[CONTRIBUTION FROM THE FOOD RESEARCH DIVISION, BUREAU OF CHEMISTRY AND SOILS, UNITED STATES DEPARTMENT OF AGRICULTURE]

ISOCITRIC ACID[1]

BY E. K. NELSON

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The predominating acid of the blackberry is an optically active form of isocitric acid,[2] an acid which was synthesized by Fittig,[3] and later in the form of its ester, by Wislicenus and Nassauer.[4]

The optical activity of the triethyl isocitrate obtained on fractionating the ethyl esters of the acids from various lots of blackberries was not, however, found to be constant. From six lots of blackberries esters were obtained having optical rotation ranging from -9.7 to $+14.75°$, indicating that the isocitric acid of the blackberry is a mixture of the optical antipodes in varying proportions, their racemic mixture, of course, also being present. As there are two asymmetric carbon atoms in isocitric acid, and no meso form is possible, four optically active forms and two racemic forms of the acid may exist.

The present investigation was undertaken for the purpose of comparing the natural isocitric acid with the acids synthesized by Fittig and Wislicenus and further studying the properties of the blackberry acid.

Accordingly Fittig's and Wislicenus' syntheses were repeated, resulting in the production of a quantity of synthetic isocitric acid and triethyl isocitrate.

Fittig's isocitric acid (which he obtained only in partly crystalline form, mixed with lacto-isocitric acid) was obtained in practically pure form, both with and without water of crystallization, while his lacto-isocitric acid was produced in pure form, melting sharply at 160–161°.

The triethyl ester of isocitric acid prepared by Wislicenus was reported by him to boil at 149–150° at 14 mm. This boiling point could not be confirmed. Triethyl isocitrate prepared according to Wislicenus was found to boil at 180–181° at 10 mm., which is more in accordance with what would be expected for an isomer of triethyl citrate, which boils at 170° at 10 mm.

In esterifying isocitric acid directly, it was found that partial dehydration took place with the formation of diethyl lacto-isocitrate. The presence of this ester causes a marked rise in the boiling point and in the saponification equivalent and affects the melting point of the hydrazide obtained from the triethyl ester.

Better results were obtained by treating barium isocitrate, containing one molecule of water of crystallization, with absolute alcohol containing

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[1] Food Research Contribution No. 77.

[2] E. K. Nelson, THIS JOURNAL, **47**, 568 (1925).

[3] Fittig, *Ann.*, **255**, 47 (1889).

[4] Wislicenus and Nassauer, *ibid.*, **285**, 7 (1895).

enough hydrochloric acid to combine with the barium and leave about 2.5% excess. The barium chloride was filtered off and the esterification completed in the usual manner. In this way the triethyl ester was prepared from Fittig's acid. It had a saponification equivalent of 635 (calcd. 609.7) and boiled at 180–181° at 10 mm., whereas the ester prepared directly from the acid had a saponification equivalent of 664.6 and distilled only partly at 219° at 10 mm.

The ester prepared by the Wislicenus method had a boiling point of 181° at 10 mm., and the saponification equivalent was 613.

It was more difficult to prepare the natural acid or its corresponding lacto-acid in crystalline form, owing to the presence of the optically active form. However, both compounds were obtained and checked by optical crystallographic methods[5] with the acid and lacto-acid obtained by synthesis. Attempts to resolve the synthetic acid into optically active components by the use of cinchonine, cinchonidine or brucine were unsuccessful.

Experimental

Fittig's Synthesis.—Fittig's synthesis of isocitric acid was carried out: 32.4 g. of sodium succinate, dried at 140°, 29.5 g. of chloral and 20.4 g. of acetic anhydride were used. The yield of trichloromethylparaconic acid was 31 g., of barium isocitrate 46.4 g., and of isocitric acid, 20 g.

The aqueous solution of isocitric acid was evaporated to a sirupy consistency. From this, on long standing, crystals were deposited. These were separated by filtration and spread on a porous plate to dry. The acid melted at about 105° but softened much below that figure; 0.3210 g. required for neutralization 43.5 cc. of $N/10$ sodium hydroxide (in ice water) and 45.1 cc. on boiling, showing the presence of a little lacto-isocitric acid. 0.4203 g. lost 0.0815 g. of water at 100° in vacuo, corresponding to 19.39% of water (calcd. for 2.5 moles of H_2O, 20.5%). As isocitric acid is converted into lacto-isocitric acid at 100° *in vacuo*, losing $1H_2O$, 1.5 moles of water must be water of crystallization.

It was found that recrystallization from ethyl acetate gave the acid free from water of crystallization; 0.2928 g. of the acid thus recrystallized required for neutralization 44.52 cc. of $N/10$ sodium hydroxide in ice water, and the same amount on boiling, thus showing its freedom from lacto-isocitric acid; 44.52 cc. of $N/10$ sodium hydroxide represents 0.2849 g. of isocitric acid, corresponding to a purity of 99.26%. The balance is probably water and as strong drying tends to produce some lacto-isocitric acid, it was analyzed without further drying.

Anal. Subs., 0.1278, 0.1015: CO_2, 0.1736, 0.1384; H_2O, 0.0486, 0.0375. Calcd. for $C_6H_8O_7$: C, 37.50; H, 4.19. Found: C, 37.06, 37.19; H, 4.25, 4.13.

Optical Crystallographic Data.—Fittig's isocitric acid (crystallized from ethyl acetate) consists of rods and irregular fragments. Its indices of refraction are N_α = 1.519, N_β = 1.527, N_γ = 1.548, all ±0.003. On the rods the extinction is straight and the sign of elongation +. The double refraction is rather strong ($N_\gamma - N_\alpha = 0.029$).

Owing to the ease with which isocitric acid loses water, changing into lacto-isocitric acid, a sharp melting point could not be obtained.

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[5] The optical crystallographic examinations were made by George L. Keenan of the Food, Drug and Insecticide Administration.

When dried *in vacuo* at 100° the isocitric acid is completely changed into lacto-isocitric acid. This product was recrystallized from boiling chloroform, in which it is sparingly soluble, and was obtained as a crystalline white substance, melting sharply at 160–161°; 0.2511 g. required 30.0 cc. of $N/10$ NaOH when titrated in ice water and 42.2 cc. in boiling water.

Anal. Subs., 0.0676, 0.0812: CO_2, 0.1016, 0.1223; H_2O, 0.0222, 0:0252. Calcd. for $C_6H_6O_6$: C, 41.37; H, 3.47. Found: C, 41.00, 41.09; H, 3.67, 3.47.

Optical Crystallographic Data.—This material crystallizes in irregular fragments without definite habit. The indices of refraction are, $N_\alpha = 1.487$, $N_\beta = 1.505$, $N_\gamma = 1.600$, all ±0.003. The optic sign is + and the double refraction is extremely strong ($N_\gamma - N_\alpha = 0.113$).

Direct esterification of Fittig's isocitric acid by refluxing with absolute alcohol containing 2.5% of hydrochloric acid led to no satisfactory result, as the resulting product distilled only partly at about 219° at 10 mm. and this had a saponification equivalent of 664.6, the calculated saponification equivalent of triethyl isocitrate being 609.7 and of diethyl lacto-isocitrate 731.4.

It is therefore evident that dehydration had occurred. In order to avoid this, 40 g. of barium isocitrate was treated with an absolute alcohol solution of hydrochloride in sufficient quantity to liberate the isocitric acid and leave a sufficient excess to carry on the esterification. After standing for some time, the barium chloride was filtered off and the filtrate refluxed in the usual manner.

In this way an ester was obtained which boiled at 180–181° at 10 mm. and had a saponification equivalent of 635, which is still high for triethyl isocitrate. In order to prepare the pure ester and through this to obtain pure hydrazides, Wislicenus' synthesis was resorted to.

Wislicenus' Synthesis.—Oxal-succinic ester, prepared and purified as directed by Wislicenus, except that it was not distilled, was reduced by sodium amalgam, As the reduction under water was slow and incomplete, dilute alcohol was used as the solvent. When the ester no longer gave a color reaction with ferric chloride, the reduction was regarded as complete and the product was extracted with ether.

The ester, left after evaporation of the ether, gave a negative ferric chloride reaction. It boiled at 10 mm. at 181°.

Anal. Subs., 0.1075, 0.0996: CO_2, 0.2044, 0.1901; H_2O, 0.0681, 0.0620. Calcd. for $C_{12}H_{20}O_7$: C, 52.17; H, 7.24. Found: C, 51.87, 52.06; H, 7.09, 6.96. Saponification equivalent: 613.5, 613. Calcd.: 609.7.

The hydrazide was prepared from Wislicenus' ester in the usual manner by mixing the solution of the ester in absolute alcohol with a moderate excess of hydrazine hydrate, warming the mixture on the water-bath, and allowing it to stand at room temperature for several days. The hydrazide was recrystallized by solution in warm water and precipitation with alcohol. Thus recrystallized it melted, with decomposition, at 199°.

Anal. Subs., 0.0613, 0.1305: CO_2, 0.0693, 0.1256; H_2O, 0.0329, 0.0692. Subs., 0.0508, 0.0415; N, 14.23 cc., 11.63 cc. at 0° and 760 mm. Calcd. for $C_6H_{14}N_6O_4$: C, 30.76; H, 6.02; N, 35.89. Found: C, 30.84, 30.81; H, 6.00, 5.94; N, 35.03, 35.05.

Optical Crystallographic Data.—This material crystallizes in rods and needles. Its indices of refraction are $N_\alpha = 1.540$ (crosswise), $N_\gamma = 1.632$ (lengthwise), both ±0.003. The sign of elongation is plus and the extinction is straight.

The analysis shows a deficiency of nitrogen for isocitric trihydrazide. The hydrazide is unstable, is easily hydrolyzed by hot water, and has not been obtained in a state of absolute purity. For this reason and also because the natural acid in the blackberry is a mixture of optically active and inactive acids, too much dependence cannot be placed on the melting point of the hydrazide of the natural acid.

Identification by optical crystallographic methods is preferable, and this shows that the hydrazides of the natural and synthetic acids correspond.

The hydrazide of the synthetic acid is less soluble in cold water than the hydrazide of the natural acid, and, while optical crystallographic data agree, the presence in the natural acid of the optically active form undoubtedly influences the properties of the acid, including the solubility and melting point of the hydrazide derived therefrom.

This assumption is borne out by the fact that an ester of the blackberry acid with a low optical rotation (−0.85°) gave a hydrazide less soluble in water than esters from the natural source with higher optical activity.

An attempt to racemize the natural acid by boiling with strong sodium hydroxide solution was unsuccessful.

Preparation of Isocitric Acid from Blackberries.—In order to study the optical activity of the triethyl isocitrate prepared from blackberries and to obtain material for comparison with the synthetic acid, the acid from several lots of blackberries was studied during the summer of 1929.

One lot of dewberries was included, but as these had some blackberries mixed with them the data cannot be taken as representing authentic dewberries, although the results are practically conclusive that isocitric acid is also the predominating acid of that fruit.

For the sake of comparison the characteristics of the highest boiling ester from these blackberries and those examined previously[2] are given in Table I.

TABLE I

CHARACTERISTICS OF THE HIGHEST BOILING ESTER FROM BLACKBERRIES

Ethyl ester from blackberries		B. p., °C., at 10 mm.	α_D of ester	Sap. equiv.	M. p. of hydrazide, °C.
Lot 1	1925	175–180	− 7.75°	614	178–179
Lot 2	1925	175–178	+14.75°	...	170–172
Lot 3	1925	175–178	+13.10°	619.7	170–172
Lot 4	1929	180–181	+ 5.50°	621.3	173–174
Lot 5	1929	182–187	+ 1.60°	629	179–180
Lot 6	1929	188–190	− 9.70°	651.7	.....

Lot 5 was purchased for dewberries. The acids from Lots 4, 5 and 6 were probably dried too much before esterification, resulting in partial conversion into lacto-isocitric acid.

There are two reasons for the fact that the melting points of the hydrazides and the boiling points of the esters vary. The higher saponification equivalents indicate a certain amount of dehydration during esterification, no doubt resulting in the contamination of triethyl isocitrate with diethyl lacto-isocitrate and a consequent rise in the boiling point. The variations in the optical rotation of the ethyl isocitrate from blackberries, showing variations in dextro and levo forms with the racemic form, also has its effect on the melting points of the hydrazides and on the boiling points of the esters.

As resolution of inactive (synthetic) isocitric acid with various alkaloids was so far unsuccessful, it was sought to recrystallize the hydrazides and obtain a form that would check with the hydrazide prepared from Wislicenus' triethyl isocitrate.

From the hydrazide of the ester from Lot 5, a hydrazide was obtained on fractional crystallization which melted at 196–197°, showing no depression in melting point when mixed with the hydrazide of Wislicenus' ester melting at 199°, and which gave optical crystallographic data identical with those of the Wislicenus' ester. It may be taken as proved, therefore, that the racemic form of the isocitric acid of the blackberry is identical with the synthetic isocitric acid.

The hydrazide prepared from Fittig's isocitric acid melted at 197–198°, and also checked with the hydrazide of Wislicenus' acid on optical crystallographic examination.

The difficulty of preparing a pure, crystalline isocitric acid from blackberries which will check with the synthetic acid is due to the fact that the blackberry acid contains varying amounts of the optically active acid.

White, crystalline isocitric acid was prepared from the blackberry acid, but titration showed it to contain a very considerable amount of lacto-isocitric acid, so that it was not analyzed. However, optical crystallographic examination showed the presence in it of the inactive isocitric acid, and also showed the identity of the lacto-isocitric acid of the blackberry with synthetic lacto-isocitric acid. Optical crystallographic examination further showed the hydrazide of the inactive acid in the hydrazides from the isocitric acid obtained from several lots of blackberries.

As shown in Table I, an ester with a high optical activity will give a hydrazide with a low melting point. The identification of isocitric acid by means of its ester and hydrazide requires that the acid be not dried too much before esterification and that the hydrazide be recrystallized in order to isolate the higher-melting hydrazide of the racemic acid. Much loss will occur, however, due to hydrolysis of the hydrazide if the hydrazide is kept long in contact with warm water.

Summary

Fittig's isocitric acid and the lacto-isocitric acid resulting from its dehydration have been prepared. Synthetic isocitric acid has been prepared for the first time in relatively pure form, free from lacto-isocitric acid and the latter has been prepared in sufficient purity to give a sharp melting point.

Wislicenus' triethyl isocitrate was prepared and its boiling point found to be much higher than the figure given by Wislicenus. The hydrazide of isocitric acid was prepared from this ester, but not in absolutely pure form.

Comparison of the hydrazide of natural isocitric acid from blackberries with the hydrazide of the synthetic isocitric acid shows that the former, recrystallized until the melting point is raised to 196–197°, is identical with the latter. The evidence proves that the natural isocitric acid is a mixture of dextro and levo isocitric acid with one or the other optical anti-

pode predominating, and that the synthetic acid is the racemic form of the same acid.

Washington, D. C.

[Contribution from the Pearson Memorial Laboratory of Tufts College]

STUDIES IN THE DIPHENYL SERIES. III. SOME PHOSPHORUS DERIVATIVES OF DIPHENYL

By David E. Worrall

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It has been shown in previous communications[1] that 4-chlorodiphenyl condenses smoothly with arsenic and antimony chlorides in the presence of sodium. The resulting tri-arylated metalloids in general have properties similar to the corresponding phenyl compounds and are useful for the preparation of derivatives containing one, two or three diphenyl groups. The study of biphenylated substances has been extended to phosphorus, the results of which are presented in the present communication.

Tri-biphenylphosphine was obtained by the action of sodium on 4-chlorodiphenyl and phosphorus trichloride dissolved in benzene.

$$3C_6H_5C_6H_4Cl + PCl_3 + 6Na = (C_6H_5C_6H_4)_3P + 6NaCl$$

The procedure followed gave directly a yield of approximately 55% of fairly pure material. The total amount of product formed was about 75% of the theoretical. Ether[2] has been used previously in similar condensations, but the results obtained in this Laboratory indicate the superiority of benzene.

Certain differences in behavior become apparent on comparing the chemical properties of tri-biphenylphosphine with the corresponding arsenic and antimony derivatives. The heats of formation of the halides of these elements increase with rising atomic weight. Therefore it was not surprising that the halogen addition products of tri-biphenylstibine were easily formed. Tri-biphenylarsine gave more trouble and the di-iodide was not isolated. Chlorine and bromine formed addition products that separated from solution with chloroform of crystallization, as was true in the stibine series. These substances, possibly because of the presence of chloroform and insolubility in water, are much less sensitive toward moisture than the corresponding phenyl derivatives. There was every indication, using tri-biphenylphosphine, that a reaction took place with chlorine and bromine, although no apparent change was observed with iodine. Yet only oils were obtained. This failure to obtain crystalline products presumably was due to the extreme hygroscopicity of the phosphorus derivatives, followed by partial hydrolysis.

$$(C_6H_5C_6H_4)_3PBr_2 + H_2O = (C_6H_5C_6H_4)_3PO + 2HBr$$

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[1] Worrall, This Journal, **52**, 664 (1930).

[2] Michaelis and Reese, *Ber.*, **15**, 1610 (1882).