Summary

1. The following compounds have been prepared and described for the first time: (a) The lithium, calcium, barium and strontium salts of 3-acetylamino-4-hydroxyphenylarsonic acid; (b) 5-bromo-3-nitro-4-hydroxyphenylarsonic acid, 5-bromo-3-amino-4-hydroxyphenylarsonic acid and 5-bromo-3-acetylamino-4-hydroxyphenylarsonic acid; (c) 3,5-di-(formylamino)-, 3,5-di-(propionylamino)-, 3,5-di-(butyrylamino)-, 3,5-di-(chloro-acetylamino)-4-hydroxyphenylarsonic acids and their sodium salts.

2. 5-Bromo-3-amino-4-hydroxyphenylarsonic acid is neither more trypanocidal or spirocheticidal than 3-amino-4-hydroxyphenylarsonic acid or its N-acetyl derivative.

3. The least toxic of all the compounds discussed in this paper is 3,5-di-(acetylamino)-4-hydroxyphenylarsonic acid. When injected intravenously into white rats its maximum tolerated dose is 4.6 times greater than that of 3-acetylamino-4-hydroxyphenylarsonic acid.

PHILADELPHIA, PENNSYLVANIA

[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF COLUMBIA UNIVERSITY, No. 593]

THE SYNTHESIS OF 6-HYDROXYPIPERONYLIC ACID AND INCIDENTAL COMPOUNDS

BY MARSTON TAYLOR BOGERT AND FRANK ROSE ELDER[1]

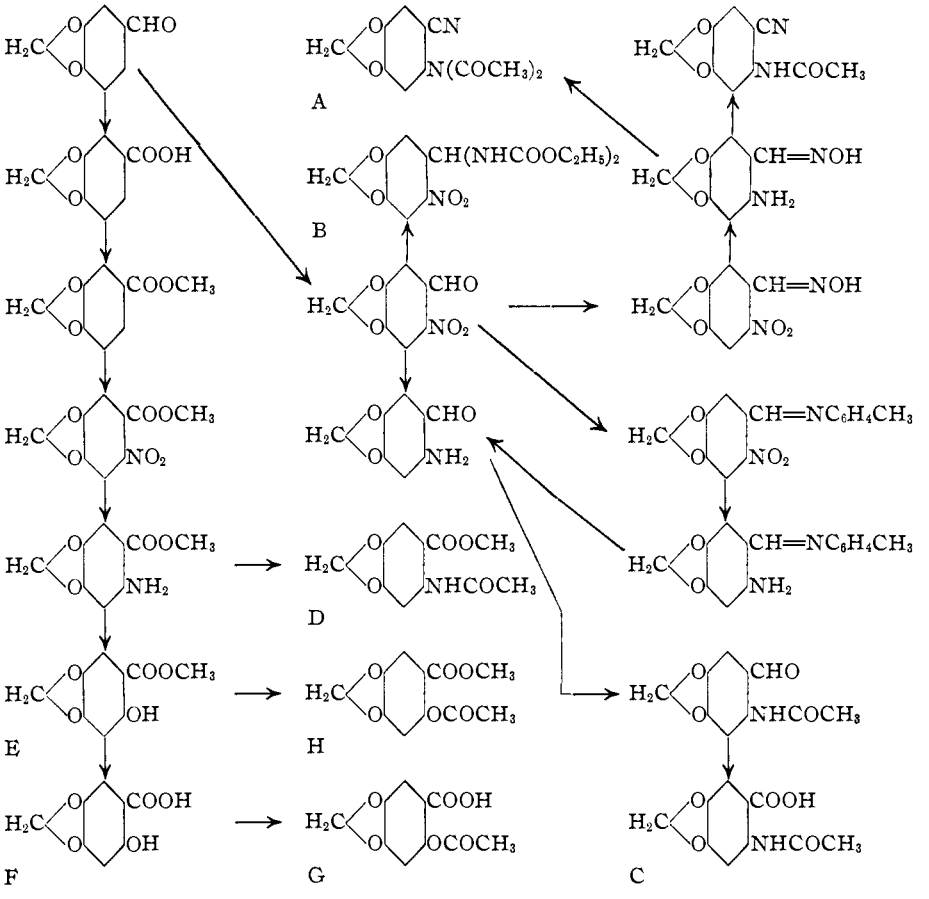
RECEIVED OCTOBER 1, 1928 PUBLISHED FEBRUARY 5, 1929

Salicylic acid and its derivatives, notably acetyl-salicylic acid (Aspirin), have proved to be of sufficient therapeutic value to justify additional experimental work for the purpose of gaining more light upon the connection between chemical constitution and physiological effect in the group of the *o*-hydroxybenzoic acids.

The experimental work which follows describes the synthesis of 6-hydroxypiperonylic acid, some of its derivatives and incidental products. This particular acid was selected because we failed to find any description of it in the literature, because it is structurally the methylene ether of a dihydroxysalicylic acid and because of the fact that the methylene-dioxy grouping is of frequent occurrence in natural products, including certain of the alkaloids.

The various lines of approach to this goal are sufficiently indicated in the flow diagram. The route via piperonylic acid proved most satisfactory, for reasons set forth in the Experimental Part.

[1] Based upon the Dissertation submitted by Dr. Elder in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University, 1927. An abstract of this paper was read before the Division of the Chemistry of Medicinal Products, at the Richmond Meeting of the American Chemical Society, April, 1927.



It is worth noting that methyl 6-hydroxypiperonylate, in contradistinction to methyl salicylate, possessed only an exceedingly faint agreeable odor, without any of the wintergreen character. Similarly, the methyl ester of the 6-aminopiperonylic acid was a colorless and practically odorless crystalline solid, with none of the aroma peculiar to methyl anthranilate.

Of the 6-hydroxypiperonylic acid, and of its acetyl derivative, sufficient material has not been accumulated as yet to proceed with the pharmacological tests.

Heretofore, 6-aminopiperonal has been obtained only indirectly from the 6-nitro compound, through the reduction of the *p*-toluidine derivative of the latter and hydrolysis of the resultant 6-aminopiperonylidene-*p*-toluidine, all attempts at the direct reduction of the nitro-aldehyde itself having failed. We have found that this direct reduction of the nitro to the amino-aldehyde can be accomplished with ferrous sulfate and ammonia, under proper conditions, with a yield of 60%.

Experimental

6-Nitropiperonal was prepared by the process of Salway,[2] with certain modifications which were found to give larger yields, as follows.

To 500 cc. of concentrated nitric acid (sp. gr. 1.42) cooled to 0°, there was added 50 g. of finely pulverized piperonal slowly and with constant stirring, the temperature being maintained below 0° during this addition and for two to three hours thereafter. If some of the piperonal still remained undissolved, the freezing mixture was removed and the mixture allowed to warm gradually, until all was dissolved. The solution was poured immediately into ice water, the yellow precipitate collected, washed free from nitric acid and stirred with sufficient 40% sodium bisulfite solution to make a thick paste. Two volumes of water were added and the mixture filtered. The residue was subjected to the same treatment and this was repeated with the two or three succeeding residues. The combined filtrates were made alkaline with sodium hydroxide, the precipitate collected, washed thoroughly and crystallized from a 1:1 mixture of ethyl acetate and alcohol. The nitropiperonal so obtained formed long, pale yellow needles, m. p. 97–98° (corr.) (Salway gives 98°); yield 46 g., or 70%. There was recovered also 14 g. of 4-nitro-1,2-methylenedioxybenzene which, taken together with the nitropiperonal, accounts for 96% of the piperonal used. The formation of this by-product, through replacement of the aldehyde group during the nitration, was noted first by Salway.

6-Nitropiperonylidene-diurethan.—In 1913 Bianchi[3] condensed piperonal with ethylurethan and obtained a product in good yield which was easily hydrolyzed. Experiments were conducted therefore with nitropiperonal, for the purpose of preparing a similar urethan condensation, to be reduced to the corresponding amino derivative and the latter hydrolyzed to the 6-aminopiperonal.

A mixture of 5 g. of nitropiperonal and 4.86 g. of ethylurethan was heated at 100–110°. To the clear yellow melt there was added 1 cc. of concentrated hydrochloric acid, the mixture was well stirred and the heating continued until the evolution of vapor ceased. The hydrochloric acid addition caused the mixture to turn first green, then brown and finally black, and nearly solid. It was left overnight, then dissolved in a mixture of alcohol, chloroform and acetone, boiled for several hours with a decolorizing carbon, filtered and the filtrate concentrated. The brownish product which separated was collected, washed with a little cold alcohol, boiled in ethyl acetate solution with a decolorizing carbon and the filtrate concentrated and cooled. There separated a fine, feathery solid, with a faint yellowish-green tinge, which melted with decomposition at 207–208° (corr.); yield, 1 g., or 10%. On long exposure to the light, its color changed to a yellowish brown. For analysis, the product was dried to constant weight at 110–115°.

Anal. Calcd. for $C_{14}H_{17}O_8N_3$: C, 47.32; H, 4.83; N, 11.83. Found: C, 47.10, 46.67; H, 5.27, 4.94; N, 11.98.

Considerable difficulty was experienced in purifying this product through failure to find any satisfactory solvent or mixture of solvents. It dissolved in both acids and alkalies, and when dry was exceedingly light, fluffy and triboelectric. Because of these unpromising results, further work along this line was abandoned.

6-Nitropiperonal Oxime.—Haber[4] described this oxime but gave no

[2] Salway, *J. Chem. Soc.*, **95**, 1155 (1909).

[3] Bianchi, *Gazz. chim. ital.*, **43**, I, 237 (1913).

[4] Haber, *Ber.*, **24**, 625 (1891).

details concerning its preparation or yield. We found that dry potassium carbonate as a condensing agent gave better results than zinc oxide.

To an alcoholic solution of the aldehyde (10 g.) and hydroxylamine hydrochloride (3.6 g.), there was added dry potassium carbonate (3.6 g.) dissolved in a little water, and the mixture was boiled for three hours. The yellow oxime which separated as the mixture cooled, when recrystallized from alcohol, melted at 201–203° (corr.); yield, 84% (9 g.). Haber gave the m. p. as 203°.

6-Aminopiperonal oxime was prepared from the nitro oxime by reduction in alcoholic solution with ammonia and hydrogen sulfide, essentially as described by Haber.[4] Decolorized and crystallized from alcohol, it formed nearly colorless, glistening needles. m. p. 182–183° (corr.); yield, 81%. Haber recorded the melting point as 175.5°.

6-Acetaminopiperonylonitrile.—Haber[4] reported that when he heated the above amino oxime for a short time with excess of acetic anhydride and sodium acetate, the acetaminopiperonylonitrile, m. p. 216° (corr.) resulted. We found that, depending upon the duration of this heating, the product was either the mono- or the diacetyl derivative.

A mixture of 2 g. of the amino oxime, 1 g. of fused sodium acetate and 20 g. of acetic anhydride was boiled for ten to twelve minutes and then poured into an excess of cold dilute sodium carbonate solution. The precipitate (1 g.) was collected, washed, crystallized from alcohol and then from chloroform. A small amount (0.3 g.) of fine, feathery needles was thus secured, m. p. 215–217° (corr.), which was dried *in vacuo* over concentrated sulfuric acid and analyzed.

Anal. Calcd. for $C_{10}H_8O_3N_2$: C, 58.82; H, 3.92; N, 13.73. Found: C. 58.78; H, 4.22; N, 13.82.

Briefer heating gave no appreciable quantity of this mono-acetyl derivative. In boiling ethyl or *iso*-amyl acetate solution, the acetylation failed wholly, and the amino oxime was recovered unaltered.

The yield of mono-acetyl derivative noted did not seem to us sufficient to make this route of approach to our goal a very promising one and the work was discontinued.

6-Diacetaminopiperonylonitrile.—When the amino oxime (2.5 g.), fused sodium acetate (2 g.) and acetic anhydride (15 g.) were boiled together for an hour, the mixture poured into the dilute carbonate solution and the precipitate crystallized from alcohol, thin plates (1.5 g.) were obtained, m. p. 115–122° (corr.). These were dissolved in chloroform (10 cc.) cold. Spontaneous evaporation of the solvent yielded colorless, thin rectangular plates, m. p. 146–147° (corr.), which were dried *in vacuo* over concentrated sulfuric acid and analyzed.

Anal. Calcd. for $C_{12}H_{10}O_4N_2$: C, 58.54; H, 4.07; N, 11.38. Found: C, 58.64; H, 4.18; N, 11.92, 11.93.

6-Nitropiperonylidene-*p*-toluidine.—Rilliet and Kreitmann,[5] after reviewing the failure of other workers to obtain 6-aminopiperonal by direct reduction of the nitro-aldehyde, succeeded in preparing it indirectly through the *p*-toluidine derivative of the latter.

On repeating this work, we found that the use of a 20% excess of *p*-toluidine gave a much better yield of the condensation product (90% instead of 75%). Since this product is very sensitive to the light, rapidly turning dark red, the condensation and subsequent crystallization were carried out in the dark, and long glistening yellow needles

[5] Rilliet and Kreitmann, *Compt. rend.*, **157**, 782 (1913).

were thus secured, m. p. 121–122° (corr.). Rilliet and Kreitmann gave the m. p. as 121.5°.

6-Aminopiperonylidene-*p*-toluidine was prepared from the nitro derivative by reduction in alcoholic solution with sodium sulfide, as described by Rilliet and Kreitmann.[5] The crude product (yield, 60%) was crystallized twice from alcohol and then once from benzene. Small rosets of fine, silky yellow needles resulted, m. p. 137–138° (corr.); yield, 12%. Rilliet and Kreitmann reported a yield of 54% and a melting point of 134.5°. Our product was dried *in vacuo* over concentrated sulfuric acid and analyzed.

Anal. Calcd. for $C_{15}H_{14}O_2N_2$: C, 70.82; H, 5.60; N, 11.02. Found: C, 70.61; H, 5.85; N, 10.89.

We tried also the reduction method of Cobenzl,[6] using sulfur and sodium sulfide in alcoholic solution, but the yield of amino derivative was less.

6-Aminopiperonal.—Rilliet and Kreitmann[5] stated that they hydrolyzed the *p*-toluidine derivative by boiling it with dilute sodium hydroxide. Judging by our own experience, they must have meant an alcoholic and not an aqueous alkaline solution, for we found that twenty hours' boiling with a 10% aqueous sodium hydroxide solution was without effect. However, it was easily hydrolyzed as follows.

A solution of 6 g. of the *p*-toluidine derivative and 15 g. of potassium hydroxide in 300 cc. of alcohol was boiled under a reflux condenser for three to four hours, an equal volume of water added and steam blown through as long as any *p*-toluidine came over. The residual liquid was filtered hot and as the filtrate cooled yellowish needles of the aminopiperonal separated which were crystallized from water and then melted at 107–108° (corr.); yield, about 50%. Rilliet and Kreitmann gave the m. p. as 107°.

The direct reduction of 6-nitropiperonal has been essayed unsuccessfully by various investigators,[7] and we were equally unsuccessful when we attempted to apply the method used by Bamberger and Demuth[8] for the reduction of *o*-nitrobenzaldehyde to the amino-aldehyde, by the use of titanous chloride,[9] or by the action of hydrogen sulfide and ammonia. We were more fortunate, however, when we tried ferrous sulfate and ammonia.[10]

To a solution of 100 g. of ferrous sulfate in 500 cc. of boiling water there was added a hot aqueous solution of 10 g. of nitropiperonal, followed by about 100 cc. of concentrated ammonium hydroxide solution in small portions, until the solution was alkaline to litmus. After boiling the mixture for five minutes, it was filtered, the precipitate washed thoroughly with boiling water and the filtrate and washings were combined and cooled. The crude amino-aldehyde which separated was recrystallized from water and then formed long yellow needles, m. p. 107–108° (corr.), identical with that obtained by the Rilliet and Kreitmann method; yield, 5 g., or 60%.

Attempts to replace the amino group by hydroxyl, through the diazo reaction, proved futile.

---

[6] Cobenzl, *Chem.-Ztg.*, **39**, 859 (1915).

[7] (a) Haber, *Ber.*, **24**, 625 (1891); (b) Friedländer and Schreiber, *ibid.*, **28**, 1385 (1895); (c) Oertley, "Thése," Université de Genève, 1910.

[8] Bamberger and Demuth, *ibid.*, **34**, 1330 (1901).

[9] (a) Knecht, *ibid.*, **36**, 166 (1903); (b) Sachs and Sichel, *ibid.*, **37**, 1862 (1904).

[10] Jacobs and Heidelberger, THIS JOURNAL, **39**, 1435 (1917).

Acetyl Derivative.—Colorless needles (from water), m. p. 161–162° (corr.). Rilliet and Kreitmann gave the melting point as 160°.

6-Acetaminopiperonylic Acid.—The 6-acetaminopiperonal (3 g.) was oxidized by potassium permanganate in the presence of magnesium sulfate, and the crude product crystallized from dilute alcohol in faintly yellowish, long feathery needles, m. p. 124–125° (corr.), soluble in alcohol but difficultly soluble in water; yield, 0.4 g. It was dried *in vacuo* over concentrated sulfuric acid and analyzed.

Anal. Calcd. for $C_{10}H_9O_5N$: C, 53.81; H, 4.03. Found: C, 52.97, 54.13; H, 4.09, 4.81.

While these analytical results are not very satisfactory, they are sufficiently close, if considered in conjunction with the method of preparation, to support the assumption that the product was the acid stated. No material was left for further analyses and the preparation was not repeated.

Piperonylic acid was prepared most conveniently as follows. To 3 liters of water, there was added 50 g. of finely powdered piperonal and the mixture was heated to 40–50°, stirred mechanically and potassium permanganate solution run in slowly until the odor of piperonal was no longer perceptible. This required generally about 125 g. of the permanganate. Excess of the latter, if any, was destroyed by the addition of a little alcohol, the mixture filtered hot and the filtrate acidified with hydrochloric acid. The piperonylic acid which separated as the solution cooled was practically pure; yield, 36 g., or 65%.

When the oxidation was conducted in acetone, instead of in aqueous solution, the yield was about the same. Oxidation in the presence of magnesium sulfate, however, resulted in considerably lower yields.

Methyl 6-Aminopiperonylate.—Piperonylic acid was converted into its methyl ester, the latter nitrated and the nitro ester reduced to the 6-amino ester, as described by Oertley and Pictet[11] and with similar results.

Acetyl Derivative.—Colorless, fine fluffy needles (from alcohol), m. p. 183–184° (corr.); soluble in alcohol, acetone, chloroform or benzene, less so in ether, and but slightly soluble in water. It was dried *in vacuo* over concentrated sulfuric acid and analyzed.

Anal. Calcd. for $C_{11}H_{11}O_5N$: C, 55.70; H, 4.64. Found: C, 55.63, 55.88; H, 4.83, 4.93.

Methyl 6-Hydroxypiperonylate.—The conversion of the amino into the hydroxy ester proved to be a rather troublesome task. When the diazotized ester was boiled with water, subjected to a current of steam, or dropped into a boiling borax solution, a dark red product resulted from which we were unable to recover any of the desired hydroxy ester. The problem was finally solved as follows.

To a well-cooled solution of 5 g. of the amino ester in 10 g. of concentrated sulfuric acid and 25 cc. of water, there was added 2.15 g. of potassium nitrate in 10 cc. of water and the diazo solution was dropped slowly into a boiling solution of 25 g. of copper sulfate in 25 cc. of water. The resultant solution was poured into 400–500 cc. of water and the mixture left overnight at room temperature. The reddish-brown precipitate was collected, washed thoroughly with water, decolorized in boiling alcoholic solution and the filtrate allowed to cool. There separated colorless rosets, m. p. 97–98° (corr.); yield, 50%. This proved to be a mixture of the desired ester with a small amount of 6-hydroxypiperonylic acid. It was therefore shaken first with a dilute sodium car-

[11] Oertley and Pictet, *Ber.*, **43**, 1336 (1910).

bonate solution, to remove the free hydroxy acid, and the residue then extracted with dilute sodium hydroxide solution. The use of too strong a caustic alkali solution must be avoided. The caustic soda extracts were acidified, the colorless precipitate was collected, washed, dried, dissolved in a mixture of alcohol and ether and the solvent allowed to evaporate spontaneously. The hydroxy ester crystallized in small colorless rosets, m. p. 99–100° (corr.), which were practically odorless and gave a deep blue color with ferric chloride solution. These crystals were dried *in vacuo* over concentrated sulfuric acid and analyzed.

Anal. Calcd. for $C_9H_8O_5$: C, 55.10; H, 4.11. Found: C, 55.19; H, 4.28.

The acetyl derivative, from the ester and acetyl chloride, was decolorized in ether solution and then separated in nearly colorless crystals, m. p. 50–60°. Recrystallized from a mixture of alcohol and ether, it formed rosets of nearly colorless needles, m. p. 97–98° (corr.); yield, 3.5 g. or 60%. For analysis, it was dried *in vacuo* over concentrated sulfuric acid.

Anal. Calcd. for $C_{11}H_{11}O_5$: C, 55.46; H, 4.20. Found: C, 55.29; H, 4.25.

6-Hydroxypiperonylic Acid.—The methyl ester was hydrolyzed by boiling it for about an hour in dilute (5%) potassium hydroxide solution until a test portion no longer yielded a precipitate when saturated with carbon dioxide. The slightly reddish solution was acidified with dilute hydrochloric acid, the precipitate collected, washed and dried; yield, 7 g. or 80%. Recrystallized from either chloroform or dilute alcohol, it formed nearly colorless fluffy needles, m. p. 211–212° (corr.), easily soluble in alcohol, but only slightly in water, ether, chloroform or benzene. For analysis it was dried *in vacuo* over concentrated sulfuric acid; (a) was crystallized from alcohol, and (b) from chloroform.

Anal. Calcd. for $C_8H_6O_5$: C, 52.74; H, 3.29. Found: C, (a) 52.38, (b) 52.45; H, (a) 3.24, (b) 3.26.

With ferric chloride solution, this acid gave the same deep blue color as its methyl ester.

6-Acetoxypiperonylic Acid.—Very great difficulty was experienced in the preparation of this aspirin by direct acetylation of the hydroxy acid and no satisfactory method was worked out, although a great deal of experimental work was done and many different processes were tried.

About equal parts of the hydroxy acid and fused sodium acetate were dissolved in acetic anhydride, an excess of acetyl chloride was added, the mixture boiled for six to eight hours, cooled, poured into a large volume of cold water and the precipitate removed, washed and dried. Fractional crystallization of this product from acetic anhydride, repeated 5 or 6 times, washing the crystals each time with a little cold ether, finally yielded a small quantity of nearly colorless needles, which melted at 155.5–156.5° (corr.), with slight preliminary softening, were freely soluble in alcohol, glacial acetic acid or acetic anhydride and dissolved but slightly in water, ether, chloroform or benzene.

Anal. Calcd. for $C_{10}H_8O_6$: C, 53.57; H, 3.57. Found: C, 53.77; H, 3.96.

Summary

1. 6-Hydroxypiperonylic acid and its acetyl derivative, the former a derivative of salicylic acid and the latter of aspirin have been synthesized from piperonal, for the purpose of pharmacological investigation.

2. It has been shown that 6-nitropiperonal can be reduced directly to the amino aldehyde by the use of ferrous sulfate and ammonia.

3. Other derivatives of piperonal and of piperonylic acid have been prepared and described.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE MOLECULAR WEIGHT OF THE HEMOCYANIN OF LIMULUS POLYPHEMUS

BY THE SVEDBERG AND FRANCIS F. HEYROTH[1]

RECEIVED OCTOBER 2, 1928 PUBLISHED FEBRUARY 5, 1929

Gross differences have been reported by several workers[2] in the copper content, oxygen-combining curves, isoelectric points and various other properties of the hemocyanins obtained from different animals. This investigation was undertaken to determine whether a similar difference could be observed in the molecular weights of the hemocyanins or respiratory pigments of two species, the vineyard snail, *Helix pomatia*, and the horseshoe crab, *Limulus polyphemus*. Measurements in the ultracentrifuge by Svedberg and Chirnoaga have shown the former protein to have a molecular weight of 5,000,000 in solutions containing 0.05–0.1% of hemocyanin near the isoelectric point, 5.2. The copper contents of the hemocyanins of *Limulus* and *Helix* are 0.17 and 0.28%, respectively.[3]

Experimental

Preparation of Material.—We are indebted to Dr. A. C. Redfield for a sample of the hemocyanin of *Limulus* prepared by him at Woods Hole in 1926. He has described its preparation as follows:[3] "The fresh serum from a large number of animals was salted out by the addition of ammonium sulfate to half saturation, and in this condition preserved for some six months. The precipitated hemocyanin was separated by filtration, redissolved in 5% saturated ammonium sulfate containing 0.001 *N* ammonium hydroxide, filtered free of all insoluble residue, and reprecipitated by adding just sufficient saturated ammonium sulfate. This precipitate was separated by centrifugation. In order to secure a satisfactory separation, it was found desirable to bring the reaction to approximately *P*H 8.0 by the addition of ammonium hydroxide. The process of redissolving and salting out was then repeated two more times."

The material so prepared was dissolved in 5% saturated ammonium sulfate and further purified by dialysis before beginning this series of experiments. The dialysis was conducted in collodion bags in the ice chest against *N*/10,000 and later *N*/1000

[1] Fellow in Medicine of the National Research Council.

[2] References in Svedberg and Chirnoaga, THIS JOURNAL, **50**, 1399 (1928), and in the papers of Stedman and Stedman, *Biochem. J.*, **20**, 938, 949 (1928).

[3] Redfield, Coolidge and Shotts, *J. Biol. Chem.*, **76**, 185 (1928).