THE DETERMINATION OF INORGANIC PHOSPHATE IN URINE BY ALKALIMETRIC TITRATION.

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In an investigation of the excretion of phosphate and other substances in short periods (chiefly of 1 hour) now being conducted in this laboratory, it has been necessary to make many inorganic phosphate determinations with limited quantities of urine. In most instances the accuracy possible with colorimetric and nephelometric methods has been sufficient, and, after a considerable amount of time had been spent in attempting to adapt then existing methods to the purpose at hand, the routine determinations were for a time made by precipitating the phosphate with magnesia mixture and determining the phosphate content of the redissolved precipitate nephelometrically (with strychnine molybdate). The necessity of resorting to this none too satisfactory arrangement has now fortunately been removed by the calorimetric method recently devised by Bell and Doisy, and this has since been used instead. In view of the empirical

1 Bell, R. D., and Doisy, E. A., J. Biol. Chem., 1920, xlv, 55. In connection with this very welcome addition to the supply of micro methods for inorganic phosphate, added emphasis should perhaps be given to the authors' remarks on the necessity of replacing the contents of the standard cup by fresh solution each time the unknown in the other cup is changed. This must be done even when several determinations are being made seriatim with a single standard. Observing this precaution, I have made several comparisons with the titration method described in this paper, as well as with the von Lorenz method, with a maximum difference of less than 2 per cent. When the rate of phosphate excretion is very small, the amount of urine necessary to give a color comparable with the standard recommended may be sufficient to give a distinct precipitate with the molybdate acid solution, and the error may then be greater. This difficulty may be avoided by repeating the determination with a smaller quantity of urine, using, if necessary, a weaker standard.
character of colorimetric and nephelometric analyses, particularly when they are applied to complex mixtures without any preliminary isolation of the substance to be determined, it seemed unwise to rely exclusively on such methods without confirmation by some procedure relatively free from empirical features. This position has fully justified itself, since the method to be described in this paper (designed partly to obtain such confirmation, and partly to secure more accurate data, when desired, without being obliged to use time-consuming gravimetric methods involving double precipitation) has assisted materially in defining more closely the conditions under which the method of Bell and Doisy is most reliable, and at an earlier date was the means of showing that the direct application to urine of the nephelometric method (as it is used for blood filtrates) gives results that are quite erroneous.

The principle of the present method is the titration, on a small scale, of the magnesium ammonium phosphate precipitate. This titration, introduced many years ago by Stolba, has recently been adapted to urine, on the basis of samples containing about

Fig. 1. Titration curves of 0.05 M (I) and 0.01 M (II) monopotassium phosphate solutions (at 20°C) on the alkaline side of the end-point. The ordinates represent the excess of alkali in terms of the method described in this paper; e.g., 1 per cent of excess alkali corresponds with the conversion of 2 per cent of the primary phosphate into secondary phosphate.

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40 mg. of phosphorus, by Bauzil and Angiolani. These investigators, as well as others who have proposed modifications of this method, adhere to the use of methyl orange, and it is particularly in this respect that the method requires alteration before it can be used for smaller quantities of material.

Electrometric $\text{CH}_2$ determinations on solutions prepared from purified monopotassium phosphate have shown that the titration curves for 0.05 $\text{M}$ and 0.01 $\text{M}$ phosphate, in the vicinity of the end-point, are not far from parallel, and that the change in pH is rapid enough to permit the titration of 0.01 $\text{M}$ solutions with greater accuracy than is generally stated to be possible with much more concentrated phosphate solutions, provided an indicator with a sufficiently sharp color change is used. 25 cc. of a 0.01 $\text{M}$ phosphate solution can in fact be titrated, using methyl red and a standard color for comparison, with an error of only about 0.01 mg. of phosphorus.

The titration curve in Fig. 2 was calculated by means of a formula derived in a manner similar to that proposed by Prideaux, but taking into account the repression of the ionization of phosphoric acid by the large excess of primary phosphate present:

$$R = \frac{(\text{NaH}_2\text{PO}_4) + 2(\text{Na}_2\text{HPO}_4)}{(\text{NaH}_2\text{PO}_4) + (\text{Na}_2\text{HPO}_4) + (\text{H}_3\text{PO}_4)}$$

$$= \frac{1 + 2 (\text{Na}_2\text{HPO}_4)}{1 + (\text{Na}_2\text{HPO}_4) + (\text{H}_3\text{PO}_4)}$$

In this equation, $\frac{(\text{Na}_2\text{HPO}_4)}{(\text{NaH}_2\text{PO}_4)}$ may be considered equal to $\frac{k_2}{[\text{H}^+]}$, without further correction for incomplete ionization of the two salts, by making use of the apparent dissociation constant of pri-
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Mary phosphate when the concentration is 0.01 M. Interpolation from the data of Michaelis and Garmendia\(^8\) gives the value 10\(^{-7}\) for this constant (\(k_3\)).

It has been found experimentally that, in the vicinity of the end-point, the ratio \(\frac{\left[H_2PO_4\right]}{\left[NaH_2PO_4\right]}\) may, with sufficient accuracy, be considered equal to the \(\left(H^+\right)\) divided by a constant \(K\). The value of \(K\) found for the circumstances under consideration is \(4\times10^{-3}\).

The equation may therefore be written:

\[
R = \frac{1 + 2 \times 10^{-7}}{1 + \frac{k_3}{\left[H^+\right]} + \frac{\left(H^+\right)}{K}} = \frac{1 + 2 \times 10^{-7}}{1 + \frac{10^{-7}}{\left[H^+\right]} + \frac{\left(H^+\right)}{4 \times 10^{-3}}}
\]

The curve (Fig. 2) was drawn from points calculated on this basis, while the circles represent points determined experimentally with 0.01 M KH\(_2\)PO\(_4\) solutions containing various amounts of added NaOH or HCl. The agreement is quite satisfactory.

Entirely apart from the matter of time consumption, titration has one distinct advantage over direct weighing of the magnesium ammonium phosphate (without double precipitation), for most of the substances that are inclined to contaminate the precipitate (calcium phosphate, magnesium phosphate, and magnesium ammonium phosphates other than MgNH\(_2\)PO\(_4\)) do not alter the titration figure, whereas they may seriously affect the weight.\(^9\)

Owing to the small scale of the new method, it has been necessary to devise special means of handling the precipitate. This is accomplished by the use of a filtration tube, consisting simply of a glass tube, about 8 mm. in internal diameter and 120 mm. long, shrunken at the lower end to a bore of 2 mm., and flanged at the upper end. (The capacity of the tube should be well over 5

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\(^8\) Michaelis, L., and Garmendia, T., Biochem. Z., 1914, lxvii, 431.

\(^9\) Mathison (Mathison, G. C., Biochem. J., 1929, iv, 237) infers that the presence of citrate prevents contamination with calcium, but there is no difficulty in demonstrating calcium in precipitates from human urine by dissolving in dilute hydrochloric acid, precipitating with oxalate, and applying the microchemical test with sulfuric acid (Chamot, E. M., Elementary chemical microscopy, New York, 1915, 288).
This device, supported by a rubber stopper in the neck of a suction flask large enough to contain a test-tube with a capacity of about 50 cc., and provided with a thin mat of paper pulp, makes it possible to filter and wash small precipitates in a very short time, and to transfer the washed precipitate (through the hole in the lower end) to a flask for titrating. The use of a proportionately small filtration tube has the further advantage that the amount of washing required by the filter is almost negligible.

**Special Reagents Required.**

*Magnesium Citrate Mixture.*—Dissolve 80 gm. of citric acid in 100 cc. of hot water. Add 4 gm. of magnesium oxide, and stir until dissolved. Cool, and add 100 cc. of ammonium hydroxide (density 0.90). Dilute to 300 cc., let stand 24 hours, and filter. (If the magnesium oxide contains much carbonate, it should be...
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fresherly ignited.) This is essentially the reagent used by Mathison and others.

95 Per Cent Alcohol.—This reagent must be neutral, since it is used for washing the MgNH₄PO₄ precipitate. 5 cc. of it should not more than slightly alter the color of 50 cc. of water containing a little methyl red and previously adjusted with very dilute alkali to an intermediate orange color. Ordinary alcohol will meet this requirement only after distillation from alkali.

Methyl Red Solution.—0.004 per cent solution in 50 per cent alcohol.

Standard Acetate Mixture.—Mix 50 cc. of 2 N acetic acid and 35 cc. of 2 N NaOH (free from carbonate), and dilute to 100 cc.

Standard Color.—To 2 cc. of the above acetate mixture (in a 100 cc. Erlenmeyer flask) add 2 cc. of the methyl red solution and 21 cc. of water.

The Method.

Transfer to a large lipped test-tube (200 X 20 mm.) an amount of urine containing between 2 and 7 mg. of inorganic phosphorus. Add water, if necessary, to make the total volume about 10 cc. Then add 1 cc. of magnesium citrate mixture and 2 cc. of ammonium hydroxide (density 0.90). Shake the tube until crystallization begins (with the larger amounts this occurs almost immediately), then shake very gently, but continuously, for 15 minutes longer. At least eight samples can be precipitated simultaneously in this way.

10 Within these limits the method is, of course, more accurate the larger the amount used.

11 When 10 cc. of urine contain less than 2 mg. of inorganic phosphorus, a larger volume should be used. In this event, the reagents should be increased in proportion, and the same is true when it is desired to use more than 10 cc. of urine for the sake of greater accuracy. If it is necessary to use more than 20 cc. of urine, the prescribed 15 minutes shaking may not be sufficient for complete precipitation. With urines as dilute as this the method is hardly more accurate than Bell and Doisy’s.

12 Merely keeping the mixture in motion (and that is all that is really necessary) tremendously accelerates the precipitation, presumably because of the much larger number of nuclei formed under these conditions. With urine, standing undisturbed over night may not be sufficient for complete precipitation.
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With a glass tube transfer to the filtration tube (described above) a sufficient amount of paper pulp to make a mat just thick enough to cover the bottom of the tube, and suck dry. Filter off the precipitate on this mat, using only very gentle suction. Wash with 10 cc. of 2.5 per cent ammonia, and then with four 5 cc. portions of 95 per cent alcohol, making no attempt to dislodge the part of the precipitate that adheres to the wall of the test-tube. Throughout these operations the lower end of the filtration tube should drain into a large test-tube set inside the suction flask, since it is better to keep the flask dry. Between the second and third washings with alcohol, it is well to wash out the test-tube that serves as a receiver.

Remove the filtration tube from the suction flask, and support it (by a clamp) with its lower end inserted into the mouth of a 100 cc. Erlenmeyer flask. Pipette into the test-tube 0.1 N HCl, 1 cc. at a time, until the precipitate dissolves completely on shaking, and pour the resulting solution into the filtration tube. By means of a stiff, sharpened nichrome wire, poke the precipitate and mat through the 2 mm. hole into the Erlenmeyer flask. Rinse with 2 cc. of methyl red solution and 13 cc. of water. To the contents of the Erlenmeyer flask add 0.1 N HCl, 1 cc. at a time, until the solution remains distinctly red after it has been shaken thoroughly. (At least 0.5 cc. of 0.1 N HCl should be added in excess of the amount necessary to decompose the precipitate.) Pour the solution into the lipped test-tube and back until the precipitate is completely dissolved, finally rinsing the few drops remaining in the test-tube into the flask with 5 cc. of water. The solution is now ready for titration with 0.1 N NaOH from a

13 Shake one 15 cm. ashless filter paper (e.g., Schleicher and Schüll No. 589, black ribbon) with 200 cc. of water in a stoppered bottle until the paper is thoroughly broken up. 2 or 3 minutes vigorous shaking should suffice (Jodidi, S. L., and Kellogg, E. H., J. Ind. and Eng. Chem., 1916, viii, 317).
14 With a calibrated Ostwald pipette.
15 If one prefers, more dilute alkali can be used (with an ordinary burette), although it would then be necessary to use less water for rinsing, since the total volume must not be much greater than 25 cc. More dilute alkali offers no particular advantage, and has the disadvantage of being less stable.
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micro-burette provided with an accessory tip. Run in the alkali until the color begins to turn, and complete the titration by adding 0.01 cc. of alkali at a time until one such quantity makes the solution definitely yellower than the standard. This method, rather than accurate matching of the colors, is necessary to compensate for the error that would otherwise be introduced because of the fact that the theoretical end-point varies with the concentration of phosphate.

The difference between the volumes of acid and alkali used gives the amount of 0.1 N acid neutralized by the precipitate. The phosphorus content of the sample (in mg.) is obtained by multiplying this figure by 1.552.

Analysis of Phosphate Solution.

A sample of monopotassium phosphate, purified by recrystallization, was dried to constant weight at 110°. A portion of this, ignited to KPO₃, showed the theoretical loss in weight.

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<th>TABLE I. Analysis of Phosphate Solution.</th>
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Various quantities of a solution, prepared from this material and containing 0.7 mg. of phosphorus per cc., were analyzed by the method described. The results are shown in Table I.

17 If a small glass bead is placed in the rubber tube connecting the burette with the accessory tip, the control of the delivery at the end of the titration is made easier.
Provided enough citrate is present during the precipitation to prevent the appearance of an amorphous precipitate of calcium phosphate, the results are the same as in the absence of calcium. When the urine contains a large amount of calcium in proportion to the phosphate present, more magnesium citrate must be added, but in doing so it is necessary to increase the volume of the solution and the amount of ammonium hydroxide. If, for example, after the addition of the reagents in the manner stated in the preceding description of the method, the solution immediately develops a turbidity that is obviously not crystalline, a new sample should be measured out, and water added to make the total volume 20 cc. (instead of 10), followed by 2 cc. of the magnesium citrate mixture and 4 cc. of ammonium hydroxide. This has so far never been found necessary with human urines.

On adding the reagents in the usual manner to 6 cc. of the above potassium phosphate solution to which had been added 30 mg. of calcium in the form of calcium chloride, an amorphous precipitate immediately appeared. Nevertheless, on doubling the volume and the quantity of reagents, this precipitate largely disappeared, and triple phosphate crystals began to separate; on continuing the analysis as usual, the result was only slightly wrong (4.22 instead of 4.20 mg. of phosphorus). The determination was then repeated, this time diluting to 20 cc. at the start and adding twice the usual amounts of reagents; no sign of an amorphous precipitate appeared, and the result was entirely correct (4.20 mg.).

Analysis of Urine.

Probably the best evidence of the accuracy of the method with urine is the fact that a second precipitation of the magnesium ammonium phosphate, after dissolving the first precipitate with acid, does not significantly alter the results. The figures obtained after double precipitation do tend to be slightly lower, as may be expected from the additional manipulation necessary, but the difference is only of the magnitude of the difference between duplicates with pure phosphate solutions (Table I).
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To show that the method is subject to no gross error, it has been compared with the von Lorenz\(^8\) method. Samples of urine (human) containing about 15 mg. of inorganic phosphorus were precipitated with magnesium citrate mixture and ammonia by shaking for 15 minutes, followed by standing over night (although the last step has never been found to alter the result). The precipitate of magnesium ammonium phosphate was then filtered off, washed with dilute ammonia, dissolved in acid, and precipitated as ammonium phosphomolybdate according to the directions given by von Lorenz. The precipitates were filtered on a platinum mat, as recommended by Neubauer and Lücker.\(^9\)

On the basis of preliminary determinations with phosphate solutions, the amount of phosphorus was obtained by multiplying the weight of the precipitate by the factor 0.0144 (the corresponding factor for \(\text{P}_2\text{O}_5\) would be 0.03296, agreeing with von Lorenz's figure 0.03295).

Table II contains a series of representative results covering somewhat more than the range recommended (2 to 7 mg. of phosphorus). The first five urines were collected without respect to time, but they were obtained during the morning when the


inorganic phosphate output is likely to be low.\textsuperscript{20} Since the amount of phosphate under these circumstances is small compared with other urinary constituents, urines of this sort offer the most rigid test of the method from the standpoint of the possibility of errors due to contamination of the precipitate by other basic substances. Attention is called particularly, in this connection, to Urine 7, which was a 3 hour sample collected during the morning, and shows an average phosphate excretion of only 5.5 mg. of phosphorus per hour.

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