **20**, I, 638 (1887); (c) Emmerling, *Ber.*, **32**, 541 (1899).

(11) (a) H. J. Waterman, *Centr. Bakt. Parasitenk.*, [2] **38**, 451 (1913); (b) Hermann and Neuschul, *Biochem. Z.*, **233**, 129 (1931); (c) Sazerac, *Compt. rend.*, **137**, 90 (1903).

(12) (a) Bernhauer and Gorlich, *Biochem. Z.*, **280**, 375 (1935); (b) Fernbach, *Compt. rend.*, **151**, 1004 (1910).

(13) (a) Kluyver and DeLeew, *Tydschr. verg. Geneesk.*, **10**, 170 (1924); (b) Virtanen and Nordlund, *Biochem. J.*, **27**, 442 (1933).

(14) (a) Sjollema and Seekles, *Rec. trav. chim.*, **44**, 827 (1925); (b) Virtanen and Bärlund, *Biochem. Z.*, **169**, 169 (1926); (c) Furth and Marion, *ibid.*, **167**, 135 (1926).

(15) visser't Hooft, "Bio chemurche onderzoekingen over het Geslacht Acetobacter," Thesis, Delft, 1925.

(16) Underkofler and Fulmer, THIS JOURNAL, **59**, 301 (1937).

(17) Neuberg and Hofmann, *Biochem. Z.*, **279**, 318 (1935).

(18) Rappaport and Reifer, *Mikrochimica Acta*, **2**, 273 (1937).

(19) U. S. P.—C. P., Merck grade.

(20) Difco Yeast Extract (powdered product), Digestive Ferment Co., Detroit, Michigan.

(21) The fermentation apparatus consisted of a 2-liter Erlenmeyer flask through the cork stopper of which passed one arm of an inverted glass U-tube (6 mm. diam.) so arranged that the end almost touched the flask bottom. Most of the vertical outside arm of the U-tube was made of tubing of larger diameter (15 mm.). This contained cotton. The stopper was also fitted with a short piece of 10-mm. tubing through which the bacterial inoculum was introduced. Near the bottom of the flask and sealed to it (a few cm. above the flat bottom), a stopcock (length of tubing on both sides of cock about 2 cm.) provided the means by which samples were conveniently removed without contamination of the fermentation solution. All openings were plugged, and the cork stopper region completely covered, with cotton.

(22) The color change of the indicator during the titration with alkali was gradual. In all later experiments, better control of the *p*H of the fermentation solution was obtained by titration with a more dilute alkali to the color tint given by a solution of equal volume and indicator concentration, and a *p*H of 6.8. The volume of 0.5 *N* sodium hydroxide which it was found necessary in this way to add to 2 l. of fermentation solution to bring the *p*H to 6.8 was 59.6 ml.

(23) The analytical accuracy of the periodate method was confirmed by the determination of the glycerol in this fermentation solution at "0" time. The solution was found, by periodate analysis, to be 2.22 *N* with respect to glycerol.

(24) The term "activated culture" here means a culture made in highly favorable environment prior to use.

(25) Glycerol-agar slants were obtained from the American Type Culture Collection at Georgetown University in Washington, D. C. Agar slant transfers were made every six or seven days (incubation temperature 20 to 26°). The purity of these slant cultures was verified by the Gram stain, by microscopic characteristics, and by the rapidity of their growth at a temperature of 20–21°, before use as the source of inoculum for the media in which they became activated.

The agar slants were composed of the following relative quantities of ingredients: 30 g. of glycerol, 5 g. of yeast extract, 20 g. of agar and 1000 ml. of distilled water. The entire mixture was heated to complete liquefaction on the water-bath, filtered, and the filtrate poured into test-tubes which were then plugged with non-absorbent cotton and sterilized. While still hot, the test-tubes were inclined and permitted to cool.

The activating media were prepared from 7.5 g. of yeast extract, 90 g. of glycerol and 1500 ml. of distilled water. In each activating flask (see footnote 26), 100 ml. of this solution was used. The flask was properly plugged, and the contents were sterilized before inoculation. After inoculation, the medium became turbid after two days of standing at room temperature (20–21°) without aeration; abundant surface growth indicated the beneficial effect of oxygen. Before inoculum from this activating solution was introduced into the fermentation solutions, a loopful of it was transferred aseptically to a Petri plate-agar medium. The characteristics of the isolated colonies, rapidity of growth at room temperature, and microscopic examination, indicated that the culture was pure.

(26) The agar-slant samples were first cultured in 250-ml. Erlenmeyer flasks. Through one hole of the rubber stopper passed a glass tube (6-mm. diam.) the bottom end of which was drawn out to a capillary which, in turn, was submerged beneath the activating medium. The other hole was fitted with a short outlet tube. Both tubes had bulbs which contained cotton near their outside ends. Aseptic inoculation of the sterile activating medium in the flask with *Acetobacter suboxydans* was made through a small 10-mm. glass tube which had been sealed to the flask just above the level of the medium. This side tube also served as the passageway through which the activated solution inoculum was poured into the entrance-tube of the large fermentation flask. Both the stopper region and the inoculation tube were protected by cotton

to permit the thorough washing of a 1-ml. pipet and to supply the 1-ml. analysis sample. The residual fluid in the portion of the tube beyond the stopcock was washed out with water, alcohol, and finally ether. Dry, sterile air was then directed with a capillary nozzle into this exposed portion of the tube to dry it, after which a plug of flamed cotton was inserted into the end. The fermentation flask was then placed in the incubator at 27 ± 2°.

The 1-ml. analysis sample was pipetted into a flask and 5 ml. of 8 *N* sulfuric acid added. The time at which this addition was made was recorded. Next 100 ml. of standard potassium periodate solution (0.02925 *N*) was pipetted into the mixture which was then heated in a boiling water-bath for fifteen minutes with occasional shaking. It was then cooled, 25 ml. of potassium hydrogen phosphate (K_2HPO_4) solution (495 g. per 1000 ml. solution) added, the mixture well shaken, 5 ml. of potassium iodide solution (1 g. potassium iodide per 5 ml. solution) added, the mixture again well shaken and the liberated iodine titrated with standard sodium thiosulfate (0.03129 *N*). With no fermentation solution added, *i. e.*, in the blank, 100 ml. of the standard potassium periodate solution required 93.47 ml. of the standard sodium thiosulfate solution for the liberated iodine.

Samples of the solution were withdrawn from time to time as the fermentation progressed. The elapsed time between the inoculation of the solution and the addition of 8 *N* sulfuric acid to the samples withdrawn for analysis is recorded in the first column of Table I. This time in the case of the first sample was made as short as possible because the assumption was made, in the calculation of the extent of conversion, that in this sample no glycerol had been converted. In all cases, the interval of time between the withdrawal of the sample and the addition of the 8 *N* sulfuric acid (which stopped fermentation) was kept below five minutes.

After inoculation both activating and fermentation solutions became more and more turbid with time. Finally, however, clarification of the liquid occurred. This clarification served as additional evidence of the cessation of conversion.

TABLE I

Elapsed time, hr.	$Na_2S_2O_3$,[a] ml.	Me. KIO_4 unreduced	Me. KIO_4 reduced (*X*)	% Glycerol converted to dihydroxyacetone
0.0	22.66	0.709	2.216[b]	0
27.8	22.82	0.714	2.211	0.5
313.5	33.35	1.043	1.882	30
458.2	38.07	1.191	1.734	44
555.8	42.18	1.320	1.605	55
700.3	45.96	1.438	1.487	66
863.2	48.93	1.531	1.394	74
1085.2	55.99	1.752	1.173	94
1685.0	57.11	1.787	1.138	97
1972.0	57.14	1.788	1.137	97

[a] The volume of thiosulfate which measures the periodate reduced in the oxidation is obtained by subtracting the proper volume in column two from 93.47 ml. [b] At 0 time no glycerol is converted to dihydroxyacetone and the 1-ml. sample would require 2.216 me. of periodate for oxidation. When all of the glycerol has been converted, the 1-ml. sample would contain only dihydroxyacetone and therefore require only 1.108 me. of periodate for oxidation. Between these two extremes the % glycerol converted (column 5) is given by the expression 100[2.216 − *X* (from column 4)]/1.108.

A second experiment was run in which a fermentation solution of approximate *p*H 6.8 was used but in which the solution was aerated with sterile air throughout the conversion. Analyses of samples revealed that some increase in conversion rate had occurred (approx. 15%) but that the total glycerol converted remained identical with that found in the first experiment, namely, 97%.

The rapidity with which the activating solution became turbid after inoculation led to the study of the rate of conversion of glycerol in this neutral, unbuffered medium without aeration. The rate in this solution was comparable to that in the buffered *p*H-adjusted one until approximately 65% conversion had occurred, after which it slowly diminished to zero at a conversion of approximately 83%. The cause of this incomplete conversion apparently lay in gradual bacterial inhibition due to hyperacidity.

Isolation of Crystalline Dihydroxyacetone.—After the fermentation solutions exhibited 97% conversion, each liter was treated with 10 g. each of Norite, calcium carbonate and diatomaceous earth, well shaken, and filtered. The combined filtrate was concentrated at 10 mm. and 35° to a volume of approximately 150 ml. per liter of original solution. To the solution was added four volumes of absolute alcohol. The well-shaken mixture was subjected to filtration and the filtrate concentrated at 10 mm. and 35° to a thin sirup which was slowly poured, with vigorous stirring, into ten volumes of acetone. After standing overnight the turbid solution was treated with Norite, filtered, and concentrated to a sirup at 10 mm. and 30°. The flask containing the sirup was transferred to a bell-jar desiccator which contained sulfuric acid and stored at 2 mm. pressure. The sirup usually became crystalline within two days, with or without seeding, but when large batches were handled, about two weeks were necessary for complete crystallization. During this storage at low pressure, colorless crystals of dihydroxyacetone were deposited, and grew to considerable size by sublimation on the sides of the flask.

When the mass had solidified, absolute alcohol was added in just sufficient quantity to dissolve it and the solution transferred to a smaller flask which was immersed in a carbon dioxide–acetone bath. The crystalline precipitate was separated rapidly by suction filtration and then washed successively with carbon dioxide–acetone cooled absolute alcohol and acetone. The solid was then dissolved in acetone and the solution filtered. Medium boiling ligroin was added until a faint turbidity persisted. Just enough acetone was then added dropwise to clarify the solution, after which it was placed in the refrigerator for twenty-four hours. Goniometrically-measurable colorless crystals were obtained in yields of approximately 70% based upon the original glycerol; m. p. 74–75°.

The preparation of the dibenzoyl derivative[27] of m. p. 121° and the 2,4-dinitrophenylhydrazone of m. p. 159°[28] confirmed the identity of the product as dihydroxyacetone.

A polarizing microscope was employed to observe a few optical properties of the large crystals. The crystal is biaxial with 2V = 20–25°. The use of an acute bisectrix interference figure and quartz wedge showed the substance to be optically negative, alpha being normal to the acute bisectrix figure.

Preparation of Trihydroxy-*iso*butyric Acid.—To 413 g. of crude and only partially crystallized dihydroxyacetone in a 1-liter round-bottom flask, immersed in an ice-bath, was added 310 g. (440 ml.) of ice-cold anhydrous liquid hydrogen cyanide.[29] The flask was then attached to a reflux condenser, the ice-bath removed, and about 2 g. of sodium cyanide added through the condenser in small portions over a twenty-minute period. The rate of reaction, as indicated by the refluxing, was controlled by frequent immersion of

(27) H. O. L. Fischer and Taube, *Ber.*, **60**, 479 (1927).

(28) Bell and Baughan, *J. Chem. Soc.*, 1947 (1937).

(29) The hydrogen cyanide was prepared by the method of Ziegler, "Organic Syntheses," Vol. VII, p. 50, 1927, with these modifications: (a) a large-bore stopcock was sealed to the bottom of the round-bottom flask to provide for periodic removal of the sodium bisulfate-sulfuric acid solution; (b) both the sodium cyanide and the sulfuric acid solutions were siphoned into the reaction chamber from storage bottles, their respective flows regulated by stopcocks; (c) the hydrogen cyanide, after having been liquefied, was collected in a glass-jacketed graduated tube to the botton end of which was sealed a small stopcock. This tube permitted both convenient cooling (with ice water) and withdrawal of known volumes of the liquid hydrogen cyanide. The apparatus was, of course, set up in an efficient hood.

the flask in the ice-bath. After refluxing no longer occurred at room temperature, the mixture was stored at 20–25° for two hours after which it was concentrated to a sirup at this temperature and at 10 mm. pressure with the use of a water pump through which the excess hydrogen cyanide was safely removed. The residue was washed into a 2-liter flask with 500 ml. of water, the solution treated with Norite, the filtrate cooled in an ice-bath, and saturated with dry hydrogen chloride. The solution was stored at 0° for twelve hours and then at room temperature for two and a half days. The liquid became almost black in color and needle-like crystals appeared. The entire mixture was transferred to a 2-liter flask and the solvent removed by vacuum distillation at room temperature (water pump). To the residue was added 1.5 liters of water, and the solution treated with Norite. The amber-colored filtrate was divided into 2 equal portions, each of which was poured into a flask (fitted with reflux condenser and soda-lime tube) which contained a hot solution of 2000 g. of barium hydroxide octahydrate in 3 liters of water. The contents of each flask were boiled for fifteen hours during which time much ammonia was expelled through the soda-lime tube and a flaky pink solid precipitated. The solid basic barium salt was separated from the hot solution by rapid suction filtration, washed several times with boiling water and air-dried at room temperature: weight 735 g., a yield of about 60% calculated from the crude dihydroxyacetone.

The 735 g. of salt was introduced into a solution of 148 ml. of concentrated sulfuric acid in 2.5 liters of water. For the adjustment of the solution so that neither barium nor sulfate ion was present, 1-ml. samples were centrifuged and the clear supernatant liquid tested. After the adjustment, the mixture was centrifuged (2400 ml. at a time), the supernatant liquid decanted, and treated with Norite. Suction filtration yielded a clear colorless filtrate which was divided into 2 equal portions each of which was treated with 185 g. of calcium carbonate. The mixtures were then boiled for one hour under reflux, subjected to suction filtration while still hot, and the solid washed with boiling water. The entire filtrate was completely concentrated at 10 mm. and 40–45°. The white crystalline residue was treated with small portions of boiling water until no more solid dissolved, after which the solution was filtered by suction. The filtration was slow due to the formation of a gelatinous mass on the filter paper as the solution in the Büchner cooled. When filtration was finished, about 1 liter of boiling water was passed through the solid on the Büchner under suction. The filtrate was then cooled and stored in a refrigerator overnight, the white crystals separated by suction filtration, washed with ice-cold water and air-dried; weight 195 g. The filtrate was treated with 95% alcohol to near-turbidity, after which the solution was stored at room temperature overnight. The crystals were then separated by suction filtration, washed with absolute alcohol and air-dried; weight 91 g. The filtrate, on treatment with absolute alcohol finally yielded 20 g. more of the calcium salt. A total of 306 g. of the hydrated calcium salt was thus isolated, a yield of 63% calculated from the basic barium salt.

The 306 g. of salt in 2 liters of water was treated with a solution of 102 g. of hydrated oxalic acid in 1 liter of water and final adjustment made in the usual manner so as to leave a slight excess of calcium. The solution was filtered by suction and then completely concentrated at 10 mm. and below 30°. During the concentration process, small quantities of precipitate—apparently calcium oxalate—appeared; these were periodically separated from the solution by filtration. Approximately 7 volumes of acetone was added to the viscous colorless residual liquid after which crystallization occurred immediately. Water was added to the mixture in portions until the solution became clear and then acetone was again added until a permanent turbidity prevailed. The turbid solution was permitted to stand for one-half hour after which the small amount of white precipitate present—apparently a mixture of the calcium salts of oxalic acid and the hydroxy acid—was separated by filtration. To the clear filtrate was added 0.5 liter of acetone and the well-mixed solution stored in the refrigerator overnight. The small quantity of precipitate which had formed was separated and the filtrate completely concentrated at 10 mm. and room temperature. Ten volumes of absolute alcohol was added to the residual clear liquid, the mixture well stirred, passed through a filter, and completely concentrated at reduced pressure and room temperature. During the concentration process, white crystals formed and, by the end of the evaporation, the entire residue was crystalline; m. p. 115–116°. The residue was treated with 500 ml. of absolute alcohol and dissolved by warming. The hot solution was filtered quickly by suction and divided into two equal portions each of which was treated with medium boiling ligroin to near-turbidity (6 volumes). Crystallization occurred as the solution cooled. The mixtures were cooled in an ice-bath and the crystals separated; total weight 183 g.; m. p. 115–116°; yield 84% calculated from the hydrated calcium salt, and 29.3% calculated from the crude dihydroxyacetone.

A small portion of the acid was recrystallized twice from mixtures of absolute alcohol and medium boiling ligroin as described above and then dried over phosphorus pentoxide in a vacuum desiccator. The m. p. of the recrystallized product was 117°. Fischer and Tafel[6] report the m. p. as 116° and Widiger[7] as 117°. When the acid was heated above its m. p., a gas was evolved (probably water vapor) from the clear melt with the formation of another clear melt (lactid?).

Anal.[30] Calcd. for trihydroxy-*iso*butyric acid, $C_4H_8O_5$: C, 35.30; H, 5.92; neut. equiv., 136.1. Found: C, 35.71; H, 5.90; neut. equiv., 138.0, 137.7.

The Phenylhydrazine Salt.—Two grams of the acid was dissolved in 50 ml. of absolute alcohol and the solution filtered. To the clear filtrate was added about 2 g. of phenylhydrazine and the mixture shaken. Crystallization began at once and seemed to be complete in about thirty minutes. The solid was separated by suction filtration and washed twice with cold absolute alcohol: yield almost quantitative (calculated as the phenylhydrazine salt); m. p. of the crude material was 120–122°.

A small portion of the crystals was dissolved in 50 ml. of hot absolute alcohol, a little Norite added, the hot solution filtered by suction, the crystals obtained from the ice-cooled filtrate brought upon a filter and washed twice with absolute alcohol; m. p. 121–122°. This substance was very soluble in water and its aqueous solution instantly reduced silver ion.

Anal. Calcd. for the phenylhydrazine salt of trihydroxy-*iso*butyric acid, $C_{10}H_{16}O_5N_2$: N, 11.47. Found: N, 11.51.

The *p*-Toluidine Salt.—One gram of the acid was dissolved in 50 ml. of absolute alcohol and the solution passed through a filter into a flask which contained a solution of 1 g. of *p*-toluidine in 15 ml. of absolute alcohol. The well-shaken mixture was stored at room temperature for twelve hours. The white crystalline solid was then separated by suction filtration and washed with cold absolute alcohol. The yield was practically quantitative (calculated as the *p*-toluidine salt). A small portion was recrystallized from hot absolute alcohol and dried over phosphorus pentoxide in a vacuum desiccator for twenty-four hours; m. p. 126.5–127.0°. This substance was very soluble in water.

Anal. Calcd. for the *p*-toluidine salt of trihydroxy-*iso*butyric acid, $C_{11}H_{17}O_5N$: N, 5.76. Found: N, 5.57.

2,2'-Dibenzoyl-trihydroxy-*iso*butyric Acid.—One gram of acid was dissolved in 1.5 ml. of pyridine and the solution cooled to 0°. Three grams of benzoyl chloride was treated with enough pyridine to form a clear solution. The acid chloride solution was then added, in small portions and with cooling, to the pyridine solution of the acid. The reaction mixture was heated for one hour on a boiling water-bath under an air condenser fitted with a calcium

(30) The micro-analyses recorded in this work were performed by Dr. T. S. Ma of the University of Chicago Microchemical Department.

chloride tube and then cooled in an ice-bath. Pyridine hydrochloride was removed by suction filtration and the filtrate added dropwise, and with vigorous stirring, to an ice-cold solution of approximately 50 ml. of concentrated hydrochloric acid in 600 ml. of water. A white material separated which, when brought to room temperature became oily. Hence, after the addition was completed, the reaction mixture was stored in the refrigerator for three days during which time the tendency of the white material to become oily when brought to room temperature became less. The supernatant liquid was decanted and ice-cold water was added to, and decanted from, the residual solid a few times to remove the water-soluble impurities. The white residue was then quickly placed on a porous plate which was, in turn, placed at once in a vacuum desiccator which contained phosphorus pentoxide. After several hours of drying, the solid weighed 1.5 g. It was insoluble in water and in carbon tetrachloride; insoluble in cold benzene, toluene and medium-boiling ligroin; very soluble in alcohol, hot benzene, hot toluene and in dilute alkali; and fairly soluble in dry ether. The material was recrystallized by a procedure which consisted of solution in warm benzene, filtration of the warm solution, addition to the filtrate of carbon tetrachloride to near-turbidity, cooling of the mixture in an ice-bath and storing at this temperature for about one hour. A white solid separated which, after having been dried over phosphorus pentoxide in a vacuum desiccator for twelve hours, had a m. p. of 132–133°. A repetition of this procedure yielded a solid which had a m. p. of 135–136°. A final recrystallization from hot toluene alone yielded a solid of sharp m. p., 137°.

Anal. Calcd. for dibenzoyl-trihydroxy-*iso*butyric acid, $C_{18}H_{16}O_7$: C, 62.79; H, 4.68; sapon. equiv., 114.8. Found: C, 62.76; H, 4.70; sapon. equiv., 114.6, 115.0.

The Phenylhydrazine Derivative (Salt?) of 2,2'-Dibenzoyl-trihydroxy-*iso*butyric Acid.—Treatment of the dibenzoylated hydroxy-acid in absolute ether solution with phenylhydrazine yielded crystals which, after recrystallization from mixtures of ether and medium-boiling ligroin, had a sharp m. p. of 110°. This substance, while dissolving slowly, was quite soluble in water.

Anal. Calcd. for the phenylhydrazine salt of dibenzoyl-trihydroxy-*iso*butyric acid, $C_{24}H_{24}O_7N_2$: N, 6.19. Found: N, 5.13.

The Phenylhydrazine Derivative (Salt?) of Tribenzoyl-trihydroxy-*iso*butyric Acid.—A solution of 3 g. of the acid in enough pyridine to dissolve it, was added, in small portions, to a pyridine (75 g.) solution of benzoyl chloride (9.5 g.). The mixture was then heated in an oil-bath at 130–135° for a total of twelve hours. The procedure as described in the preparation of the dibenzoylated acid was then followed except that the filtrate from the pyridine hydrochloride was poured into a mixture of 1200 ml. of water, 80 ml. concentrated hydrochloric acid and cracked ice. The dried material finally obtained weighed 8 g. It was dissolved in benzene, Norite added, the mixture warmed and the solution filtered. Carbon tetrachloride was added to the filtrate but no turbidity occurred. The mixture was then cooled in an ice-bath for a few hours during which time some viscous material separated. The solution was passed through a filter and completely concentrated at 30° at reduced pressure, the residue dried over phosphorus pentoxide in a vacuum desiccator, and then dissolved in absolute ether. A few drops of phenylhydrazine was added. A white precipitate separated in a few minutes, which, after recrystallizations from a hot benzene–toluene–ligroin mixture, from hot toluene, and finally from a toluene–ligroin mixture, had a m. p. of 137.0–137.5° (red melt). This substance was very insoluble in water.

Anal. Calcd. for the phenylhydrazine salt of tribenzoyl-trihydroxy-*iso*butyric acid, $C_{31}H_{28}O_8N_2$: N, 5.03. Found: N, 4.79.

Triacetyl-trihydroxy-*iso*butyric Acid.—A mixture of 5 g. of the acid and 25 g. of acetic anhydride was heated for fifteen hours on a water-bath in a flask under an air condenser fitted with a calcium chloride tube, after which the excess acetic anhydride and acetic acid were removed at 100° by distillation at reduced pressure. The residue was then distilled at 1 mm. and a bath temperature of 200–240°. The colorless oily distillate was treated with 40 ml. of water and left for several days. During this time most of the oil went into solution. The small portion that did not dissolve became solid. This crude solid melted at 83–85°. After recrystallization from ether to which some medium-boiling ligroin had been added, it melted at 86.0–86.5°. The substance was very insoluble in water and in dilute alkali and may have been a compound of lactid-like structure such as tetraacetyl-tetrahydroxymethyl-glycolide.[31]

Anal. Calcd. for the dimeric tetraacetyl-tetrahydroxy-methyl-glycolide, $C_{32}H_{40}O_{24}$: C, 47.53; H, 4.99; mol. wt., 808. Found: C, 47.68; H, 5.05; mol. wt., 805 (Exaltone), 762 (triphenylmethane).

A mixture of 2 g. of the acid and 6 g. of freshly distilled acetyl chloride was heated for three hours in an oil-bath at a temperature of 65° under a reflux condenser fitted with a calcium chloride tube. The mixture became homogeneous and clear. The excess acetyl chloride was then removed by distillation at 60° and 2 mm. pressure. The distillation was frequently interrupted to permit the addition of small amounts of absolute ether, the evaporation of which aided in the complete removal of volatile products. The flask, with its viscous colorless residue, was then placed in a vacuum desiccator which contained phosphorus pentoxide and sodium hydroxide pellets and stored for many days. The acetylated product did not crystallize. This oily substance was soluble in water, alcohol, benzene, xylene, ether and carbon tetrachloride.

Anal. Calcd. for triacetyl-trihydroxy-*iso*butyric acid, $C_{10}H_{14}O_8$: C, 45.80; H, 5.38; sapon. equiv., 65.5. Found: C, 45.38; H, 5.43; sapon. equiv., 65.4, 65.9.

The Phenylhydrazine Derivative (Salt?) of Triacetyl-trihydroxy-*iso*butyric Acid.—Treatment of an absolute ether (2 ml.) solution containing 5 drops of the acetylated acid with an absolute ether (3 ml.) solution containing 5 drops of phenylhydrazine led to rapid precipitation of a substance, which, after several recrystallizations from warm ether, had a sharp m. p. of 94.0° (red melt). This substance was soluble in water, ethyl alcohol, toluene, chloroform, dioxane and insoluble in cold absolute ether. It was fairly soluble in warm ether. An aqueous solution of this substance reduced silver ion instantaneously. Analysis indicated that this substance may have been a compound in which 2 moles of substituted acid were in combination with 3 moles of phenylhydrazine, a phenomenon which has been reported before.[32]

Anal. Calcd. for the phenylhydrazine salt of triacetyl-trihydroxy-*iso*butyric acid (mole ratio of triacetylated acid to phenylhydrazine = 2/3), $C_{38}H_{52}O_{16}N_6$: N, 9.90. Found: N, 9.59, 9.85.

Summary

1. A new procedure for determining the rate and extent of conversion of glycerol to dihydroxyacetone by *Acetobacter suboxydans* was developed. It was found that, in buffered solutions of optimum *p*H, almost 100% conversion occurred.

2. Some new optical properties of dihydroxyacetone crystals are reported.

3. Trihydroxy-*iso*butyric acid was, for the first time, prepared from *pure* dihydroxy-acetone by treatment with sodium cyanide-catalyzed liquid anhydrous hydrogen cyanide followed by hydrolysis of the nitrile to the acid.

4. Three derivatives of trihydroxy-*iso*butyric acid were prepared, the exact nature of which remains undetermined.

(31) Blaise and Bagard, *Ann. chim. phys.*, [8] **11**, 115 (1907).

(32) Lockemann and Weiniger, *Ber.*, **41**, 3102 (1908).

5. Five derivatives of trihydroxy-*iso*butyric acid, hitherto unreported, were prepared and their more important properties determined.

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[CONTRIBUTION FROM THE DEPARTMENT OF ANIMAL AND PLANT PATHOLOGY OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Size and Shape of Tobacco Mosaic Virus Particles[1]

BY MAX A. LAUFFER

I. Introduction

Several physico-chemical techniques have been developed for the determination of the size and physical characteristics of protein particles. These include ultracentrifugation, diffusion and viscosity studies. From various combinations of data obtained by at least two of these methods used in conjunction with the partial specific volume, it is possible to determine not only the molecular weight, but also the size and shape of protein particles. In general, the conclusions drawn from various combinations of the above methods are self consistent. Nevertheless, confirmation of the results by some direct method would enhence the value of such results greatly. It has been pointed out that tobacco mosaic virus protein affords an excellent medium through which to obtain such a confirmation, because the particles of this protein are extremely anisometric and because they are large enough to be seen and measured with fair precision with the electron microscope.[1a] It was shown from the data available in the literature that in a general sort of way the conclusions regarding the size and shape of tobacco mosaic virus protein particles drawn from viscosity, diffusion, and sedimentation results were in agreement with the dimensions measured directly from electron micrographs.[1a] Nevertheless, these data suffered from the limitation of having been obtained in several laboratories on different virus preparations probably in different states of aggregation. It was therefore thought worth while to carry out viscosity, sedimentation and diffusion studies on a limited number of preparations and to compare these results directly with electron micrographs on the same preparations. This report is a description of such studies carried out for the primary purpose of affording a critical test of the general validity of the indirect methods of determining the size and shape of protein particles.

II. Materials

Five preparations of tobacco mosaic virus were used in this study. Preparations A and B were isolated from frozen and ground fourteen-week-old Turkish tobacco plants which had been inoculated with tobacco mosaic virus at the age of nine weeks. Preparation A was isolated by two alternate high and low speed centrifugation cycles. The high speed runs were made for one hour at 24,000 r. p. m. in a Bauer and Pickels air-driven quantity centrifuge.[2] No chemical agent was used at any stage in the treatment. In the isolation of Preparation B, 3 g. of potassium biphosphate was added per 100 g. of pulp before the extraction of the juice, and the two centrifugation cycles were carried out with 0.1 *M* phosphate buffer at *p*H 7 as the solvent instead of water as for Preparation A. Preparation C was an old preparation of unknown history. Preparation D was isolated from a different crop of plants in a manner identical with that for Preparation A. It was used without further purification in viscosity and sedimentation studies. For use in specific volume determinations it was further purified by precipitation with ammonium sulfate and by isoelectric precipitation. It was finally brought to the isoelectric point and dialyzed against distilled water. The ash content was determined to be 1.83%. This further purified material is referred to as Preparation D′. Preparation E, which was used only in specific volume studies, was obtained by subjecting the juice from diseased plants to three high-speed centrifugations, two isoelectric precipitations, two ammonium sulfate precipitations, and, finally, electrodialysis. It was titrated to *p*H 5 with sodium hydroxide to bring it back into solution. The ash content was found to be 1.22%.

III. Partial Specific Volume

In order to be able to determine molecular weight and particle dimensions by indirect physico-chemical procedures and in order to determine particle weights from direct measurements with the electron microscope, it is necessary to know the partial specific volume. Three different values for the partial specific volume of tobacco mosaic virus have been reported. Eriksson-Quensel and Svedberg[3] obtained a value of 0.646. Stanley found a value of 0.77.[4] Bawden and Pirie obtained 0.73 for tobacco mosaic virus and

(1) The data described in this paper were discussed in two papers presented before the Divisions of Biological Chemistry and Colloid Chemistry at the 107th meeting of the American Chemical Society, Cleveland, Ohio, April, 1944.

(1a) M. A. Lauffer and W. M. Stanley, *Chem. Rev.*, **24**, 303 (1939); *Kolloid-Z.*, **91**, 62 (1940).

(2) J. H. Bauer and E. G. Pickels, *J. Exptl. Med.*, **64**, 503 (1936).

(3) I. Eriksson-Quensel and T. Svedberg, THIS JOURNAL, **58**, 1863 (1936).

(4) W. M. Stanley, *J. Phys. Chem.*, **42**, 55 (1938).