METHODS OF BIOCHEMICAL ANALYSIS

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PREFACE TO VOLUME I

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need for material achievement to keep in sight of the advance of useful ideas.

The current volume is the first of a series which is designed to try to meet this need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological and, if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who have either originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

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Determination of ORGANIC PHOSPHORUS COMPOUNDS by Phosphate Analysis

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I. INTRODUCTION

The realization of the biological significance of phosphorylation processes stimulated and concentrated a great deal of interest on the problem of phosphate analysis. Consequently, the majority of the analytical methods in this field have been elucidated by biochemists. The existence of a great variety of such methods can be ascribed not only to the large number of combinations in which phosphates occur in living material, but also to the very manifold situations with which the analyst is faced when attempting to transform his substance into a form suitable for quantitative estimation. The two problems of

determining methods for the analysis of a compound in pure solution, and the analysis of a single compound present in a biologically complex mixture of proteins, lipids, carbohydrates, etc., are certainly of quite different natures.

The present paper contains a summary of the methods by which organic phosphorus compounds can be estimated by means of phosphate analysis. These estimations can only be accomplished when the compound in question can be degraded in such a way as to give rise to inorganic phosphate. In the first section, methods for the determination of inorganic phosphates are reviewed and critically examined. In the second section, some methods for the fractionation of biological systems with respect to organic phosphorus compounds are treated.

II. DETERMINATION OF INORGANIC PHOSPHATES

1. Orthophosphate

Most of the early methods of phosphorus analysis were based on the principle of bringing orthophosphate into an insoluble form, after which it could be estimated gravimetrically (42, 43, 114, 132, 149) or nephelometrically (12, 70, 87, 88), or in an indirect way by analyzing its precipitating counterpart. The latter could be done, for example, by titration of magnesium in $MgNH_4PO_4$ (51, 54, 136) or by an estimation of strychnine (86) or uranium (66, 95, 137) in the respective phosphomolybdates, or, as was suggested more recently, by a polarographic determination of molybdenum (125, 143).

Many of these methods took advantage of the property of orthophosphate to form a complex compound with molybdic acid according to the reaction,

$$H_3PO_4 + 12H_2MoO_4 \longrightarrow H_3Mo_{12}PO_{40} + 12H_2O$$

the insoluble salts of which are especially suited to the above types of analysis, as their phosphorus content is relatively low. Nevertheless, all of these methods suffer the weakness that they require relatively large amounts of sample and thus hardly correspond to the sensitivity demands of modern biochemical analysis.

Another property of early interest of the phosphomolybdic acid complex, recognized in 1914 by Taylor and Miller (151), is that it

could be converted by reduction into a deep blue substance called molybdenum blue. The mechanism of this reaction was later studied in detail by Berenblum and Chain (9) who state:

All the reducing agents tested reduce molybdic acid in the absence of phosphate. The reduction velocity is greatest at about 0.2 *N*, and diminishes rapidly to zero with increasing acidity. Phosphates accelerate the rate of reduction of molybdic acid. This acceleration occurs over a wide acid range, but decreases with increasing acidity.

The rate of reduction of molybdic acid, the accelerating effect of phosphate and the end points reached in these reactions are all dependent on the concentration of molybdate and on the concentration and nature of the reducing agent. The stronger the reducing agent, the greater the number of molecules of MoO_3 reduced per atom of phosphate.

The theory (by Feigl (49)*) that the reduction of molybdic acid is catalytically activated by phosphate, has been confirmed. The reaction does not go to completion, however, but reaches definite end points, because the product of the reduction inactivates the catalyst.

Further studies of the reaction mechanism have been presented by Woods and Mellon (163). The spectrophotometric properties of molybdenum blue were investigated by Fontaine (58).

Unfortunately, some early authors (7, 34, 151) failed to find reproducible experimental conditions, the elusive point being the important of a suitable acidity. Thanks to the work of Briggs (16) and of Fiske and Subbarow (52), this problem could be overcome and the latter authors succeeded in elaborating the first fully reproducible method which is even today one of the most frequently employed.

A. THE FISKE AND SUBBAROW METHOD

Principle. Phosphate is converted into phosphomolybdic acid in 0.5 *M* sulfuric acid and reduced with 1-amino-2-naphthol-4-sulfonic acid.

Reagents.

A. 5 *M* H_2SO_4.

B. 2.5% ammonium molybdate.

C. 0.5 g. recrystallized 1-amino-2-naphthol-4-sulfonic acid is dissolved in 15% $NaHSO_3$ and diluted to 195 ml. with 15% $NaHSO_3$. Add 5 ml. 20% Na_2SO_3. Warm until all material is dissolved. Store well stoppered and protected from light in the cold.

* Insert by the Reviewers.

Procedure. The sample containing 0.1–12 μM phosphate is mixed with 0.4 ml. of A, 0.8 ml. of B, and 0.4 ml. of C, respectively. Add water to 10 ml. Mix and let stand at room temperature for 10 minutes. Measure color intensity at 660 mμ. Precision ± 0.005 μM.

A great number of modifications have been suggested, most of which imply a substitution of the amino-naphthol-sulfonic acid by other reducing agents. Among these hydroquinone (41), 2,4-diaminophenol (122), monomethyl-*p*-aminophenol (67, 148, 153), ascorbic acid (3), thiosulfate (78), stannous chloride (14, 36, 75, 93, 94), ferrous sulfate (133, 147, 150) may be mentioned. Some slight modifications in other respects have also been proposed (8, 68, 74, 106, 152). A critical survey of the Fiske and Subbarow and its modifications has recently been published by Furukawa *et al.* (60).

Other variants of the molybdenum blue method have been necessitated, not so much because of unsatisfactory sensitivity and reproducibility of the Fiske and Subbarow method, but rather because of some special circumstances that may prevail on analyzing biological material. For example, when desiring to determine the total phosphorus content of a sample the organically bound phosphates must be brought into inorganic form. This is usually accomplished by wet incineration in the presence of a strong acid and an oxidizing agent. Here the problem often arises that an uncontrolled amount of acid remains in the sample after incineration, which may hazard the success of the phosphate determination if this is performed according to a method where an accurate level of acidity is required. In order to avoid difficulty, King (85) suggested the use of perchloric instead of sulfuric acid whereby the Fiske and Subbarow procedure can be rendered less sensitive to variations in acidity. The procedure also has the advantage that perchloric acid is also a deproteinizer of choice when it is desired to obtain an extract suited for spectrophotometric analysis.

B. KING'S METHOD

Principle. The phosphate is converted into phosphomolybdic acid in the presence of perchloric acid and is reduced according to Fiske and Subbarow (52).

Reagents.

A. 60% perchloric acid.

B. 5% ammonium molybdate.

C. 0.5 g. 1-amino-2-naphthol-sulfonic acid, 30 g. $NaHSO_3$ and 6 g. Na_2SO_3 dissolved to 250 ml. Filtered and stored well sealed.

Procedure. The sample (containing phosphate in the same concentration range as in the case of the Fiske and Subbarow method) is pipetted into a 15 ml. volumetric flask. 1.2 ml. of A, 1 ml. of B and 0.5 ml. of C respectively are added. Water is added to 15 ml. The sample is mixed by inverting and shaking. Color intensity is measured after 5 minutes.

When the total phosphate is to be determined, combustion is performed in the above amount of perchloric acid, the small losses occurring during this procedure being without significance.

Slight modifications of King's method have been suggested by Allen (2) and by Kuby *et al.* (92). A variant proposed by Nakamura (124) is able to increase the sensitivity of the method about four times. A critical examination of the King procedure has recently been undertaken by Mitsuhashi and Nakanishi (120).

Another weakness of the Fiske and Subbarow method (also shared by the King variant) is that it proceeds under conditions which may cause a splittage of acid labile organophosphate compounds. This danger is even more acute since molybdate is known to accelerate the acid catalyzed hydrolysis of such compounds (111, 158, 159). This problem became realized in connection with the discovery of creatine phosphate by Fiske and Subbarow. These authors thus proposed (53) a precipitation procedure based on the difference in solubility of ortho- and creatine phosphates as the calcium salts in order to avoid the interference with phosphate analysis caused by breakdown of creatine phosphate. A similar separation of the two compounds can also be accomplished by precipitating orthophosphate as the magnesium ammonium (104) or the barium salt (40). The same problem was met some years later by Lipmann when attempting to identify and estimate acetyl phosphate (102). Again, in this case, a procedure has been suggested in which orthophosphate was precipitated under suitable conditions as the calcium salt while acetylphosphate remained in solution. A further extension of this method for a selective determination of both acetyl- and creatine phosphate in the presence of orthophosphate has also been described (103). All these procedures are, however, seriously limited in their possibilities by the fact, discussed in greater detail in the following section, that they need relatively large amounts of sample and are not suited for serial work.

A promising approach to an elimination of the interference caused by acid labile phosphate compounds, and thus to a determination of true orthophosphate, has been provided by Lowry and Lopez (110).

These authors succeeded in working out conditions under which the formation and reduction of phosphomolybdate can be accomplished at a pH as high as 4. The reaction is performed in acetate buffer with ascorbic acid as the reducing agent. Under these conditions (and if the previous deproteinization has been performed in a sufficiently cautious manner, e.g., with saturated ammonium sulfate at pH 4) labile phosphate compounds, such as creatine phosphate, acetyl phosphate, or ribose-1-phosphate, are only split at a rate of 5% or less of that observed under the conditions of the Fiske and Subbarow method.

C. THE LOWRY AND LOPEZ METHOD

Principle. The phosphate is converted into phosphomolybdate at pH 4 in acetate buffer and the complex reduced by ascorbic acid.

Reagents.

A. Acetate buffer pH 4 (0.1 *M* acetic acid, 0.025 *M* sodium acetate).

B. 1% ascorbic acid.

C. 1% ammonium molybdate in 0.025 *M* H_2SO_4.

Procedure. In order to take care of the advantages of this method, the deproteinization must be performed under conditions which do not hydrolyze the particular labile compound, e.g., with ice-cold trichloroacetic acid (5%), perchloric acid (3%), or, in the case of very labile compounds, with saturated ammonium sulfate, which is 0.1 *M* in acetic acid and 0.025 *M* in sodium acetate (pH 4). The concentration of phosphate in the extract should be in the range 0.075—0.5 mM. The extract is diluted five times with 0.1 *M* sodium acetate in the case of acid precipitation and with A in the case of ammonium sulfate. To the diluted sample 0.1 volume of B and 0.1 volume of C are added. Readings are made at 5 and again at 10 minutes after the addition of molybdate at 700 mμ (or anywhere between 650 and 950 mμ). The two values are extrapolated to zero time.

Slight modifications of the method have been suggested by Potter (128), and by Ferrari (50). Some effects of cyanide perchloric acid and trichloroacetic acid on the color intensity obtained in the procedure have been discussed by Potter *et al.* (129).

An embarrassing problem in all the above procedures is their bad tolerance for certain kinds of interfering substances of nonphosphate character, possibly occurring in biological test systems. According to the thorough investigation of Berenblum and Chain (9) three main classes of such interfering substances can be distinguished: (*A*) those which alter the acidity of the medium, e.g., acids, alkalis,

buffers; (*B*) those which form molybdenum complexes, e.g., fluorides, citrates, oxalates, etc.; (*C*) those which change the concentration of the reducing agent, e.g., nitrates, hypochlorite, etc. The brilliant idea of these authors to take advantage of the extreme solubility of phosphomolybdic acid in organic solvents offered a general solution of the problem.

According to the original method described by Berenblum and Chain (10), the phosphomolybdic acid formed in an aqueous solution of sulfuric acid is extracted by shaking with isobutanol. The two layers are separated and, after some washings with dilute sulfuric acid, the phosphomolybdic acid in the organic solvent is reduced by shaking with an aqueous solution of stannous chloride. After separating the two phases once more, the color intensity is determined in the isobutanol solution. In addition to being almost completely independent of interfering substances, the method also has the great advantage of allowing broad variation of the reagents involved; moreover, the color intensity is stable within a wide range of time (cf. ref. 127). Furthermore, the principle of separating orthophosphate by solvent distribution has received important application for studies involving radioactive phosphate, as will be discussed in a later section.

A method implying a simplification of the Berenblum and Chain procedure has been described by Martin and Doty (117). These authors have replaced isobutanol with a mixture of equal parts isobutanol and benzene, which decreases the time required for the separation of the two layers. Furthermore, they perform the reduction of the phosphomolybdic acid in an aliquot of the organic layer, diluted with acid ethanol, by adding an aqueous solution of $SnCl_2$. Repeated shakings and separations are thus avoided. A valuable novelty introduced by these authors is that they render permissible the presence of limited amounts of protein in the sample to be analyzed by including silicotungstic acid into the reaction mixture. This makes the method very well suited for routine analysis of phosphate in enzyme incubation mixtures.

D. THE BERENBLUM AND CHAIN METHOD AS MODIFIED BY MARTIN AND DOTY

Principle. The phosphomolybdic acid formed in acid solution is extracted by shaking a mixture of isobutanol and benzene. An aliquot of the organic layer is diluted with acid ethanol and treated with an aqueous solution of $SnCl_2$.

Reagents.

A. 5 *M* H_2SO_4.

B. 10% ammonium molybdate.

C. isobutanol-benzene mixture (equal parts).

D. 10% $SnCl_2 \cdot H_2O$ in concentrated HCl. Freshly diluted 200 times with 0.5 *M* H_2SO_4.

E. 3.2% (v/v) sulfuric acid in absolute ethanol.

Procedure. (As adapted for animal material by Ernster *et al.* (47)): 3 ml. of the deproteinized sample containing between 0.05–10 μM phosphate are mixed in a test tube with 0.5 ml. of A, 5 ml. of C and 0.5 ml. of B. The mixture is shaken for 15 seconds. After separation of the two layers a suitable amount (0.1–2.5 ml.) is removed by means of an all-glass syringe and diluted with E to 5 ml. 0.5 ml. of D is added and mixed immediately. Color intensity is measured at 625 or 730 mμ (or any intermediate wave length).

An advantage of this method is that it can be employed even when the sample contains small amounts of protein (cf. p. 16). In this case 1 ml. of silicotungstic acid is to be included in the water phase. This reagent is prepared as follows: 26.7 g. $Na_2SiO_3 \cdot 8H_2O$ and 397.0 g. $Na_2WO_4 \cdot 2H_2O$ are dissolved in 2.5 l. of water. 75 ml. concentrated H_2SO_4 are added and the solution refluxed for 5 hours. After cooling, it is filtered and diluted with water to 5 l.

Slight modifications of the above procedures have been proposed by several workers (5, 47, 90, 108, 159). A technical improvement in the form of a mixing vessel has been offered by Davidson and Waymouth (33). An ultra micro procedure based on the Berenblum and Chain principle has recently been worked out by Schaffer *et al.* (138); this allows the determination of phosphate in an amount of 0.002 μg. P.

Ennor and Rosenberg (44) have criticized the reliability of the Martin and Doty method in the presence of creatine phosphate, by claiming that orthophosphate from labile phosphates by hydrolysis after the separation of the two layers might migrate into the organic layer and thus interfere with the analysis. This could be refuted, however, by Ernster *et al.* (48) who demonstrated that these objections were exaggerated.

During recent years some methods have appeared which are based on a colorimetric determination of phosphorus, without involving a reduction of phosphomolybdic acid. A direct measurement of the greenish-yellow color of phosphomolybdic acid has been suggested by Boltz and Mellon (15). Another procedure, implying a

measurement of the color change occurring in a solution of the dye quinaldine red in the presence of phosphomolybdate, has been described by Soyenkoff (140, 141).

A promising new approach (which, it seems, has not yet reached the majority of laboratories interested in phosphate metabolism) is that based upon Misson's reaction (119) involving a determination of phosphorus as the beautifully yellow phospho-vanadio-molybdate (63, 64, 65, 89, 112).

Finally, attempts have been made to determine phosphorus in the flame spectroscope (37); this method is, however, too sensitive to disturbances.

2. Pyro- and Metaphosphates

There are three principles described in the literature for specific determinations of inorganic pyrophosphate.

According to the method of Cohn and Kolthoff (22), pyrophosphate can be quantitatively separated from a solution containing orthophosphate and phosphate esters in an excess of 16 times by precipitation at pH 3.6 as the crystalline cadmium or manganous salt. This method allows the separation of as little as 2 μM of pyrophosphate within an error of 10%. The quantitative determination of the precipitate is accomplished by means of orthophosphate analysis after hydrolysis of the dissolved precipitate.

A modification of this method, taking into account the fact that, under certain conditions, ATP may have a solubilizing action on manganous pyrophosphate, has recently been reported (20).

A specific colorimetric method for the determination of pyrophosphate has recently been described by Flynn *et al.* (57). It is based on the old observation by Davenport and Sacks (31) that, in the presence of cystein, pyrophosphate reacts with molybdic acid in a way similar to orthophosphate. The amount of pyrophosphate is estimated as the difference obtained with the Fiske and Subbarow reaction mixture supplemented with cystein after 90 and 7 minutes. The method is as yet to be regarded as approximate since it is disturbed by orthophosphate and by labile phosphate esters undergoing hydrolysis during the long period of color development.

Finally, attempts have also been made to determine pyrophosphate after hydrolysis by means of specific inorganic pyrophosphatase (1).

A method for separating pyrophosphate from nucleotides has been proposed by Crane and Lipmann (29). It is based on an adsorption

of the nucleotides on charcoal. The content of pyrophosphate in the sample is given by the difference in acid labile P before and after charcoal treatment.

Owing to the occurrence of many polymeric forms with widely shifting chemical properties (79), no uniform methods are available for the determination of metaphosphates. Some forms are suited for analysis based on measuring the metachromasia induced by these compounds in the dye toluidine blue. The method, which initially was used as a qualitative one (1), has recently been thoroughly investigated and refined by Damle and Krishnan (30).

Differential analysis of inorganic phosphate, including ortho-, pyro-, and metaphosphates in mixtures, have been based on fractional precipitation, taking advantage of the very low solubility of the barium salt of metaphosphate at a pH as low as 3 (76, 80, 160, 164). The procedures imply, thus, a combination of barium precipitation with Cohn and Kolthoff method. In view of the rather narrow pH ranges, however, these methods must mostly be regarded as semiquantitative.

A method for the differential determination of meta- and pyrophosphate, based on a measurement of color changes induced by these compounds in an isoamyl alcohol-ether solution of a ferrithiocyanate complex, has been described by Wirth (161).

A paper chromatographic separation procedure of inorganic ortho-, pyro-, trimeta-, and tetrametaphosphates has recently been described by Growther (69).

III. DETERMINATION OF ORGANIC PHOSPHORUS COMPOUNDS

1. Tissue extracts

A. FRACTIONATION BY ACID HYDROLYSIS

The original interest in the estimation of organic phosphorus compounds was concerned with gaining a quantitative picture of their occurrence in biological material. During the early 1930's, when this problem arose, the number of known phosphate esters was relatively low, and the organic radicals included in them were rather similar from the chemical point of view. It was therefore natural that the first attempts at a quantitative estimation of these compounds was

based on the characteristics of the linkage by which the phosphate was bound to the organic radicals. A splittage of this linkage by means of acid-catalyzed hydrolysis, followed by orthophosphate determination, seemed at this time to afford a promising basis for a differential estimation of phosphoric esters occurring in biological extracts.

The standard conditions generally used for acid hydrolysis consist of heating a sample in a boiling water bath in the presence of 1 *M* hydrochloric acid. When treating a tissue extract in this way, three fractions usually are distinguished: (*1*) "free phosphate," i.e., that reacting as orthophosphate under the conditions of the Fiske and Subbarow method; (*2*) acid labile phosphate, i.e., that split after 7 minutes of boiling in hydrochloric acid (or 15 minutes with sulfuric of the same concentration); (*3*) "residual" phosphate, i.e., that withstanding this treatment. To the first category belongs, besides orthophosphate, acetyl phosphate, ribose-1-phosphate, carbamyl phosphate, and, partially, creatine and arginine phosphates. The second group includes inorganic pyro- and metaphosphates, nucleotidepolyphosphates, glucose-1-phosphate, and, partially, phospho*enol*pyruvate, glyceraldehyde phosphate, dihydroxyacetone phosphate and the phosphate group in the 1-position of the hexose-diphosphates and of diphosphoglyceric acid. The third category comprises all true phosphate esters, such as glucose-6-phosphate, ribose-5-phosphate, glycerol phosphate, adenosine-5-phosphate, etc.

Evidently, none of the fractions may represent a uniform type of compound and in this form, thus, fractional hydrolysis in itself is hardly suited for a differential analysis of individual phosphate compounds in a tissue extract. It may be useful, on the other hand, under refined conditions of hydrolysis, especially if an extremely labile fraction is to be distinguished from orthophosphate (45, 81, 98, 105, 113), or when the hydrolysis procedure is preceded by a fractionation of the tissue extract by precipitation or other means.

B. FRACTIONATION BY PRECIPITATION

Fractionation by means of precipitation of organic phosphorus compounds as the metal salts has for some time been the conventional method of characterizing and estimating these compounds in tissue extracts. The principle is based on a difference in solubility of the Ba, Ca, Mg, Hg, Ag, Pb, etc., salts of these compounds. Thus, it