[A CONTRIBUTION FROM THE FOOD RESEARCH INSTITUTE, STANFORD UNIVERSITY, AND THE AGRICULTURAL EXPERIMENT STATION, UNIVERSITY OF CALIFORNIA]

The Bitter Glucoside of the Olive

BY W. V. CRUESS AND C. L. ALSBERG

The bitter glucoside of olives, olive leaves and olive bark was studied by Bourquelot and Vintilesco[1] in 1908. They described it as a noncrystalline, intensely bitter substance, readily soluble in alcohol, fairly soluble in water and practically insoluble in ether. Its specific rotation was given by them as −127° and its reducing power on Fehling's solution as 0.412 that of dextrose. They christened it "oleuropein."

Power and Tutin,[2,3,4] in 1908, prepared a bitter mixture of several amorphous compounds from olive leaves and bark by the method of Bourquelot and Vintilesco. They inferred that the "oleuropein" of these investigators was not a pure substance. Vanzetti,[5] in 1909, obtained an intensely bitter levorotatory amorphous substance from olive twigs. Hilts and Hollingshead[6] (1920), in a report of studies on the chemical changes that occur during the ripening and pickling of olives suggested that the bitter principle is a tannin-like substance.

Isolation and Purification.—Olives were first extracted at the boiling point with water. Boiling destroys an emulsin-like enzyme in the fruit. The extract was cooled and the oil removed by skimming and subsequent filtering with infusorial earth. The filtered, oil-free extract was concentrated principally *in vacuo* to a heavy sirup, mixed with 5 volumes of acetone, and filtered. The filtrate was evaporated almost to dryness, usually *in vacuo*, taken up in absolute acetone and again evaporated almost to dryness. Some mannite separated on allowing the partially concentrated acetone solution to stand for several days. Salting out of the bitter principle with ammonium sulfate probably separated it from any residual mannite. The acetone solution was evaporated almost to dryness and the residue was taken up in anhydrous acetone, this cycle being repeated three times to eliminate as much as possible of the material that was insoluble or only slightly soluble in acetone. The residue left on evaporating the acetone was taken up in water, neutralized with barium hydroxide, filtered, and sufficient ammonium sulfate added to salt out the bitter principle. This was further purified by precipitation fractionally with lead acetate at approximately *p*H 4.5; decomposition of the precipitate with hydrogen sulfide; evaporation to dryness; solution in absolute acetone; concentration to dryness; dissolving in water; electrodialysis until free of electrolytes; concentration to dryness; dissolving in absolute acetone; addition of diethyl ether to precipitate most of the bitter principle and drying of the precipitate. The last few operations were repeated until there was no further change in optical rotation and copper-reducing power.

The substance purified as described was creamy-white in color and amorphous. Attempts to crystallize it from various solvents and mixtures of solvents failed.

Solubility.—The bitter principle is very soluble in ethyl alcohol, methyl alcohol, acetone, pyridine, glycerin and glacial acetic acid. It is quite soluble in hot water and to the extent of about 4% at room temperature. It is practically insoluble in common fat solvents such as ether, chloroform, carbon disulfide, etc., and only slightly soluble in ethyl acetate. On boiling an aqueous solution an insoluble resin forms, probably a phlobaphene (an anhydride) similar to that formed by pyrocatechol tannins.[7]

Qualitative Reactions.—It is salted out of aqueous solution by high concentrations of sodium chloride and several other salts, while ammonium sulfate causes the bitter principle to separate at as low a concentration as 0.025 saturated ammonium sulfate. The bitter principle is free from nitrogen. It is mildly acid in reaction.

It is precipitated by lead acetate, mercuric chloride and by silver nitrate, the silver in the precipitate being rapidly reduced to metallic silver. A green color, turning purple on addition of sodium bicarbonate is obtained with ferric chloride, indicating the presence of a pyrocatechol or orthodihydroxy grouping. It is precipitated by copper salts in dilute ammonia but not in acid solutions. It forms precipitates with nitrous acid and with bromine water, reactions for pyrocatechol tannins.

It is adsorbed by decolorizing carbons but not by freshly precipitated aluminum hydroxide, fuller's earth or infusorial earth. It is easily recoverable from the carbon by treatment with ethyl alcohol. It was not precipitated by gelatin nor adsorbed by hide powder; therefore it is not a tannin in the usual sense of this word.

It reduces Fehling's solution and potassium permanganate solution. It is levorotatory and its aqueous solution has a slightly higher refractive index than a sucrose solution of equal concentration.

On boiling aqueous solutions a resinous precipitate forms, probably a phlobaphene, characteristic of certain tannins (see Freudenberg[7]).

Specific Rotation.—The specific rotation of 0.5 to 3% solutions of the more highly purified samples in water ranged from −145 to −148° as compared to −127° reported by Bourquelot and Vintilesco. The specific rota-

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(1) E. Bourquelot and J. Vintilesco, *Compt. rend.*, **147**, 533–535 (1908): see also *J. Pharm. Chim.*, 303–314 (1908).

(2) F. B. Power and F. Tutin, *Pharm. J.*, **81**, 714–715 (1908).

(3) F. B. Power and F. Tutin, *J. Chem. Soc.*, **93**, 891–904 (1908).

(4) F. B. Power and F. Tutin, *ibid.*, **93**, 904–917 (1908).

(5) B. L. Vanzetti, *Atti. Accad. Lincei.*, **2**, 189–190 (1908).

(6) R. W. Hilts and R. S. Hollingshead, U. S. D. A. Dept. Bull., **903** (1920).

(7) K. Freudenberg, section on the tannins in Abderhalden, "Handbuch der biologischen Arbeitsmethoden," Abt. I, Teil 10, Heft 2, pp. 459–462 (1921).

tion was of similar magnitude in dilute solution in ethyl alcohol, pyridine and acetone, these solvents having little to no effect on this property.

Copper Reducing Power.—The average reducing power upon Fehling's solution was 0.47 of that of dextrose using the Munson–Walker method.

Potassium Permanganate Reducing Power.—By the usual method of titration of tannin solutions with standard potassium permanganate solution as described in "Methods of Analysis of the Association of Official Agricultural Chemists," using indigo carmine indicator, the reducing power was found to be approximately 45% of that of tannin.

Combustion Analysis.—Combustion analyses of the more highly purified samples gave the following average values for C, H and O: C, 53.65; H, 6.51 and O, by difference, 39.84.

Molecular Weight Determination.—By depression of the freezing point in glacial acetic acid the indicated molecular weight was 433 and by the boiling point method in alcohol, 470. Bumping interfered with the determination in alcohol. In water the substance apparently hydrates and may associate as the solution exhibits colloidal properties.

OH Groups.—It was readily acetylated and benzoylated in dry pyridine; 5 g. of bitter principle bound 3.85 g. of acetic anhydride, showing the presence of a number of OH groups. These represent the phenolic groups of the pyrocatechol nucleus and the OH groups of dextrose.

Acid and Enzyme Hydrolysis.—Emulsin from almonds slowly hydrolyzed the bitter principle, eventually reducing the optical rotation nearly to zero and causing a marked increase in copper reducing power. In olive leaves a much more active hydrolytic enzyme was found that carried the hydrolysis beyond zero rotation to a distinctly dextro rotation.

Pectinol, a commercial mold enzyme preparation furnished by Röhn and Haas Company, also gave rapid hydrolysis changing the rotation from levo to dextrorotation. Yeast invertase had no action. Evidently the substance is a beta glucoside. After enzyme hydrolysis the solution was still intensely bitter.

Mineral acids readily hydrolyzed the bitter principle at the boiling point. In order to study the products of acid hydrolysis an aqueous solution was boiled with 2% sulfuric acid for five hours under a reflux condenser. The solution after hydrolysis was neutralized with barium hydroxide and filtered to remove the barium sulfate. The filtrate was clarified with lead acetate to remove nonsugars. The copper-reducing power of the filtrate was determined with Fehling's solution. Calculated as dextrose, the maximum yield of sugar obtained in a comparative series in which the boiling period was varied from one-tenth to twenty-four hours was 30.00% by weight and for a period of five hours of boiling.

In the clarified samples copper reducing power and dextrose by polarization agreed closely, indicating absence of other optically active products of hydrolysis. The sugar present was isolated in crystalline form from the clarified filtrate; recrystallized from alcohol and identified by means of its osazone, specific rotation, fermentability by yeast and x-ray spectrum as alpha-*d*-glucose.

The non sugar fraction recovered from the lead precipitate was a bitter, optically inactive, water-soluble, noncrystalline substance that gave on combustion analysis: C, 65.65, 65.84; H, 6.80, 7.20. It was soluble in ether and gave a strong positive color reaction for the pyrocatechol group. It tended to polymerize and decrease greatly in solubility in water.

Hydrolysis with Alkali.—The bitter taste of the glucoside is destroyed by sodium hydroxide at approximately *p*H 10.5 to 11.5 depending on the temperature and time of treatment, although the solution changes but little in optical rotation unless treatment is very severe.

By hydrolysis of a 3% solution at 80 to 100° for ten minutes with either 4% $Ba(OH)_2 \cdot 8H_2O$ or 3% sodium hydroxide, acidification with hydrochloric or sulfuric acid to *p*H 3.5, and extraction with ether there was obtained a crystalline acid. This acid purified by recrystallization from 50% alcohol and finally from benzene–alcohol solution by the method of mixed melting points of the acids concerned comparison of x-ray spectra,[8] and by various qualitative tests proved to be caffeic acid, which has the formula $C_6H_3(OH)_2CH{=}CHCOOH$. The caffeic acid used for comparison was made from green coffee by the method described by Charaux.[9]

The OH groups are in the ortho position with respect to each other, *i. e.*, caffeic acid possesses an ortho dihydroxy or "catechol" group as well as one double bond. Its presence accounts in part at least for the strong pyrocatechol color reactions given by the bitter principle.

A small quantity of an unidentified crystalline phenol was obtained by subliming the crude phenol fraction from severe alkaline hydrolysis. This phenol melted at 91.5° and did not give color reactions for pyrocatechol.

From the product of mild alkaline hydrolysis in the cold there was obtained a water soluble substance, not bitter in taste, evidently a glucoside, that was strongly levorotatory and readily hydrolyzed by emulsion, Pectinol or by dilute sulfuric or hydrochloric acid, changing on hydrolysis to dextrorotation. Dextrose was recovered from the products of acid hydrolysis. The glucoside possessed approximately 0.4 the reducing power of dextrose on Fehling's solution.

Phlobaphene.—The water-insoluble resin formed on boiling a water solution of the bitter principle was purified by partial decolorization with vegetable decolorizing carbon in 95% alcohol. The dry substance contained 60.93% carbon, as compared with 53.65% in the original glucoside. Evidently as indicated by Freudenberg[7] the phlobaphene is in the nature of an anhydride; loss of water would increase the carbon content of the substance.

The average specific rotation in alcohol of several samples was −116.8° at approximately 20°. The reducing action on Fehling's solution and on permanganate was similar to that of the original glucoside. A crystalline acid was obtained on alkaline hydrolysis; this appeared from melting point and qualitative tests to be caffeic acid.

Oleuropein Content of Olives.—The bitter principle was extracted from the finely ground fresh flesh with acetone; this solution was filtered, concentrated almost to dryness in a round-bottomed flask, the final stage under vacuum;

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[8] Kindly obtained by Dr. W. H. Dore, Division of Plant Nutrition, University of California.

[9] C. Charaux, *J. pharm. chim.*, **2**, 292–298 (1911). Caffeic acid was prepared by C. Smith, of the University of California.

the residue was taken up in dry acetone, filtered, run almost to dryness as above, taken up in water, the bitter principle salted out with ammonium sulfate, dissolved in acetone, again taken almost to dryness as above, taken up in 95% alcohol and diluted to volume with water. Its concentration was determined by means of Fehling's solution by the Munson–Walker method. The oleuropein content of the flesh of twenty samples of olives, representing several varieties and several degrees of maturity, was found to range from 0.59 to 2.28%. The Mission variety was highest in content of bitterness and the Sevillano lowest. Manzanillo and Barouni varieties were also rich and the Ascolano poor in bitterness content. Green fruit was richer than the ripe in content of the bitter glucoside. The average concentration of all samples was about 1.0%. Bourquelot and Vintilesco reported 2% oleuropein in the fruit used by them.

Olives frozen on the trees in December, 1932, were found still to be intensely bitter ten days later, but devoid of the levorotatory oeluropein. On standing for another ten days the bitter taste disappeared. The fruit contains an emulsin-like enzyme that was liberated by freezing, and possibly another enzyme that split or oxidized the bitter ester remaining after hydrolysis of the glucoside.

Summary and Conclusions

1. The bitter principle was separated from olives and purified by a method that gave a product of considerably higher specific rotation than obtained by the method of Bourquelot and Vintilesco.[1]

2. It was found to be a bitter glucoside readily hydrolyzed by an enzyme occurring in olive leaves and by "Pectinol," a commercial Penicillium mold preparation. Emulsin hydrolyzed it slowly; invertase did not attack it.

3. Acid hydrolysis liberated *d*-glucose and an ether soluble, bitter ester not present before hydrolysis. During hydrolysis by Pectinol enzyme or by mineral acid the optical rotation changed from strongly levo to dextro, as the glucoside was split and dextrose liberated. The bitter ester was readily hydrolyzed by alkalies with the loss of its bitter taste.

4. The glucoside when treated with sodium hydroxide lost its bitter taste, but retained its levorotation if the treatment was not too severe.

5. From the products of alkaline hydrolysis was isolated a crystalline acid that gave the same qualitative reactions, same x-ray spectrum, and possessed the same melting point as caffeic acid. The melting point was not depressed by mixing the two acids. An unidentified crystalline phenol (not pyrocatechol) was also isolated.

6. From consideration of the properties determined it might be concluded that the bitter principle appears to have both a glucoside linkage and an ester linkage. The former is split by mineral acids, emulsin and other enzymes of emulsin-like nature, and the ester linkage by alkalies and apparently by an enzyme occurring in olives.

7. The bitter glucoside was found to be present in olive flesh to the extent of about 1% on the average, but its concentration in green fruit of the Mission and Manzanillo varieties was considerably higher, and in ripe fruit of the Ascolano and Sevillano varieties, considerably less than this general average.

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The Preparation of Derivatives for the Identification of Alkyl Chlorides[1]

BY H. W. UNDERWOOD, JR., AND JOHN C. GALE

No satisfactory method for the identification of alkyl chlorides is at present available. Marvel, Gauerke and Hill[2] prepared crystalline derivatives of a number of alkyl bromides and iodides and of five chlorides by the preparation of the Grignard compounds and the reaction of these with the corresponding mercuric halides to give the alkyl mercuric halides. Gilman and Furry[3] characterized several organomagnesium halides, including benzyl chloride, by reaction with α-naphthyl isocyanate to give the α-naphthalides. They stated that these derivatives were less soluble, better crystallized, higher melting, and more easily purified than the corresponding anilides. Schwartz and Johnson[4] identified in a corresponding manner sixteen alkyl bromides and three chlorides, namely, *t*-butyl, *t*-amyl and benzyl by converting the halides into the anilides. These anilides were in general satisfactory derivatives.

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[1] Because of the death of Professor Underwood the present paper has been prepared by the junior author.

[2] C. S. Marvel, C. Gauerke and E. Hill, THIS JOURNAL, **47**, 3009 (1925).

[3] H. Gilman and M. Furry, *ibid.*, **50**, 1214 (1928).

[4] A. M. Schwartz and J. R. Johnson, *ibid.*, **53**, 1063 (1931).