[CONTRIBUTION FROM THE FEDERAL POLYTECHNIC INSTITUTE, ZURICH, SWITZERLAND.]

PSEUDO-MUSCARINE ("SYNTHETIC MUSCARINE.")

BY ALBERT B. WEINHAGEN.

Received March 26, 1920.

Muscarine is a very powerful base occurring in the fly-agaric, (*Amanita muscaria*). A very small amount stops a frog's heart in diastole, an action which can be blocked or promptly relieved by atropine. In the fungus, the base is associated with choline, and since muscarine differs from choline merely by one additional atom of oxygen, Harnack[1] gave it the formula of the choline derivative, $OHN(CH_3)_3CH_2CH(OH)_2$. As a matter of fact, by evaporating choline with nitric acid, Schmiedeberg and Harnack[2] soon after obtained a base (synthetic or pseudo-muscarine) which stopped the heart in a similar manner to muscarine. This seemed to prove the supposition that natural muscarine and the oxidation product of choline are identical.

However, Boehm[3] and Honda[4] and H. Meyer[5] showed that there are differences between the pharmacological actions of the natural and the synthetic base, the most marked difference being that the latter has a distinct curare action upon the motor nerve terminations which the natural base lacks entirely. With a view towards a solution of this discrepancy, numerous investigations have concerned themselves with the compound obtained by evaporating choline with nitric acid. The compound was generally examined and described in the shape of the chloroplatinate itself.

Schmiedeberg and Harnack[6] thus obtained the chloroplatinate of "synthetic muscarine" to which they gave the formula $(ClN(CH_3)_3CH_2\text{-}CH(OH)_2)_2PtCl_4$, and when using more dilute acid, a by-product which they looked upon as nitro-oxyethyl-dimethylamine.

Nothnagel[6] isolated the same "synthetic muscarine" as Schmiedeberg and Harnack, and as a by-product obtained the nitrous acid ester of choline, $(ClN(CH_3)_3CH_2CH_2ONO)_2PtCl_4$. When using more dilute acid he obtained a by-product agreeing in many respects with that of Schmiedeberg and Harnack.

Schmidt and Wagner[7] found only the nitrous acid ester of choline.

Boehm[3] formulated his chloroplatinate of "synthetic muscarine" as Schmiedeberg and Harnack did theirs. This formula was furthermore corroborated or accepted by numerous other investigators.[8]

[1] Harnack, *Arch. exp. Path. Pharm.*, 4, 168 (1876).

[2] Schmiedeberg and Harnack, *ibid.*, 6, 101 (1877).

[3] Boehm, *ibid.*, 19, 87 (1885).

[4] Honda, *ibid.*, 65, 454 (1911); 64, 72 (1910).

[5] Nothnagel and H. Meyer, *Arch. Pharm.*, 231, 261 (1893); *Ber.*, 26, 804 (1893).

[6] *Loc. cit.*

[7] Schmidt and Wagner, *Ann. Chem.*, 337, 37 (1904).

[8] Boehm, Honda, Nothnagel and Meyer, *loc. cit.* See also later references to Fuehner, Harmsen, Straub, Walter and Schott.

The problem was reinvestigated in 1914 by A. J. Ewins[1] and H. H. Dale[2] Ewins found but one chloroplatinate, *i. e.*, that of the nitrous acid ester of choline, $(ClN(CH_3)_3CH_2CH_2ONO)_2PtCl_4$. By a critical examination of the analytical data, Ewins showed that the "synthetic muscarine" of former investigators had all consisted of the nitrous acid ester of choline. Dale showed that the nitrous acid ester of choline exhibits precisely those pharmacological properties which previous investigators had ascribed to "synthetic muscarine." Ewins concludes that the action of conc. nitric acid upon choline in all cases leads to the formation of but one compound, the nitrous acid ester of choline.

My own investigation was originally based upon the supposition that "synthetic muscarine" might indeed be identical with the natural base, and that the additional curare-action and other divergences of the synthetic product might be due to the presence of active by-products. Some support had been lent to this supposition by the fact that I had previously isolated even trimethylamine from the products when preparing "synthetic muscarine," and, therefore, suspected the presence of intermediate decomposition products as well. In this paper are submitted the data of the most typical of a series of attempts with the above-mentioned action of nitric acid upon choline.

Experimental.

Pure choline chloroplatinate was prepared from the choline of egg lecithine, and from choline obtained by the method of Renshaw.[3] The characteristic 6-sided crystals contained by analysis 31.57 and 31.62% of platinum, respectively, as compared with the calculated 31.65%.

Sample I.

Here, as well as with the following samples, choline chloroplatinate was treated with nitric acid according to the original directions of Schmiedeberg and Harnack. The chloroplatinate (5.5 g.) was dissolved in nitric acid (sp. gr. 1.4) on the water bath and then evaporated to dryness on the sand bath. The resultant product was extracted successively with alcohol and several times with small amounts of cold water (these extracts being preserved for subsequent examination). The undissolved portion was fractionally recrystallized from hot water.

First Fraction (2.4 g.).—This, the least soluble fraction of chloroplatinate, crystallized in the shape of very minute octahedra and the combination of octahedron and cube. These small crystals could in no way be obtained larger. At times the octahedra intergrew end-to-end forming chains or large aggregates, generally in the shape of 4 papal crosses joined concentrically at right angles. By altering the concen-

[1] A. J. Ewins, *Biochem. J.*, 8, 209 (1914).

[2] H. H. Dale, *J. Pharm. expt. Therap.*, 6, 147 (1914).

[3] Renshaw, THIS JOURNAL, 32, 128 (1910).

tration, etc., the aggregates could be converted into the small separate octahedra and *vice versa*. The salt was anhydrous. It melted with decomposition at 234° (bath 200°). One part dissolves in 103 parts of water at 20°. The chloroplatinate gave a distinct nitroso reaction (dark blue) with diphenylamine and sulfuric acid, and the chloride gave the Liebermann reaction.

Subs. (vacuum dried), 0.1853, 0.2199, 0.2125: Pt, 0.0534, 0.0637, 0.0613. Subs. 0.1628, 0.1688: AgCl, 0.2060, 0.2130.

Calc. for $(ClN(CH_3)_3CH_2CH_2ONO)_2PtCl_4$: Pt, 28.92; Cl, 31.57. Found: Pt, 28.81, 28.98, 28.83; Cl, 31.30, 31.28.

This was evidently the chloroplatinate of the nitrous acid ester of choline as described by Ewins.[1]

The salt is anhydrous and loses weight neither in vacuum over sulfuric acid nor when heated to 100°. The loss of weight occurring above 100° is evidently due to decomposition. A sample of 0.1450 g. kept at 130–140° lost 2.75% in 2 hours, 3.51% in 5 hours, 8.41% in 8 hours, and 12.62% when heated to 150°.

The Hydrochloride.—Several grams of chloroplatinate was decomposed with potassium chloride, and the hydrochloride extracted with alcohol. It is best recrystallized by adding ether to a hot concentrated alcoholic solution until the solution just begins to cloud. Upon cooling, the hydrochloride crystallized in clear, transparent, small prismatic needles and prisms which melted at 165°, and contained $2H_2O$. It gave the nitroso reaction.

Subs., 0.1153, 0.1380: AgCl, 0.0808, 0.0964. Found: Cl, 17.39, 17.28.
Calc. for $ClN(CH_3)_3CH_2CH_2ONO:2H_2O$: Cl, 17.34; H_2O, 17.60.

The water of crystallization cannot be determined directly, seeing that it is given off slowly and only at temperatures approaching the melting point, whereas at such temperatures the hydrochloride also splits off chlorine, *viz.*,

0.1206 g. vac. dry, dried 1 hr. at 130° lost 0.0000 g.
0.1206 g. vac. dry, dried 1 hr. at 160° lost 0.0106 g. = 8.79%.
0.1206 g. vac. dry, dried 3 hr. at 160° lost 0.0247 g. = 20.53%.
0.1206 g. vac. dry, dried 6 hr. at 160° lost 0.0302 g. = 25.04%.

The residue then gave 0.0447 g. AgCl, *i. e.*, 9.18% Cl. It will be noted that the loss of weight at 160° plus the chlorine left in the residue practically amount to the sum of chlorine plus water of crystallization required by the formula of the hydrochloride.

The Aurichloride.—This crystallized from very dil. hydrochloric acid in small, pointed flakes.

Subs., 0.1756, 0.1867: Au. 0.0732, 0.0784.
Calc. for $ClN(CH_3)_3CH_2CH_2ONO.AuCl_3$: Au, 41.71. Found: 41.68, 41.98, 41.71.

The Free Base.—The base was prepared by decomposing its phos-

[1] *Loc. cit.*

photungstate. It represented a clear, faintly yellowish oil with a distinctly basic odor; it was hygroscopic; it rapidly absorbed carbon dioxide from the air; it is soluble in water and in alcohol, but insoluble in ether. When kept in vacuum over sulfuric acid, it crystallized in short, broad needles and in spear-shaped crystals which tended to join 4 concentrically at right angles. The base gave a distinct nitroso reaction with diphenylamine and sulfuric acid.

The Sulfate and the Perchlorate.—The former crystallized from water in hair-like crystals, the latter in thin, transparent flakes.

The Second, Third and Fourth Fractions (1 g.).—These 3 fractions of chloroplatinate proved to be identical with the first fraction.

Subs., 0.2092, 0.2659, 0.2726: Pt, 0.0602, 0.0770, 0.0785.
Found: Pt, 28.77, 28.97, 28.80.

The Fifth Fraction (0.4 g.).—This chloroplatinate crystallized in small, well-defined, clear-cut, 4-, 5- and 6-sided plates and in 3-cornered prisms. The crystals were orange-red, transparent and glistening, and did not effloresce in a vacuum over sulfuric acid. M. p. 204–205° without charring but with voluminous orange foam. The salt gave an intense nitroso reaction with diphenylamine and sulfuric acid.

Subs., 0.1332, 0.1121: Pt, 0.0385, 0.0324. Found: Pt, 28.83, 28.90.

Although the platinum content practically agrees with that of the nitrous acid ester of choline (see first 4 fractions), the distinct differences in crystal forms, pharmacological properties (see Summary), and other properties disprove such identity. This compound was probably identical with the nitro-oxyethyl-dimethylamine isolated by Schmiedeberg and Harnack.[1] $((CH_3)_2NCH_2CH_2ONO_2)_2H_2PtCl_6$ requires 28.79% Pt.

The Sixth Fraction (0.7 g.).—This, the last fraction, consisted entirely of trimethylamine chloroplatinate. It crystallized in what at first seemed to be medium-sized octahedron-cube combinations, but after some time could be recognized as thick 5- and 6-sided plates and combinations resembling the octahedron-cube type. It melted with decomposition at 235°, and was thus in all respects identical with trimethylamine chloroplatinate prepared for the sake of direct comparison. This chloroplatinate did not give a reaction with diphenylamine and sulfuric acid, and split off trimethylamine when treated with alkali.

Subs., 0.1777, 0.2682: Pt, 0.0657, 0.0988.
Calc. for $((CH_3)_3N)_2H_2PtCl_6$: Pt, 36.97. Found: 36.97, 36.84.

The Alcoholic Extraction.—The extracts resulting at the outset when the product of the reaction was washed with alcohol and with cold water were examined. The aqueous extract contained only unchanged choline chloroplatinate (0.2 g.). The alcoholic extract furnished 0.5 g. of chloroplatinate which crystallized from water in small plates, some pointed,

[1] *Loc. cit.*

some oblong with indentations on the 2 shorter sides, some almond-shaped, and some like miniature arrow-heads. When recrystallized, it at times represented short prisms and prismatic needles and at times wedge-shaped flakes. It melted without charring at 186°. The chloroplatinate thus differs distinctly from that of the nitrous acid ester of choline and from that isolated in the fifth fraction.

Subs., 0.2283, 0.1347: Pt, 0.0656, 0.0385. Found: Pt, 28.73, 28.60.

It gave a distinct nitroso reaction with diphenylamine and sulfuric acid. As noted, only a very small amount of this compound was isolated.

Sample II.

Choline chloroplatinate was here treated with nitric acid as described in the case of Sample I, the sole difference being that it was evaporated with the acid 5 times instead of only once.

The First and Second Fractions.—The chloroplatinate of these fractions crystallized from water in cubes and short prisms, which could at times be converted into small plates, large striated flakes, and spear-shaped forms. It was anhydrous and melted with decompositions at 208°.

Subs., 0.1368, 0.1312, 0.1724: Pt, 0.0411, 0.0393, 0.0515.
Found: Pt, 29.97, 29.93, 29.88.

It will be noted that this chloroplatinate differs distinctly from that of the nitrous acid ester of choline as well as from that obtained in the remaining fractions of Sample I. The platinum contents, as a matter of fact, agrees very well with Schmiedeberg and Harnack's muscarine formulation, *viz.*, $(ClN(CH_3)_3CH_2CH(OH)_2)_2PtCl_4$, which requires 30.15%.

The Third and Fourth Fractions.—This chloroplatinate crystallized from water in very clear-cut, transparent, 4-, and 6-sided plates which effloresced in a vacuum over sulfuric acid. It will be noted that the chloroplatinate obtained in the fifth fraction of Sample I crystallized in plates which although very similar did not effloresce. The chloroplatinate gave a distinct nitroso reaction with diphenylamine and sulfuric acid. It melted without charring at 204°. The exact nature of this compound could not be determined.

Subs., (3) 0.1463, (4) 0.1130: Pt, (3) 0.0414, (4) 0.0320. Found: Pt, (3) 28.30. (4) 28.33.

The Fifth, Sixth, and Seventh Fractions.—These fractions, representing by far the greatest portion of compound here obtained, consisted of trimethylamine chloroplatinate.

Subs., (5) 0.1598, (6) 0.1454, (7) 0.1216: Pt, (5) 0.0588, (6) 0.0534, (7) 0.0448. Found: Pt, (5) 36.81, (6) 36.71, (7) 36.92.

Sample III.

Here 5 g. of choline chloroplatinate was treated with nitric acid precisely as described in the case of Sample I.

The First, Second, and Third Fractions.—These yielded 3.4 g. choline nitrous acid ester chloroplatinate (see Sample I, first fraction).

The Fourth and Fifth Fractions.—These yielded 1.3 g. of a chloroplatinate crystallizing from water in oddly-shaped aggregates formed exactly like miniature bird feathers, no matter how often recrystallized. The compound gave the nitroso reaction with diphenylamine and sulfuric acid. It melted with decomposition at 207°.

Subs., (4) 0.1711, (4) 0.1881, (5) 0.1566: Pt, (4) 0.0568, (4) 0.0625, (5) 0.0556.
Found: Pt. (4) 33.20, (4) 33.24, (5) 33.14.

The amount of this compound did not suffice for a definite chemical characterization, but it will be noted that the value found agrees for the nitrous acid ester of amino-ethyl alcohol (see Summary), *viz.*, $(NH_2CH_2CH_2ONO)_2H_2PtCl_6$, which requires 33.10% of platinum.

The alcoholic extraction yielded a very small amount of the same compound as the alcoholic extraction in the case of Sample I.

Subs., 0.1088: Pt, 0.0313. Found: 28.77.

Sample IV.

Choline chloroplatinate was here treated with nitric acid precisely as in the case of Sample I.

The first 4 fractions yielded choline nitrous acid ester chloroplatinate (see Sample I, first fraction).

Subs., 0.1789: Pt, 0.0417. Found: Pt, 28.87.

The fifth and last fraction consisted exclusively of trimethylamine chloroplatinate.

Subs., 0.1866: Pt, 0.0687. Found: Pt, 36.81.

No further compound could be isolated in this attempt.

Sample V.

Here the nitrous acid ester of choline was obtained by the direct action of nitrous anhydride gas upon the free base choline suspended in dry chloroform. Upon evaporation at low temperature there was obtained a clear oily residue, which was dissolved in dil. hydrochloric acid and freed from traces of insoluble wax-like by-products by filtration. The chloroplatinate obtained from the filtrate was fractionally recrystallized.

The first 2 fractions (about 10% yield) consisted of the chloroplatinate of the nitrous acid ester of choline (see Sample I, first fraction).

Subs., 0.1614: Pt, 0.0568. Found: 28.99.

All subsequent fractions yielded unchanged choline chloroplatinate.

Subs., 0.2800, 0.1207: Pt, 0.0885, 0.0381.
Calc. for $(ClN(CH_3)_3CH_2CH_2OH)_2PtCl_4$: Pt, 31.65. Found: 31.62, 31.57.

It appears that the yield of choline nitrous acid ester by this method is at all times comparatively very small.

Summary.

These results show that the action of nitric acid upon choline chloroplatinate according to the original directions of Schmiedeberg and Harnack does not produce a single new compound, but that the product generally contains several new compounds as by-products. Furthermore, even small unintentional variations in the reaction cause a variation in the product resulting; and finally a deviation even merely to the extent illustrated by Sample II of this investigation can cause the formation of quite a different main product. Considering these facts, it would seem that many of the numerous exact qualitative and quantitative pharmacological examinations of "synthetic muscarine" thus prepared from choline (among these Honda,[1] Harmsen,[2] Boehm,[1] Fuehner,[3] Straub,[4] Meyer[1] Walter,[5] Schott,[6] etc.), should be considered with these facts in mind.

In the majority of cases, to be sure, when the original directions for the reaction are observed (Samples I, III and IV), the greater portion of the resultant product consists of the nitrous acid ester of choline. One cc. containing chloroplatinate equivalent to 0.8 mg. of the hydrochloride of this compound stopped the heart of a frog (subcutaneously; frog 38 g.) in diastole within 13 minutes. The arrest was momentarily relieved by atropine. This was found to be the minimum dose for complete arrest Boehm[1] and Honda[1] quote 0.5 to 1.5 mg. as the dose for "synthetic muscarine."

In addition to trimethylamine, there was isolated a secondary decomposition product (fifth fraction of Sample I), which was probably nitrooxyethyl-dimethylamine. This compound was without influence upon the rate of the frog heart. One cc. containing chloroplatinate equivalent to one mg. of hydrochloride of this compound (subcutaneously; frog 40 g.) did not affect the heart of a frog except that it showed a very slight and merely very temporary decrease of its rate. The successive injection of 6 additional mg. showed absolutely no influence upon the rate which had again become normal, whereas, as has been noted, as little as 0.8 mg. of choline nitrous acid ester suffice to stop the heart.

Another secondary decomposition product (fourth and fifth fractions of Sample III) seemed to be the nitrous acid ester of amino-ethyl alcohol, $NH_2CH_2CH_2ONO$. Seeing that choline is readily formed by methylating amino-ethyl alcohol,[7] it seems but natural that the reverse process

[1] *Loc. cit.*

[2] Harmsen, *Arch. expt. Path, Pharm.*, **50**, 361.

[3] Fuehner, *ibid.*, **59**, 179 (1906); **61**, p. 283.

[4] Straub, *Pflueger's Arch.*, **119**, 127 (1907); **110**, 492 (1905).

[5] Walter, *ibid.*, **78**, 597 (1899).

[6] Schott, *Arch. expt. Path. Pharm.*, **65**, 239 (1911).

[7] Trier, *Z. physiol. Chem.*, **80**, 409 (1912).

might readily be induced by nitric acid in this instance. One cc. containing chloroplatinate equivalent to one mg. of the hydrochloride of this compound merely succeeded in decreasing the rate of a frog's heart (subcutaneously; frog 41 g.) from 40 to 32 per minute at the end of half an hour. The successive injection of 8 additional mg. merely decreased the rate to 24 per minute. Finally an attempt was made to stop this heart by injecting choline nitrous acid ester, of which 0.8 mg. normally suffice to stop a frog's heart. With this heart the injection of 0.8 mg. merely decreased the rate from 24 to 16; additional 1.6 mg. furthermore decreased it to 12, and this rate could not be further decreased even by 2 additional injections of 1.6 mg. each. The heart thus continued at the rate of 12 beats per minute even after 5.6 mg. of choline nitrous acid ester had been injected. The compound evidently had an action antagonistic to the muscarine action of choline nitrous acid ester. The test was repeated with similar results.

A further by-product was isolated from the alcoholic extracts (Samples I and II). The platinum contents were very similar to those of choline, nitrous acid, ester, chloroplatinate, but the crystal forms, melting points, etc., disprove such identity. The nature of this compound could not be established.

By deviating from the original directions as illustrated by Sample II, the formation of the nitrous acid ester of choline was circumvented entirely. The main product here (first and second fractions) agreed in platinum contents with the requirements of Harnack's muscarine formula, $(OH)N(CH_3)_3CH_2CH(OH)_2$. Seeing that the greater portion of choline was decomposed to trimethylamine, the yield of base was very small.

As an additional product there was in this case (third and fourth fractions, Sample II) obtained a chloroplatinate which differed from all the by-products so far obtained. In spite of great similarity, it differs (efflorescence and platinum contents) also from the chloroplatinate of the fifth fraction of Sample I. One cc. containing chloroplatinate equivalent to one mg. of the hydrochloride of this compound (subcutaneously; frog 36 g.) had no influence upon the rate of a frog's heart. Similarly, the successive injection of 4 additional mg. had practically no effect, whereas the final injection of one mg. of choline nitrous acid ester quickly brought diastolic arrest, thus showing that there was here no antagonism, as opposed to the compound of the fourth fraction of Sample III.

In Sample V it was shown that choline nitrous acid ester can be obtained by the interaction of nitrous acid anhydride and the base choline, but that the yield by this method is very small.

It may be noted that the chloroplatinate of the first and second fractions of Sample II, although agreeing therewith tolerably well as regards

platinum contents, differ distinctly from the chloroplatinate of natural muscarine in crystal forms, the latter crystallizing in octahedra which have no definite melting point.[1]

[CONTRIBUTION FROM THE DIVISION OF AGRICULTURAL BIOCHEMISTRY, MINNESOTA AGRICULTURAL EXPERIMENT STATION.]

THE COLORIMETRIC ESTIMATION OF TYROSINE BY THE METHOD OF FOLIN AND DENIS.[2]

BY ROSS AIKEN GORTNER AND GEORGE E. HOLM.

Received April 12, 1920.

Folin and Macallum,[3] in 1912, noted that uric acid and phenols produced a deep blue color in solutions of phosphotungstic acid when alkali was subsequently added. This observation was rapidly followed by a paper by Folin and Denis[4] in which they give detailed directions for the preparation of 2 reagents, one of which contains phosphotungstic acid and the other both phosphotungstic and phosphomolybdic acids. The former reacts with uric acid but not with monohydric phenols including tyrosine, while the latter reagent produces a beautiful blue color with phenol solutions, the reaction, according to Folin and Denis, being unmistakably positive with 1 part of tyrosine in 1,000,000 parts of water.

In a third paper Folin and Denis[5] apply the phenol reagent to the colorimetric estimation of tyrosine in proteins, without, however, reporting any careful quantitative study of the reagent when pure tyrosine was used. In all some 27 proteins were tested for tyrosine content by the new reagent and in every instance more tyrosine was found than was recorded in the literature from gravimetric determinations. The average percentage of tyrosine found was 5.065% by the colorimetric method and 2.647% by the gravimetric method for the 20 proteins where both figures are available. Folin and Denis express their belief that tyrosine is the only component of the proteins which reacts with the phenol reagent and that the colorimetric method gives an accurate measure of the amount of tyrosine present.

Shortly after Folin's paper appeared Abderhalden and Fuchs[6] and Abderhalden[7] stated that tryptophane, oxytryptophane and oxyproline

[1] A. J. Ewins, *Biochem. J.*, 8, 209 (1914).

[2] Published with the approval of the Director as Paper No. 203, Journal Series of the Minnesota Agricultural Experiment Station. Presented before the Division of Biological Chemistry at the Spring Meeting of the American Chemical Society, St. Louis, April 12–16, 1920.

[3] *J. Biol. Chem.*, 11, 265–6 (1912).

[4] *Ibid.*, 12, 239–243 (1912).

[5] *Ibid.*, 12, 245–251 (1912).

[6] *Z. physiol. Chem.*, 83, 468 (1913).

[7] *Ibid.*, 85, 91 (1913).