them by the addition of sodium hydroxide resulted in precipitation of the free base. This acidity was responsible for the hyperemia and marked irritation which were noted with all of the compounds, and possibly tended to shorten the duration of the anesthesia because of the excessive lacrimation. It is hoped that further modification of the structure will eliminate this objectionable feature.

Summary

Thes yntheses of several 1,3,4-trisubstituted piperidines have been described and some of their properties noted.

The 1-alkyl-3-carbethoxy-4-piperidyl benzoates have very marked local anesthetic action. They show an increase in anesthetic action and a decrease in toxicity as the size of the alkyl radical attached to the nitrogen increases. The amyl derivatives possess a greater anesthetic power than cocaine and show only about one-thirtieth of its toxicity.

As pointed out, the salts of these compounds are acid in aqueous solution and cause irritation and hyperemia.

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A MICRO-COLORIMETRIC METHOD FOR THE ESTIMATION OF PHOSPHOLIPINS IN SEEDS[1]

BY N. B. GUERRANT

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Introduction

The attention of the biological chemist has been attracted for a number of years to the presence of those phosphorus-containing lipoid substances found in both plant and animal tissues. The universal distribution of these substances seems to indicate that they play an important part in the metabolic processes of the living cell. There is little doubt that they serve as a source of the phosphorus required for building up the complex nucleoproteins of cell nuclei. Indeed it might be said that these substances play an important role in the making of the essential substratum of living matter.

This important class of substances has been designated by Leathes[2] as phospholipins. The exact chemical structure of none of the phospholipins has been definitely established, and the constituents of only a few of them have been approximated.[3] However, a considerable amount of

[1] Published with the permission of the Director of the Oklahoma Agricultural Experiment Station.

[2] Leathes, "The Fats," Longmans, Green and Co., London, 1910.

[3] Maclean, "Lecithin and Allied Substances," Monograph of Biochemistry, Longmans, Green and Co., 1918.

work has been done upon these substances, most of which has been qualitative in nature rather than quantitative.[4] There is much literature relating the details of the numerous investigations, but we will note only those references pertaining to those investigations which are strictly comparable to ours. We have special reference to a number of articles appearing in the literature concerning the quantitative estimation of the various forms of phosphorus in blood and urine.

While such investigations have helped materially in clearing up the mystery concerning the nature, composition and function of this type of chemical complex, the field of investigation has by no means been exhausted. It is generally conceded that phospholipins occur in varying amounts in all seeds; but we have found no record of an attempt to devise a method for the quantitative estimation of the amount present. Since the phospholipins in seeds appear to play an important part in the metabolism of seeds, and the physiological changes during germination, we have undertaken this investigation in order to devise a quick and comparable method for the estimation of the total phospholipins in various seeds.

Procedure

After much preliminary work in the selection of a suitable solvent, the method of extraction, technique of oxidation, reducing reagent and time allowed for color formation, the following method was adopted.

The material for analysis is first ground until it passes through a 40-mesh sieve. One g. of the dried material is placed in a centrifuge tube and 25 cc. of a 20:80 mixture by volume of ether and alcohol added. The tube is then corked tightly and placed in a shaker where it is shaken vigorously for ten minutes. At the end of this time, the tube is removed from the shaker and centrifuged until all insoluble matter is thrown down. Five cc. of the supernatant liquid is then transferred to a 30–35cc. test-tube which is graduated at 10, 15, 20 and 25 cc. and bent slightly above the last mark. The tube is placed in a hot water-bath until the extract is reduced to a small volume. At this point, a similar tube containing a known amount of a standard phosphate solution is entered in the procedure. One cc. of dil. sulfuric acid (1 part of concd. acid to 2 parts of water) is added to each tube. The tubes are then placed upon a small hot-plate in a somewhat inclined position in such a manner that only the rounded portion of the tube rests upon the hot-plate. The hot-plate should be capable of furnishing sufficient heat to volatilize a portion of the sulfuric acid, but not enough to drive the fumes from the tube. When the contents show the first signs of darkening, small drops of 30% hydrogen peroxide (Superoxol) are carefully added to each tube by means of a small capillary pipet. The hydrogen peroxide is added from time to time as long as any coloration is visible.

[4] Brauns and MacLaughlin, THIS JOURNAL, **42**, 2238 (1920).

When the last trace of color has disappeared, the heat is gradually increased until the tubes are half filled with sulfuric acid fumes, and they are maintained at this temperature for six minutes. During this period all of the excess of oxidizing agent has been expelled, which is essential to the final development of the true blue color. The tubes are then cooled, the sides rinsed down with water, and the volumes made up to the 10cc. mark. The contents of the tubes are then maintained at a boiling temperature for ten minutes, which is essential to convert all of the phosphate into the ortho form. The tubes are then placed in a water-bath where they are cooled to room temperature. One cc. of a 5% ammonium molybdate solution is added to each tube and the contents are well mixed by shaking. The tubes are removed from the water-bath and 1 cc. of a solution of a 0.4% aminonaphtholsulfonic acid repetition is added. The contents are then mixed thoroughly by shaking and allowed to stand for five to ten minutes depending upon the amount of phosphate present. At the end of this time the tubes are diluted to the mark, and the colors compared. The whole determination can be completed within a period of 60 to 80 minutes.

Reagents

Ether-Alcohol Mixture.—This is made by adding one part by volume of anhydrous ether to four parts of absolute alcohol, and preserving the mixture in a tightly-stoppered container.

Molybdate Solution.—Five g. of C. P. ammonium molybdate is dissolved in 95 cc. of water and the solution preserved in a glass-stoppered container.

Sulfuric Acid.—One part by volume of concd. sulfuric acid is poured into 2 parts of water. (The concentration of this mixture is approximately 12.5 *N*.)

Hydrogen Peroxide.—This is a 30% solution of hydrogen peroxide, which is put on the market by Merck and Company, under the trade name "Superoxol."

Standard Phosphate Solution.—This solution contains sufficient monopotassium phosphate to furnish 0.1 mg. of phosphorus per cc. of solution. The solution is preserved by the addition of a few drops of concd. sulfuric acid before the final dilution.

20% Sodium Sulfite Solution.—This is made by dissolving 20 g. of C. P. sodium sulfite in 80 cc. of water. The solution is unstable and for best results should be made up at 10-day intervals.

20% Sodium Bisulfite Solution.—This is made by dissolving 100 g. of the pure salt in 400 cc. of water. The solution is kept in a tightly stoppered bottle and filtered just before use.

Aminonaphtholsulfonic Acid Reagent.—One g. of purified 1,2,4-aminonaphtholsulfonic acid is added to 225 cc. of 20% sodium bisulfite solution and brought into solution by the addition of small quantities of 20% sodium sulfite solution. The volume is then made up to 250 cc. by the addition of bisulfite solution. This solution is comparatively stable but will darken on standing for a period of several months. For best results, a fresh solution should always be used.

Discussion

The method depends upon the intensity of the color formed as a result of the reduction of the phosphomolybdic acid. The principle was formerly

suggested by Taylor and Miller,[5] and later the theory of its action was more fully explained by Wu.[6] Since that time it has been made the basis of a number of determinations pertaining to both blood and urine.[7]

There are some minor details connected with this technique which cannot be stated definitely and this method, as with other methods, must be practiced a number of times before proficiency can be attained. However, in the process of its development several factors have been observed which considerably affect the final results of the determination. Some of these have been reported by investigators working with other materials, while others are noted from our own observations.

One of the first precautions to be observed is in the preparation of the solvent. A number of solvents and combinations of solvents were tried but none proved so satisfactory as a mixture of anhydrous ether and absolute alcohol in the ratio of one part of ether to four parts of alcohol by volume. A mixture of these two solvents had previously been recommended by Schulze.[8] The proportion of the solvents necessary for optimum extraction was obtained by estimating the amount of phosphorus extracted from an equal amount of material by various proportions of the two liquids. The following data give the number of milligrams of phosphorus extracted from 1 g. of material by 25 cc. of a mixture of various percentages of the two components.

TABLE I

RESULT OF EXTRACTING WITH VARIOUS PROPORTIONS OF THE TWO SOLVENTS

Ether by vol., %....	100	90	80	70	60	50	40	30	20	10	0
Alcohol by vol., %..	0	10	20	30	40	50	60	70	80	90	100
Phosphorus, mg.....	11.9	14.6	16.3	19.2	24.1	28.0	33.4	37.6	41.2	31.4	17.9

The exact amount of solvent required to extract 1 g. of material cannot be fixed definitely, as this depends upon the nature and lipin content of that particular seed. With the grain sorghums, it was found that 20 cc. of solvent per gram of material was apparently sufficient to extract all of the lipoid phosphates, but in the determinations reported, this amount was increased by 25% to insure complete extraction. The use of alcohol or ether containing traces of water will result in the extraction of material other than phospholipins. It was found that inorganic phosphates are sufficiently soluble in such a solvent to be responsible for a considerable portion of the color. For this reason, 95% alcohol and ordinary ether can not be used.

The effect of increasing the time of shaking was also noted, but there appeared to be no advantage in shaking longer than ten minutes.

[5] Taylor and Miller, *J. Biol. Chem.*, **18,** 215 (1914).

[6] Wu, *ibid.*, **43,** 189 (1920).

[7] Bell and Doisy, *ibid.*, **44,** 55 (1920).

[8] Schulze, *Z. physiol. Chem.*, **20,** 225 (1895); *Chem.-Ztg.*, **28,** 751 (1914).

Another phase of the determination which offers a fruitful source of errors is the method of oxidation, but with a small amount of material as is required in this determination, it was found to be a simple and reliable operation, when the several factors are reasonably controlled. A deficient amount of sulfuric acid may result in charring and over-heating[9] while the use of an excess of acid will interfere in the final color development.[10] For this reason the amount of acid used must range within rather narrow limits. It is essential that all tubes contain practically the same amount of acid at the end of the digestion, and since the amount of organic material in each test-tube is small, equal volumes of acid are added to each tube before they are placed on the hot-plate. By digesting on such an apparatus an equal amount of heat can be applied to all tubes. One feature of this method of digestion is that the heat is applied to only the bottom of the tube, thus preventing the over heating and subsequent volatilization of the phosphates, which is both possible and probable over a free flame. Another advantage is that several tubes can be digested, at the same time, at practically the same temperature, which is almost impossible with the usual method of digestion. An additional advantage of this method is that bumping is almost entirely eliminated, thus making it unnecessary to use beads or other materials to prevent this usual disturbance. The use of bent test-tubes reduces the chance for loss by bumping to a minimum.

Several oxidizing agents were tried, but hydrogen peroxide proved to be the most efficient and satisfactory. Fuming nitric acid was found to react too violently, while nitric acid and nitrates when not completely expelled by heating, caused a greenish-yellow tint in the solution which interfered with the color comparison. The time required to expel completely all traces of nitric acid is much greater than that required to expel the excess of peroxide.

A number of possible reducing agents were tried, among which were stannous chloride, hydroquinone,[11] β-naphthol, α-naphthol, α-naphthylamine, p-aminophenol, and 1,2,4-aminonaphtholsulfonic acid.[12] The hydroquinone and the aminonaphtholsulfonic acid responded most favorably. Of the two, the aminonaphtholsulfonic acid was used, since it reacts in a shorter period of time and produces more color.

Table II gives a comparison of the results obtained by this method with those obtained by the usual gravimetric method, which required a hundred times as much material. These values are the average of three determinations and express the number of milligrams of phosphorus from 1 g. of material.

[9] Baumann, *J. Biol. Chem.*, **59**, 667 (1924).
[10] Whitehorn, *ibid.*, **62**, 133 (1924–25).
[11] MacCallum, *Proc. Roy. Soc.*, **63**, 467 (1898).
[12] Fiske and Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

TABLE II

MILLIGRAMS OF PHOSPHORUS FROM THE PHOSPHOLIPINS OF 1 G. OF MATERIAL

Subs.	Darso	Red kafir	White kafir	White milo	Feterita
Grav. method.....	31	42	33	36	26
Micro-col. method.....	30	40	32	36	27

In conclusion, I take pleasure in thanking Dr. C. T. Dowell, Dr. V. G. Heller, and Mr. W. D. Gallup for their suggestions and coöperation, during this investigation.

Summary

A quick and accurate method for the estimation of the relative amounts of phospholipins in various seeds is described. Results obtained by this method check very satisfactorily with those obtained by the usual gravimetric method, which requires a hundred times the amount of material. The phospholipin content of a number of seeds has been determined. A few results are given here in order to show the accuracy of the method, and other results will be published in due time.

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BETA-XENYLHYDROXYLAMINE AND ITS REARRANGEMENT

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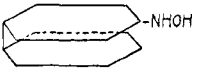
Introduction

The reactions of a number of diphenyl derivatives have been interpreted on the basis that the two phenyl groups have at times a bi-planar and not a co-planar configuration.[2] If this is the case with β-xenylhydroxylamine, then one of its bi-planar formulas can be represented as follows.



Aryl hydroxylamines undergo ready rearrangement, particularly under the influence of mineral acids, to give aminophenols, the hydroxyl group rearranging to the *para* and, to a less extent, to the *ortho* position. Be-

[1] This paper is an abstract of a part of a thesis presented by James E. Kirby in partial fulfilment of the requirements for the degree of Master of Science in Chemistry at Iowa State College.

[2] Kaufler, *Ann.*, **351**, 151 (1907); *Ber.*, **40**, 3250 (1907). Cain, Coulthard, and Micklethwait, *J. Chem. Soc.*, **101**, 2298 (1912). Cain and Micklethwait, *ibid.*, **105**, 1437 (1914). Turner, *ibid.*, **107**, 1495 (1915). Kenner and Stubbings, *ibid.*, **119**, 593 (1921). Christie and Kenner, *ibid.*, **121**, 614 (1922). Adams, Bullock and Wilson, THIS JOURNAL, **45**, 521 (1923). Raiford and Clark, *ibid.*, **48**, 485 (1926).