METHODS FOR THE QUANTITATIVE ESTIMATION
OF INORGANIC PHOSPHORUS IN VEGETABLE
AND ANIMAL SUBSTANCES

OHIO
Agricultural Experiment
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PREFACE.

That portion of this bulletin which has to do with plant substances marks the first step in a program of investigation of the phosphorus compounds of the food of animals.

This study is necessary as a preliminary to the practical application of the results of our animal metabolism experiments with pure phosphorus compounds.

The greatest difficulty in the way of a quantitative estimation of the groups of phosphorus compounds in vegetable substances has been the accurate separation of inorganic phosphates from the salts of phytic acid.

The solution of this problem opens up an important field to a new and promising line of attack, and points the way with considerable certainty to the estimation of phytin and nuclein phosphorus.

The solution of the problem of estimation of inorganic phosphorus in a wide range of animal tissues makes judgment possible as to the availability of the phosphorus of the food for the nutrition of the functional substance of these parts.
INTRODUCTION.

In studying the nutritive value of vegetable foods, either in their usual state, or as modified by the chemical constitution of soils or fertilizers, it is important that we distinguish between organic and inorganic phosphorus, and in considering the specific effects of nutrients on the development of animals and the composition of their organs and tissues, it is a matter of great interest and importance that we know whether or not variations in their phosphorus content involve the simple inorganic salts in solution in their liquids, or the complex organic phosphorus compounds of the living structures.

This distinction is of significance in animal nutrition especially, because of the limited usefulness as nutrients of mineral elements in an inorganic condition.

We have no reason to expect that variations in the food will produce especially marked variations in the inorganic phosphorus content of animal tissues. In order, therefore, to get the results desired, it is necessary that the method used for the estimation of inorganic phosphorus be as nearly as possible chemically and mechanically perfect.

On these accounts, and as a part of the general program of study of the nutritive value of compounds of phosphorus, which is in progress in this department, we have undertaken a solution of the problem of quantitative estimation of inorganic phosphorus in plant and animal substances generally.

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A number of methods for these determinations have been published but have been found to be either inaccurate or cumbersome, and therefore we have given the subject some attention.

Conclusions have been reached which give us convenient and approximately accurate methods for the estimation of inorganic phosphorus in a wide range of plant and animal substances.

As a problem in quantitative chemical analysis the estimation of inorganic phosphorus in vegetable substances is rendered particularly difficult by the facts that in each substance under observation we have a different mixture of phosphates, variously related, both chemically and physically, to different groups of organic compounds.

The problem of complete extraction may be influenced by the mechanical relation of the phosphates to insoluble and impenetrable structures, and the possibility of hydrolysis of organic phosphorus compounds by the extractive reagents introduces into the problem an element of uncertainty for the removal of which our present knowledge of the relations of phosphorus to organic compounds in plants does not begin to suffice.

Further, combinations of inorganic phosphorus with organic compounds, such as may occur without the agency of a vital process in a living organism, introduce an element of embarrassment into the discussion, as also does the fact that there is no clear-cut and essential distinction between organic and inorganic compounds generally, but these several objections to the general idea of such a separation as we contemplate do not have weight sufficient to prevent the solution of the problem on a useful working basis.

ESTIMATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES.

Of the various methods which have come to the attention of the authors, that used by Hart and Andrews* appeared to give the most specific starting-point for further work. The essential points of this method are as follows:

1. Extract 5 grams of sample by shaking for 15 minutes in 125 c. c. of .2 percent hydrochloric acid.
2. Filter, and wash with water until filtrate measures 500 c. c.
3. Neutralize a 200 c. c. portion of this filtrate with ammonia. Precipitate at 65° with 10 grams of ammonium nitrate, 25 c. c. of neutral ammonium molybdate solution and 2 c. c. of nitric acid, specific gravity 1.20. Keep at this temperature for 15 minutes; allow to cool gradually, and filter after one hour.
4. Treat this precipitate as is usual in a phosphorus estimation, dissolving in ammonia, precipitating with magnesia mixture, burning to the pyrophosphate, and then redissolving and reprecipitating to free from impurities.

*Bul. 238, New York Agr. Exp. Sta., p. 188.
Hart and Andrews found that the official molybdate solution decomposed nucleinic acid.\(^1\)

In order to reduce this decomposition to a minimum they used neutral ammonium molybdate solution with just sufficient nitric acid to precipitate the phosphoric acid present. This quantity they state to be 2 c. c. of 1.20 nitric acid.\(^2\)

The second precipitation as magnesium ammonium phosphate was advised by them to remove from the pyrophosphate possible contamination from magnesium oxide resulting from proteid-magnesium compounds precipitated along with the magnesium ammonium phosphate by the magnesia mixture.\(^3\)

In making use of this method we found it not entirely satisfactory, and on the following accounts:

1. The time allowed for extraction appears to be insufficient.
2. The precipitation with neutral molybdate solution and the minimum amount of acid, is rendered difficult, as a routine method, by the fact that 2 c. c. of 1.20 nitric acid are in many cases not nearly sufficient to cause precipitation of the inorganic phosphorus.
3. The bulky, flocculent precipitate which is often formed may completely mask the formation of the yellow precipitate of inorganic phosphorus, rendering it impossible to judge of the completeness of the precipitation.
4. The precipitate is, with many products, very difficult to filter.
5. This method involves the precipitation of inorganic phosphorus in the presence of phytin. A quantitative precipitation of inorganic phosphorus alone, from a solution containing phytin, appears to be impossible.
6. Nucleinic acid may be hydrolized by the nitric acid used in this precipitation, the result being the formation of inorganic from organic phosphorus.
7. Nucleinic acid, like phytin, appears to hinder the formation of the yellow precipitate.
8. A possible source of error lies in the carrying down of phosphorus-containing proteids with the yellow precipitate, the solution of these proteids in ammonia, their precipitation with magnesia mixture, and in this way their contribution to the amount of the pyrophosphate.

In an attempt to remedy the imperfections of this method we made the following observations:

The time of extraction in .2 percent hydrochloric acid may be safely lengthened, at least for most substances, to three hours.*

The decomposition of nucleinic acid by the nitric acid used in the molybdate precipitation, is obviated by substituting for this first process, a precipitation with magnesia mixture, since free nucleinic acids and some at least of their salts are soluble in ammonium hydrate.

Besides getting rid of the nucleinic acids, this precipitation largely removes the mechanical difficulty of handling the bulky, flocculent, molybdate precipitate, and obviates the necessity for the delicate determination for each substance studied, of the point of compromise between completeness of precipitation of inorganic phosphates and decomposition of organic phosphorus compounds by nitric acid.

The precipitate with magnesia mixture is very much less bulky, and is more easily filterable, and in this case there is no doubt as to the completeness of the process.

The interference of phytin with the estimation of inorganic phosphorus, which may amount to a complete suppression of the yellow precipitate if this compound be present in considerable amount, or which may, through decomposition, add to the inorganic phosphorus if the phytin be present in smaller quantity, is entirely done away with by the separation of phytin and inorganic phosphorus in the precipitate with magnesia mixture, by dissolving the inorganic phosphates out of this precipitate with acid-alcohol. The phytin is practically, if not entirely insoluble in this reagent.

Below are details of work sustaining these conclusions.

The problem of removal of nucleinic acids from the .2 percent hydrochloric acid extract was worked out by experiments with a preparation of nucleinic acid from yeast. The free nucleinic acid and some of its salts, at least the magnesium compound, proved soluble in ammonium hydrate. This fact led to the separation of inorganic from nucleinic acid phosphorus by precipitating with magnesia mixture and filtering out the precipitate.

The preliminary precipitation with magnesia mixture has the double advantage of removing the nucleinic acid and of facilitating subsequent work. After dissolving the magnesia precipitate in hydrochloric acid, the molybdate solution produces a normal yellow precipitate which is free from the bulky flocculent mass which frequently entirely obscures, if indeed it does not prevent, the

*The use of phenol in the extractive reagent would probably prevent the activity of enzymes in substances where they are particularly active, though unless proven necessary the use of phenol is inadvisable.
formation of the yellow precipitate when the hydrochloric acid extract of the substance is treated directly with the molybdate solution.

After working out this point we learned from correspondence with Parke, Davis & Co., of Detroit that this magnesia precipitation was in use by this firm in the estimation of inorganic phosphorus in their preparation of nucleic acid from yeast.

In order to determine whether or not nucleic acid was decomposed in ammonia solution during our first magnesia precipitation, we weighed out three 1-gram portions of nucleic acid which had been prepared from yeast. These were dissolved in ammonia, and allowed to stand over night with magnesia mixture, as in the first step of our method for inorganic phosphorus estimation. Slight precipitates formed, which we combined, dissolved and reprecipitated, first with molybdate solution and then with magnesia mixture, but the combined precipitates were unweighable.

That conditions were correct for precipitation of inorganic phosphorus, was proven by addition of sodium phosphate to another sample of nucleic acid, which was dissolved and treated like the three above mentioned. The phosphorus came down promptly in the usual way.

U. Suzuki and others* have found that in certain foods and fodders which they examined, including wheat bran and barley, a large portion of the organic phosphorus is present as phytin, and that accompanying this is an enzyme which decomposes phytin into phosphoric acid and inosite.

Attempts were made to prevent possible decomposition by enzymes during the estimation of inorganic phosphorus, by previous heating of the material, both by dry heat and by boiling, but the results were unfavorable to the use of this principle in a routine quantitative method.

After demonstrating that a longer time of extraction than 15 minutes was desirable, we sought to learn whether or not the action of enzymes might be expected to enter largely into the determination of results with the longer extraction.

Inorganic phosphorus was determined in two samples of wheat middlings by two different methods of procedure.

The extractions were made with .2 percent hydrochloric acid, and the extracts allowed to stand over night, two with 6.6 percent of phenol in the extractive reagent, and the other two in the .2 percent hydrochloric acid alone. The extracts were allowed to stand over night in order to give the enzymes a fair chance to demonstrate their presence.

*Bul. No. 4, Vol. 7, College of Agriculture, Tokio.
One sample gave us .006 percent of inorganic phosphorus with phenol present and .010 percent without. The other sample gave .005 percent and .012 percent with and without phenol.

While the amounts of inorganic phosphorus involved were very small the variations in the percent of inorganic phosphorus were considerable, and on the strength of this evidence we began the use of phenol in our provisional method, without having demonstrated that enzymes would make an appreciable difference in the inorganic phosphorus during our three-hour extraction, and without having determined that there was no bacterial growth in our .2 percent hydrochloric acid extract during the twenty-four hours which it stood.

These above results are comparative only, since they were obtained by precipitation in the presence of phytin.

After some preliminary work with the method of Hart and Andrews, certain modifications were compared with the original method. The results are set forth in the following table.

**COMPARISON OF MODIFICATIONS OF HART AND ANDREWS'S METHOD**

<table>
<thead>
<tr>
<th>Percent of Inorganic Phosphorus</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
<th>Method 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent of Inorganic Phosphorus</td>
<td>Hart's method, 15 minutes extraction; 2 c.c. nitric acid used in precipitation</td>
<td>Hart's method as in Column 1, but with 3 hrs. extraction; 2 c.c. nitric acid used in precipitation</td>
<td>Hart's method as in Column 1, but with 3 hrs. extraction, and 7 c.c. nitric acid used in precipitation</td>
<td>Modified by use of phenol in extractive reagent, and preliminary magnesium precipitation; phosphorus then precipitated by official molybdic solution</td>
</tr>
<tr>
<td>1 Gluten flour .......</td>
<td>.030</td>
<td>.023</td>
<td>.053</td>
<td>.044</td>
</tr>
<tr>
<td>2 Gluten feed .......</td>
<td>.052</td>
<td>.119</td>
<td>.171</td>
<td>.149</td>
</tr>
<tr>
<td>3 Cotton seed meal ...</td>
<td>.044</td>
<td>.105</td>
<td>.140</td>
<td>.118</td>
</tr>
<tr>
<td>4 Distiller's grains ...</td>
<td>.039</td>
<td>.085</td>
<td>.092</td>
<td>.087</td>
</tr>
<tr>
<td>5 Corn stover ........</td>
<td>.028</td>
<td>.091</td>
<td>.098</td>
<td>.098</td>
</tr>
<tr>
<td>6 Bermuda grass ......</td>
<td>.020</td>
<td>.101</td>
<td>.179</td>
<td>.174</td>
</tr>
<tr>
<td>7 Alfalfa hay ..........</td>
<td>.023</td>
<td>.073</td>
<td>.103</td>
<td>.091</td>
</tr>
<tr>
<td>8 Clover hay ..........</td>
<td>....</td>
<td>....</td>
<td>....</td>
<td>....</td>
</tr>
</tbody>
</table>

With the Hart and Andrews method as in Columns 1, 2, and 3, the preliminary precipitation was with neutral molybdate solution and varying amounts of nitric acid.

It will be observed that the figures in the second column are all higher than the corresponding figures in the first column, showing that 15 minutes extraction is insufficient.

There is, of course, a possibility that the greater amount of inorganic phosphorus found by the three-hour extraction, than by the fifteen-minute extraction, is due to hydrolytic decomposition of organic phosphorus compounds by the .2 percent hydrochloric acid during extraction, rather than to greater thoroughness of extraction.
In order to test this point we determined the inorganic phosphorus in phytin and in nucleinic acid, in one set of samples precipitating immediately after solution, in another set after 3 hours' standing, and in a third after 24 hours' standing in the .2 percent hydrochloric acid. We were unable to demonstrate decomposition in this reagent in either 3 or 24 hours' extraction.

The figures in the third column are, with one exception, higher than the figures in the second, which may be due either to the 2 c. c. of nitric acid used in Method 2 being insufficient to cause complete precipitation, or to the 7 c. c. of acid used in Method 3 being excessive, and augmenting the amount of inorganic phosphorus by decomposition of organic forms, or both decomposition and incomplete precipitation may have affected results.

After precipitation of inorganic phosphorus as with Method 2 with 2 c. c. of nitric acid, 5 c. c. of nitric acid were added to the filtrates to test the thoroughness of the precipitation. The characteristic yellow precipitate promptly appeared in all cases, except with gluten flour.

The figures with Method 4 are, with one exception, higher than the corresponding figures with Method 2, showing that the 2 c. c. of nitric acid used with Method 2 were insufficient. These figures are with one exception, lower than the corresponding figures with Method 3, showing that either the use of phenol in the extractive reagent, or the elimination by the magnesia precipitation of the possibility of decomposition of nucleinic acid by nitric acid, or both, tended to obviate probable inaccuracies in this method of work.

Subsequent investigation, however, showed that Method 4 was unreliable, because of the presence of phytin in the solution from which the inorganic phosphorus was precipitated; so the determinations have a comparative but not an absolute value.

In order to study the bearing of fineness of grinding of the sample on this method of work, a coarsely ground sample of bluegrass was divided into two equal parts and one of these was re-ground. Both samples were then passed through standard sieves with the following results:

<table>
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<th>PERCENT OF MATERIALS RETAINED BY SEIVES</th>
</tr>
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<tbody>
<tr>
<td>Coarsely ground............................</td>
</tr>
<tr>
<td>Finely ground......................... ....</td>
</tr>
</tbody>
</table>

Method 4 gave .106 percent of inorganic phosphorus in the finely ground sample and .109 percent in the coarsely ground sample, indicating that extreme fineness of division of such fodders as
blue-grass is probably not essential, and that our failure to extract all of the inorganic phosphorus in 15 minutes was in all probability not due to insufficient fineness of division of the material.

The method of extraction and precipitation used throughout the greater part of this study is as follows:

Place 25 c. c. of 90 percent phenol in a flask graduated to deliver 500 c. c.; add a small portion of .2 percent hydrochloric acid, and shake the flask until the phenol is dissolved. Then fill to the mark with .2 percent hydrochloric acid, and pour the contents upon 10 grams of the sample in a 600 c. c. Florence flask. Close the flask with a rubber stopper and shake at intervals of 5 minutes for three hours.

At the end of this period filter the extract through a dry paper. To a 350 c. c. portion of the filtrate add 10 c. c. of magnesia mixture and 50 c. c. of strong ammonia, and allow to stand over night.

Inorganic phosphorus is precipitated as magnesium ammonium phosphate, along with phytin if present, while nucleins, being soluble in ammonia, remain in solution. Next morning filter, and wash the precipitate free from phenol with dilute ammonia.

In the case of materials containing phytin, such as wheat bran and middlings, the precipitate consists almost wholly of phytin and magnesium ammonium phosphate. It is dark gray in color, and usually completely soluble in dilute acids. In the case of materials containing no phytin, it consists of impure magnesium ammonium phosphate, usually contaminated with variously colored unknown compounds.

Determination of inorganic phosphorus in this precipitate by dissolving in dilute acid, and precipitation with molybdate solution, did not give true results in the presence of phytin.

Experiments were now made on the action of phytin on the precipitation of inorganic phosphorus. In this work a commercial preparation of phytin was used. This material was a calcium-magnesium salt prepared by the Society of Chemical Industry, in Basle, Switzerland. It contained 19.9 percent of phosphorus. The sample was probably contaminated by a small quantity of inorganic phosphorus, since a separation from the latter is very difficult.

Definite quantities of this phytin were dissolved in 0.2 percent hydrochloric acid; a definite quantity of inorganic phosphorus in the form of di-sodium phosphate was added, and the solution precipitated with official molybdate solution in the usual way.

The following series of figures gives the results of the first trial:
The phytin in each case was dissolved in 100 c. c. of 0.2 percent hydrochloric acid, to which was added di-sodium phosphate, equivalent to 0.0420 gram of magnesium pyrophosphate. Ammonium nitrate and 40 c. c. of official molybdate solution were used in the precipitation of the inorganic phosphorus. The number of grams of phytin in each case represents approximately the percent of phytin in the solution.

### SUPPRESSION OF YELLOW PRECIPITATE BY PRESENCE OF PHYTIN

<table>
<thead>
<tr>
<th>Grams of phytin</th>
<th>Grams of pyrophosphate equivalent to inorganic phosphorus added</th>
<th>Grams of pyrophosphate recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.0420</td>
<td>0.000</td>
</tr>
<tr>
<td>0.5</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.4</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.3</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.2</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.1</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.05</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.03</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.01</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
<tr>
<td>0.00</td>
<td>&quot;</td>
<td>0.002</td>
</tr>
</tbody>
</table>

The striking fact about this series is that a one-percent solution of phytin completely suppressed the precipitation of the inorganic phosphorus present, there being no yellow precipitate after an hour's digestion at 65° C. This suppression of the yellow precipitate seemed to diminish with decrease in the phytin content of the solution, as shown by the increase in the weights of pyrophosphate as the corresponding amounts of phytin were decreased from 1 gram to .2 of a gram. With continued decrease in the phytin present, however, the results increased to quantities in excess of the blank. This may have been due to a decreased suppression of the precipitate by the lower percentages of phytin, the phosphorus in excess of the blank being contributed by the inorganic phosphorus present as an impurity in the phytin; or with decrease in the amount of phytin present, there may have been an increased splitting off of inorganic phosphorus from phytin.

The result on a .2 percent solution of phytin may have been due to a combination of two compensating errors, one the partial hydrolysis of phytin, and the other the partial suppression of precipitation by the presence of phytin, the combination of these factors resulting in a check with the blank.

Later results indicate that the high results with the low percents of phytin were partly due to the formation of inorganic phosphorus by the decomposition of phytin.
The results would naturally be higher than the blank because of the presence of appreciable quantities of inorganic phosphorus in the phytin sample, but this would not account for the low results obtained when considerable phytin is present, nor for the high results later obtained on low-phytin solutions. Duplicate series of determinations were made, all of which gave similar indications.

When using neutral molybdate, although decomposition was not so marked, as indicated by high results on low-phytin solutions, it was necessary in many cases to add more than the 2 c. c. of nitric acid, as specified by Hart and Andrews, in order to effect a separation of the yellow precipitate, while in 0.5 percent and 1.0 percent solutions of phytin, the yellow precipitate was completely suppressed as before.

It was also observed at this time that when the di-sodium phosphate solution was added to the 0.2 percent hydrochloric acid solution of phytin, a cloudiness was at first produced, which disappeared on stirring. In a one-percent solution of phytin it was found possible actually to precipitate the phytin with a strong solution of di-sodium phosphate, indicating either a partial salting out of the phytin, or a combination of inorganic phosphate with the phytin, due to a possible unsaturated condition of the latter.

Considering the results obtained thus far, it was obvious that a separation of phytin from inorganic phosphorus was necessary, if exact results were to be secured.

A quantitative separation of inorganic phosphorus from such a substance as phytin, is a task attended by considerable difficulty, because of the similarities in solubility and the difficulty of preventing hydrolysis of the phytin.

No organic liquid was found in which phytin is appreciably soluble. The only solvent seemed to be dilute acids, in which, of course, inorganic phosphates are also soluble. Phytin is precipitated from such solutions by alkalis and strong alcohol. Inorganic phosphates precipitate under the same conditions, when bases such as calcium and magnesium are present, as in the case of acid extracts of feeding materials.

Owing to the very limited number of solvents for phytin, methods of precipitation in acid solution were investigated.

It was found that aluminium salts cause a precipitation of phytin in a 0.2 percent hydrochloric acid solution of the latter. By adding ammonium nitrate and digesting at 60°C. we were able to precipitate the phytin from even a .01 percent solution of the same.
A number of series of determinations were made, using potash alum as the precipitant. The phytin was in each case dissolved in 100 c. c. of 0.2 percent hydrochloric acid in a 250 c. c. flask. Inorganic phosphorus was added as usual, and also 30 grams of solid ammonium nitrate. The flask was filled nearly to the mark with 0.2 percent acid, and then the phytin precipitated with a small excess of a saturated solution of alum. The flask was then filled to the mark, shaken, and the contents digested at 60° C. for one hour. The contents were then filtered through dry papers, and a measured portion was taken for determination of inorganic phosphorus. Under these conditions phytin was precipitated even in 0.01 percent solutions, but came down as an exceedingly soft, bulky and finely divided precipitate, which could not be held satisfactorily by any kind of filter. A series made by this method gave the following results:

**SEPARATION OF INORGANIC PHOSPHORUS AND PHYTIN BY PRECIPITATION WITH POTASH ALUM**

<table>
<thead>
<tr>
<th>Grams of phytin present</th>
<th>Grams of pyrophosphate equivalent to inorganic phosphorus added</th>
<th>Grams of pyrophosphate recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>.0672</td>
<td>.0621</td>
</tr>
<tr>
<td>0.4</td>
<td>.0672</td>
<td>.0654</td>
</tr>
<tr>
<td>0.3</td>
<td>.0672</td>
<td>.0626</td>
</tr>
<tr>
<td>0.2</td>
<td>.0672</td>
<td>.0607</td>
</tr>
<tr>
<td>0.1</td>
<td>.0672</td>
<td>.0604</td>
</tr>
<tr>
<td>0.05</td>
<td>.0672</td>
<td>.0645</td>
</tr>
<tr>
<td>0.04</td>
<td>.0672</td>
<td>.0644</td>
</tr>
<tr>
<td>0.05</td>
<td>.0672</td>
<td>.0650</td>
</tr>
<tr>
<td>0.02</td>
<td>.0672</td>
<td>.0650</td>
</tr>
<tr>
<td>0.01</td>
<td>.0672</td>
<td>.0652</td>
</tr>
</tbody>
</table>

At the same time, samples of the phytin used were treated in a similar way, without the addition of inorganic phosphate. They gave the following results:

<table>
<thead>
<tr>
<th>Grams of phytin present</th>
<th>Grams of pyrophosphate recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>.0117</td>
</tr>
<tr>
<td>0.3</td>
<td>.0095</td>
</tr>
<tr>
<td>0.1</td>
<td>.0033</td>
</tr>
<tr>
<td>0.05</td>
<td>.0009</td>
</tr>
</tbody>
</table>

Now if these latter values are assumed to represent the amount of inorganic phosphorus in the phytin sample, they should be added to the .0672 gram present in inorganic form in order to obtain the true values for inorganic phosphorus; for example, in the case of a 0.5 percent solution of phytin, .0672 gram of pyrophosphate should have been recovered, plus what the phytin itself contained, namely .0117 gram. Therefore, .0672+.0117=.0789 gram magnesium pyrophosphate, which presumably represents the correct value for inorganic phosphorus in the 0.5 percent solution.
When the series is thus worked out it is readily seen that the results are all too low.

Thinking that possibly the low results were due to a dragging down of inorganic phosphorus by the bulky precipitate of phytin, we made several series of determinations in which the precipitate was thoroughly washed with warm 0.2 percent hydrochloric acid, but in all cases the precipitate, being so finely divided, was washed through into the filtrate. It could not be held by filter paper, paper pulp or asbestos.

We next tried precipitating the phytin as the lead salt in 0.2 percent nitric acid solution. The details of the method were similar to those of the alum method, except that no ammonium nitrate was used in the digestion of the precipitated lead salt.

By the use of a 20 percent solution of lead nitrate, complete precipitation of the phytin seemed to be obtained. The precipitate was heavier and more easily filtered than the aluminium salt.

Experiments proved that 0.2 percent nitric acid was the best medium in which to make the precipitation.

This method gave results indicated by the following series:

| Separation of Inorganic Phosphate and Phytin by Precipitation with Lead Nitrate |
|---|---|---|
| Grams of phytin present | Grams of pyrophosphate equivalent to inorganic phosphorus added | Grams of pyrophosphate recovered |
| 0.4 | 0.0304 | 0.0256 |
| 0.3 | 0.0304 | 0.0292 |
| 0.1 | 0.0304 | 0.0305 |
| 0.05 | 0.0304 | 0.0311 |
| 0.04 | 0.0304 | 0.0304 |
| 0.03 | 0.0304 | 0.0327 |
| 0.01 | 0.0304 | 0.0312 |

Four determinations were also made on the phytin sample without addition of inorganic phosphorus as follows:

<table>
<thead>
<tr>
<th>Grams of phytin present</th>
<th>Grams of pyrophosphate recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>0.0035</td>
</tr>
<tr>
<td>0.3</td>
<td>0.0020</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0011</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

The low results on solutions with high phytin content again indicate either a dragging down of inorganic phosphorus by the heavy precipitate of lead phytate, or actual combination between phytin and inorganic phosphates.
It was also thought possible that the particular form of the crucial phytin used might be accountable for these varying results. To test this, several series of determinations were made with the lead precipitation on extracts of feeding materials.

The method of procedure was as follows:

From 50 to 100 grams of material were extracted for three hours with a large volume of 0.2 percent hydrochloric acid. The resulting extract was filtered through paper by suction. Measured portions of this extract were placed in beakers, and a definite amount of di-sodium phosphate added, as before. The mixture was then precipitated with magnesia mixture and ammonia, using 10 c. c. of the former to 20 c. c. of the latter. After standing over night, the precipitate was filtered off and washed with dilute ammonia. After draining well, the paper with the precipitate was placed in a flask containing 250 c. c. of 0.8 percent nitric acid, and shaken until the paper and precipitate were thoroughly broken up. This solution was then filtered through a dry filter, and 200 c. c. of the filtrate placed in a 250 c. c. flask. After making neutral with ammonia, enough nitric acid was added to make the 250 c. c. of solution contain 0.2 percent of nitric acid. Lead nitrate solution was now added in slight excess, the flask filled to the mark, and digested for one hour at 60° C. The phytin was then removed by filtration through a dry filter, and after cooling, 200 c. c. of the filtrate was precipitated with official molybdate solution. The yellow precipitate was washed with diluted molybdate solution until free from lead, and phosphorus was then determined in the usual way.

This method gave the following results on wheat bran, and was also tried on alfalfa, which is thought to contain no phytin.*

One hundred grams of bran were extracted with 1500 c. c. of 0.2 percent hydrochloric acid containing 25 c. c. of 90 percent phenol.

To measured portions of this extract was added di-sodium phosphate solution equivalent to a final weight of .0246 gram of magnesium pyrophosphate.

### SEPARATION OF INORGANIC PHOSPHORUS AND PHYTIN BY PRECIPITATION WITH LEAD NITRATE IN ACID EXTRACT OF WHEAT BRAN

<table>
<thead>
<tr>
<th>Volumes of extract</th>
<th>Pyrophosphate equivalent to inorganic phosphorus added</th>
<th>Pyrophosphate recovered</th>
<th>Calculated weight of pyrophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. C.</td>
<td>Grams</td>
<td>Grams</td>
<td>Grams</td>
</tr>
<tr>
<td>100</td>
<td>.0246</td>
<td>.0326</td>
<td>.0328</td>
</tr>
<tr>
<td>80</td>
<td>.0246</td>
<td>.0320</td>
<td>.0320</td>
</tr>
<tr>
<td>60</td>
<td>.0246</td>
<td>.0298</td>
<td>.0298</td>
</tr>
<tr>
<td>50</td>
<td>.0246</td>
<td>.0290</td>
<td>.0290</td>
</tr>
<tr>
<td>40</td>
<td>.0246</td>
<td>.0286</td>
<td>.0286</td>
</tr>
<tr>
<td>25</td>
<td>.0246</td>
<td>.0273</td>
<td>.0273</td>
</tr>
<tr>
<td>10</td>
<td>.0246</td>
<td>.0258</td>
<td>.0258</td>
</tr>
<tr>
<td>5</td>
<td>.0246</td>
<td>.0233</td>
<td>.0233</td>
</tr>
<tr>
<td>100</td>
<td>.0000</td>
<td>.0091</td>
<td>.0091</td>
</tr>
</tbody>
</table>

The fourth column refers to results which should have been obtained if .0091 gram of pyrophosphate is considered to represent the amount of inorganic phosphorus in the 100 c. c. of extract taken; and to this is added the blank, .0246 gram pyrophosphate, which had been added as di-sodium phosphate. For quantities of extract less than 100 c. c. proportional parts of the value .0091 were taken.

A similar series of determinations was made on alfalfa hay. Forty grams of alfalfa hay were extracted with 1000 c. c. of 0.2 percent hydrochloric acid containing 50 c. c. of 90 percent phenol. The inorganic phosphate added was again equivalent to .0246 gram pyrophosphate.

### RECOVERY OF INORGANIC PHOSPHORUS ADDED TO ACID EXTRACT OF ALFALFA

<table>
<thead>
<tr>
<th>Volumes of extract</th>
<th>Pyrophosphate equivalent to inorganic phosphorus added</th>
<th>Pyrophosphate recovered</th>
<th>Calculated weight of pyrophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. C.</td>
<td>Grams</td>
<td>Grams</td>
<td>Grams</td>
</tr>
<tr>
<td>100</td>
<td>.0246</td>
<td>.0343</td>
<td>.0388</td>
</tr>
<tr>
<td>80</td>
<td>.0246</td>
<td>.0330</td>
<td>.0370</td>
</tr>
<tr>
<td>60</td>
<td>.0246</td>
<td>.0312</td>
<td>.0355</td>
</tr>
<tr>
<td>50</td>
<td>.0246</td>
<td>.0290</td>
<td>.0345</td>
</tr>
<tr>
<td>40</td>
<td>.0246</td>
<td>.0274</td>
<td>.0341</td>
</tr>
<tr>
<td>25</td>
<td>.0246</td>
<td>.0255</td>
<td>.0329</td>
</tr>
<tr>
<td>10</td>
<td>.0246</td>
<td>.0236</td>
<td>.0315</td>
</tr>
<tr>
<td>5</td>
<td>.0246</td>
<td>.0220</td>
<td>.0301</td>
</tr>
<tr>
<td>100</td>
<td>.0000</td>
<td>.0006</td>
<td>......</td>
</tr>
<tr>
<td>100</td>
<td>.0000</td>
<td>.0000</td>
<td>......</td>
</tr>
</tbody>
</table>

Although these results are not exact checks with the theoretical values, still they indicate, it is thought, that it may be possible entirely to recover the inorganic phosphorus added to these extracts; this in turn indicating in the case of wheat bran, that it is possible to make a quantitative separation of phytin and inorganic phosphorus.

Working with such extracts, however, results were always complicated by the uncertain amount of inorganic phosphorus in the extract itself. Figures representing this factor were obtained, it is true, but there was no means of proving whether they represented the actual values or not. Lack of a standard resulted in an element of uncertainty as to the above values of .0091, .0096 and .0090 for pyrophosphate in 100 c. c. of the extracts, although they are believed to be approximately correct.

It was thought that if a pure phytin could be prepared, free from inorganic phosphorus, it would, by eliminating this unknown factor, be possible to prove definitely whether a separation between phytin and inorganic phosphorus could be made quantitative.
It can be readily seen that such a preparation is attended by the same difficulties as is a quantitative determination of inorganic phosphorus in the presence of phytin.

While working on the relative solubilities of magnesium ammonium phosphate and phytin, it was found that although both substances are practically insoluble in 95 percent alcohol, a decided difference exists if the alcohol contains a very small quantity of mineral acid.

It was found further, that in 100-150 c. c. of 95 percent alcohol containing 0.2 percent of nitric acid, the quantities of magnesium ammonium phosphate commonly worked with were completely soluble, when the precipitated phosphate was shaken up with the reagent. On the other hand, phytin as precipitated from feeding materials appeared to be insoluble.

This suggested a means of separation, and at the same time a means of preparing inorganic-phosphorus-free phytin. With the latter object in view, a sample of phytin was prepared as follows:

A large quantity of wheat bran was finely ground, and extracted for several hours with 0.2 percent hydrochloric acid. The coarse bran was strained out by the use of linen, and then the extract was filtered by suction through paper in a Büchner funnel. A fairly clear filtrate was thus secured. This filtrate was precipitated with a large volume of magnesia mixture, and immediately filtered, as above, by suction. Magnesia mixture was used in order to have conditions similar to those of the regular quantitative method. The precipitate was washed thoroughly with water. This gave a very white, soft mass of fairly pure phytin. This precipitated phytin was then dissolved in a large volume of very dilute hydrochloric acid, and freed by filtration from a small residue of starchy material. The filtrate was again precipitated with magnesia mixture, and washed as before. This was repeated some 8 or 10 times. The washed precipitate was then macerated in a mortar with a small quantity of 95 percent alcohol containing 0.2 percent of nitric acid. A considerable volume of the same reagent was then added, and the whole mass shaken thoroughly in a flask until the lumps were broken up. This was then filtered again by suction, washed thoroughly with the alcohol reagent, and the maceration and filtration repeated several times. Finally the precipitate, after thorough washing with the acid-alcohol, was washed free from acid with absolute alcohol, and then dried at 50-60° C.

The product thus obtained was very white, and gave only an exceedingly faint test for inorganic phosphorus, the quantity of pyrophosphate from one gram of material being unweighable.
This test of its purity was made by precipitating the phytin in 0.2 percent nitric acid solution with lead nitrate, filtering and testing the filtrate with official molybdate solution. Tested directly, the sample would of course give no test, since the yellow precipitate would be totally suppressed by the phytin.

It is thought that separation was effected by the rapid filtration of the precipitated phytin, giving any inorganic phosphorus present, insufficient time to form magnesium ammonium phosphate, and also by the solvent action of the acid-alcohol on the inorganic phosphates present.

On this prepared phytin sample, determinations were made in an attempt to recover added inorganic phosphorus.

Using the lead nitrate method, better results were obtained than before, although exact recovery seemed impossible, due again doubtless to the slight solubility of the lead phytate formed, in the acid present, with a subsequent decomposition by the acid in the molybdate solution.

The acid-alcohol separation was accordingly tried on this prepared sample.

In each case 0.1 gram of phytin was dissolved in 100 c. c. of 0.2 percent hydrochloric acid. Inorganic phosphorus in the form of di-sodium phosphate was then added. The solution was then precipitated with magnesia mixture and ammonia. The next morning the solutions were filtered, and the precipitate washed, first with dilute ammonia, and then with 95 percent alcohol. After draining, the paper with the precipitate was placed in a flask containing 150 c. c. of 95 percent alcohol, which contained in turn, 0.2 percent of nitric acid. This flask was then shaken vigorously until the paper and precipitate were thoroughly disintegrated. The contents were then filtered through a dry filter, and 100 c. c. of filtrate placed in a beaker. This filtrate was then treated as follows:

Method I. Alcoholic filtrate was made alkaline with ammonia; filtered through a Gooch crucible; washed with alcohol; dissolved in dilute nitric acid, and precipitated with molybdate solution.

Method II. Same as Method I except that alcoholic filtrate was precipitated with magnesia mixture, instead of plain ammonia.

Method III. Five grams solid ammonium chloride were added to the acid-alcohol containing the paper and precipitate, and shaken up with the latter. The solution was then treated as in Method I.

Method IV. Alcoholic filtrate was precipitated with magnesia mixture; precipitate dissolved in dilute nitric acid, made alkaline, and precipitated direct with magnesia mixture, ignited and weighed in the usual way.
Determinations were made in triplicate. The blank on the inorganic phosphorus added, was equivalent to .0272 gram magnesium pyrophosphate.

**COMPARISON OF METHODS FOR PRECIPITATING INORGANIC PHOSPHORUS FROM ACID-ALCOHOL SOLUTION**

<table>
<thead>
<tr>
<th>Method</th>
<th>Pyrophosphate equivalent to inorganic phosphorus added (Grams)</th>
<th>Magnesium pyrophosphate recovered (Grams)</th>
<th>Corresponding amounts of phosphorus (Grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>2</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>3</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>4</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>5</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>6</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>7</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>8</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>9</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>10</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>11</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
<tr>
<td>12</td>
<td>.0272</td>
<td>.0272</td>
<td>.0272</td>
</tr>
</tbody>
</table>

Since all these determinations are within the limit of mechanical error the indication is that the separation was quantitative.

The method was also used on wheat middlings. Fifty grams were extracted for 3 hours with 1200 c. c. of 0.2 percent hydrochloric acid containing 25 c. c. of 90 percent phenol. The extract was filtered through paper by suction, and varying quantities used in the determinations. Inorganic phosphorus was added, and the method followed as outlined above, the alcoholic filtrate being treated as in Method I.

**SEPARATION OF INORGANIC PHOSPHORUS AND PHYTIN BY ACID ALCOHOL**

<table>
<thead>
<tr>
<th>No.</th>
<th>Pyrophosphate equivalent to inorganic phosphorus added (Grams)</th>
<th>Volumes of extract (C. C.)</th>
<th>Magnesium pyrophosphate recovered (Grams)</th>
<th>Calculated weights of pyrophosphate (Grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>.0272</td>
<td>100</td>
<td>.0275</td>
<td>.0272</td>
</tr>
<tr>
<td>14</td>
<td>.0272</td>
<td>100</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>15</td>
<td>.0272</td>
<td>90</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>16</td>
<td>.0272</td>
<td>60</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>17</td>
<td>.0272</td>
<td>50</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>18</td>
<td>.0272</td>
<td>40</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>19</td>
<td>.0272</td>
<td>25</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>20</td>
<td>.0272</td>
<td>10</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>21</td>
<td>.0272</td>
<td>5</td>
<td>.0274</td>
<td>.0272</td>
</tr>
<tr>
<td>22</td>
<td>.0000</td>
<td>100</td>
<td>.0000</td>
<td>.0000</td>
</tr>
<tr>
<td>23</td>
<td>.0000</td>
<td>100</td>
<td>.0000</td>
<td>.0000</td>
</tr>
<tr>
<td>24</td>
<td>.0000</td>
<td>100</td>
<td>.0000</td>
<td>.0000</td>
</tr>
</tbody>
</table>
Taking into consideration mechanical errors, etc., the results are as close as could be expected.

Using Method II as detailed on page 474, some common feeding materials were tested for inorganic phosphorus. Total phosphorus was determined by the Neumann method. Determinations were made in triplicate.

These triplicate determinations were made on separate 10-gram samples of the feeding material, and not on the same solution.

**TOTAL AND INORGANIC PHOSPHORUS IN CERTAIN FOODSTUFFS.**

<table>
<thead>
<tr>
<th>Foodstuff</th>
<th>Total phosphorus</th>
<th>Inorganic phosphorus</th>
<th>Average inorganic phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>0.23</td>
<td>0.135 0.136 0.136</td>
<td>0.136</td>
</tr>
<tr>
<td>Clover hay</td>
<td>0.171</td>
<td>0.104 0.104 0.104</td>
<td>0.104</td>
</tr>
<tr>
<td>Corn meal</td>
<td>0.266</td>
<td>0.040 0.042 lost</td>
<td>0.041</td>
</tr>
<tr>
<td>Soy beans</td>
<td>0.547</td>
<td>0.054 0.054 lost</td>
<td>0.054</td>
</tr>
<tr>
<td>Cow peas</td>
<td>0.445</td>
<td>0.056 0.056 0.056</td>
<td>0.056</td>
</tr>
<tr>
<td>Oats</td>
<td>0.397</td>
<td>0.061 0.059 lost</td>
<td>0.060</td>
</tr>
<tr>
<td>Wheat</td>
<td>0.394</td>
<td>0.036 0.036 0.036</td>
<td>0.036</td>
</tr>
<tr>
<td>Brewer's grains</td>
<td>0.492</td>
<td>0.012 0.011 0.013</td>
<td>0.013</td>
</tr>
<tr>
<td>Distiller's grains, from corn</td>
<td>0.366</td>
<td>0.026 0.049 0.049</td>
<td>0.049</td>
</tr>
<tr>
<td>Rice polish</td>
<td>0.60</td>
<td>0.027 0.027</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Since these triplicates represent separate samples of the feeding material, their close agreement indicates that this method is consistently workable.

The elimination, which seems to have been attained, of organic phosphorus compounds from the solution in which the inorganic phosphorus is finally precipitated, precludes the possibility of magnesium oxide or proteid phosphorus being present as an impurity in the inorganic phosphorus precipitate. Further, in those samples
which contain phytin, the acid-alcohol separation eliminates the factors (1) of suppression of the yellow precipitate by the phytin, and (2) of decomposition of the phytin by acid in the reagents used in precipitating inorganic phosphorus. Inorganic phosphorus alone appears to be present in the final solution obtained, and a clear-cut, normal, phosphorus determination results.

After considerable use of phenol in this precautionary way, we made a further test of its value on middlings, alfalfa and distiller's grains. The results were as follows:

**EFFECT OF PHENOL IN INORGANIC PHOSPHORUS ESTIMATION**

<table>
<thead>
<tr>
<th>Foodstuff</th>
<th>Total phosphorus</th>
<th>10 c. c. phenol</th>
<th>15 c. c. phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middlings (1)</td>
<td>.762</td>
<td>.046</td>
<td>.046</td>
</tr>
<tr>
<td>Middlings (2)</td>
<td>.059</td>
<td>.061</td>
<td>.060</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>.130</td>
<td>.130</td>
<td>.128</td>
</tr>
<tr>
<td>Distiller's grains</td>
<td>.514</td>
<td>.414</td>
<td>.414</td>
</tr>
</tbody>
</table>

These determinations were made by our final acid-alcohol method. These results incline us to the belief that in these feeds, at least, there is not an appreciable splitting off of inorganic from organic phosphorus compounds by enzymes during the course of our three-hour extraction, and hence that the use of phenol is probably not necessary. In a further test of this point we got exactly the same figure, .0459 percent, for inorganic phosphorus in wheat middlings, both with and without the presence of phenol during extraction.

In another test of the same point we determined inorganic phosphorus in wheat middlings, distiller's grains and alfalfa, both with and without the presence of phenol during extraction. The differences due to the presence of phenol were immaterial. There was no evidence of benefit from the use of phenol, and hence it seems much preferable not to introduce this strong reagent into the determination.

In an attempt to shorten our provisional method we made three sets of determinations of inorganic phosphorus on wheat middlings, two on distiller's grains, and two on alfalfa, in which we sought to determine whether a precipitation with magnesia mixture should follow the separation of inorganic phosphorus from phytin in acid alcohol, or whether we might evaporate the acid-alcohol solution of inorganic phosphorus, take up with acid, and proceed with the final estimation of phosphorus in the official gravimetric way by precipitation, first with acid molybdate solution, then with magnesia mixture, and burning to the pyrophosphate.
In all cases the results by these two methods were either absolutely or practically identical, and hence we conclude that a magnesia precipitation after the acid-alcohol separation of phytin and inorganic phosphorus is unnecessary.

A similar test was made of acid and neutral molybdate solutions in the final precipitation of inorganic phosphorus, in the latter case only sufficient nitric acid being added to cause a separation of the yellow precipitate. This comparison was made by our final method, in which the phosphorus estimation is made directly after the acid-alcohol separation of inorganic phosphorus and phytin, and also by the earlier provisional method in which a magnesia precipitation intervened between the acid-alcohol separation and the phosphorus estimation. The results were practically the same, and hence our only basis for choice between these methods was ease and rapidity of manipulation.

**ROUTINE METHOD**

In detail the final method adopted for inorganic phosphorus in plant substances is as follows:

Pour exactly 300 c. c. of .2 percent hydrochloric acid onto 10 grams of the finely ground sample in a 400 c. c. Florence flask. Close with a rubber stopper, and shake at intervals of 5 minutes for three hours. Filter the extract through dry filters into dry flasks.

Owing to the character of the extracts from certain feeding materials, especially soy beans and oats, it may be necessary to use gentle suction toward the end of the filtration. To prevent rupture of the filter paper, a platinum cone may be used. A Witt filtering apparatus is a convenience in this work. Filtration of the extract is facilitated in many cases by the use of about one inch of fine sand in the point of the filter paper.

Measure out a 250 c. c. portion of this filtered extract, and precipitate in a 400 c. c. beaker with 15 c. c. of magnesia mixture and 30 c. c. of strong ammonia. Allow to stand over night, and filter through doubled 11 cm. S. & S. No. 595 filters, taking care to decant as long as possible without pouring out the precipitate. Then complete the transfer of the precipitate to the paper.

This filtration also may be hastened toward the end by suction, using a platinum cone to keep the paper intact.

This precipitate consists of magnesium ammonium phosphate, together with phytin, when this compound is present in the substance involved, and small quantities of variously colored unknowns. Wash several times with 2.5 percent ammonia, and then several times with 95 percent alcohol until free from ammonia. This result may be hastened by also washing with alcohol between the
two filters. The alcohol clears up the precipitate by dissolving out a large part of the variously colored compounds other than phytin and magnesium ammonium phosphate. Allow the remaining precipitate to drain, and then spread out the inner paper on the top of the funnel, and allow the alcohol to evaporate.

When practically dry, place this inner paper with the precipitate into an Erlenmeyer flask. Add 100 c. c. of 95 percent alcohol containing 0.2 percent of nitric acid. Close the flask with a rubber stopper and shake vigorously until the paper is thoroughly broken up. If the precipitate is flaky, and refuses to break up on shaking, allow to stand in the acid-alcohol over night.

Now filter through a dry, double filter into a dry flask. Pipette out 75 c. c. of the filtrate into a small beaker, and evaporate almost but not quite to dryness. Dissolve in dilute nitric acid, and filter if necessary; then determine phosphorus in the usual gravimetric way, by precipitation first with acid molybdate solution, later with magnesia mixture, and then burning to the pyrophosphate. Re-precipitation has been found to be unnecessary.

The result as obtained above represents 6.25 grams out of the original 10 grams of material, and so to reduce to a 1-gram basis multiply by .16.
ESTIMATION OF INORGANIC PHOSPHORUS
IN ANIMAL TISSUES

The most valuable method known to us for the determination of inorganic phosphorus in animal tissues has been that of Emmet and Grindley*. This method was worked out for use on muscular tissue, and for this purpose we have found it to be accurate and satisfactory. Attempts to use this excellent method with tissues other than muscle, however, showed further study of the problem to be necessary. After thorough trial we have adopted a method for our work, which is equally applicable to muscle, liver, kidney and brain, and which while giving identical results with Emmet and Grindley’s method when used on muscle, has the advantage over this method of being somewhat more easily workable, and of saving one precipitation which is often necessary in the use of Emmet and Grindley’s method on muscle, and which is always necessary when this method is used on liver, kidney and brain.

The difficulties encountered in our attempts to make general use of Emmet and Grindley’s method for muscle, on other tissues, were in the filtration of the cold-water extracts, and in getting uncontaminated precipitates of inorganic phosphorus.

Cold-water extracts of liver, kidney and brain are not filterable by usual methods. Coagulation by boiling renders easy the filtration of liver extract, and renders possible the filtration of the extract of kidney, but does not assist materially in the filtration of the extract of brain.

Further—after finding workable methods for coagulating and filtering the extracts, the neutral molybdate precipitation of Emmet and Grindley’s method gave us bulky, flocculent, organic precipitates, especially in the case of liver, kidney and brain, which by obscuring the formation of the yellow precipitate, rendered impossible an accurate judgment as to the amount of acid necessary to the completion of the precipitation. These bulky, flocculent precipitates redissolve in ammonia, and reappear in the precipitate with magnesia mixture. The possibility of adding phosphorus or magnesia to the pyrophosphate through their presence in the above-mentioned flocculent, organic precipitates, contributes another item of uncertainty and possible inaccuracy to the use of this method when we attempt to apply it to liver, kidney and brain.

In our study of methods of preparation of the water-extract for filtration, we found that boiling and treatment with ammonium sulphate would render the extracts of any of these tissues readily filterable.

After a trial of various methods on brain, we found most satisfactory results obtainable by boiling the extract for a few moments, the exact time being immaterial, then adding ammonium sulphate in solution, and continuing to boil for about ten minutes.

The various difficulties of precipitation and filtration, incident to the preliminary molybdate precipitation, were obviated by direct precipitation of the inorganic phosphorus, from the coagulated and filtered extracts, with magnesia mixture. This gives us a precipitate of approximate purity, results in a very great saving of time in filtration, and obviates the possibility of hydrolysis of organic phosphorus compounds by the nitric acid used in precipitation with ammonium molybdate.

This precipitate with magnesia mixture is dissolved and re-precipitated, first with official acid molybdate solution, and then again with magnesia mixture in the usual way, and the phosphorus weighed as the pyrophosphate.

The essentials of the new method are (1) extraction of the inorganic phosphates with hot water, (2) boiling the extracts with ammonium sulphate to render them filterable, (3) direct preliminary precipitation with magnesia mixture, and (4) a number of important mechanical details of procedure.

Our first work on this problem was an attempt to use Emmet and Grindley's method for muscle, on liver, kidney and brain. We proceeded as follows:

The muscle extract was prepared with cold water according to Grindley*.

The liver extract was prepared in the same way, but was heated on the steam bath in order to render it filterable through paper.

The kidney extract was prepared in the same way as the liver extract, but was not filterable through paper. This solution was filtered through sand in the cone of a linen filter such as is used in crude-fiber determinations.

The brain extract contains practically the whole of the brain substance, the addition of water simply diluting the paste. It was not filterable hot or cold, through paper, linen or sand. This solution was rendered filterable by bringing to a boil, adding ammonium sulphate, and continuing to boil for ten minutes. It was then passed through sand on linen.

We now had (1) a cold-water extract of muscle, (2) hot-water extracts of liver and kidney, and (3) a hot-water-ammonium sulphate extract of brain.

Measured portions were concentrated by boiling in order to reduce the bulk and to coagulate proteids, and were then filtered through paper, and washed with water, but not without difficulty in the case of liver, kidney and brain.

Proceeding by the method of Emmet and Grindley, we treated the extracts with neutral molybdate solution and 3 c. c. of 1.20 nitric acid, as specified, to effect the separation of the yellow precipitate.

In the case of liver, kidney and brain, it was found that 3 c. c. of nitric acid were insufficient. Two cubic centimeters in addition, five in all, were found necessary, because of the abundance of organic matter in these solutions, and 10 grams of ammonium nitrate, instead of 5 grams as specified by Emmet and Grindley, were found necessary with the brain, where we had added ammonium sulphate.

The results were not satisfactory. The liver, kidney and brain extracts gave abundant flocculent precipitates, greenish colored with the liver, nearly white in the case of the brain, and of an intermediate grayish color with the kidney. These contaminations so masked the yellow precipitate that accurate judgment as to the completeness of the precipitation was impossible.

The precipitates were filtered out, and reprecipitated with official acid molybdate solution. The contaminations persisted in all cases except with muscle.

The yellow precipitates were redissolved, and precipitated in the usual way with magnesia mixture.

These last precipitates were obviously impure. With the brain an abundant, white, flocculent contamination was present, while with the liver and kidney the precipitates were highly colored.

The results with muscle were satisfactory.

The pyrophosphates from liver, kidney and brain were boiled with nitric acid, and precipitated once more with magnesia mixture, but the results were of doubtful value.

We next attempted the precipitation of inorganic phosphates with dilute solutions of calcium and ferric chlorides. The gelatinous precipitate was difficult to wash, and probably on this account, gave slightly higher results on muscle than did Emmet and Grindley's method.

The direct precipitation of the concentrated cold-water extract of muscle with magnesia mixture was now tried, followed by precipitation, first with official molybdate solution,
and then with magnesia mixture, and this gave results identical with others obtained by the Emmet and Grindley method.

This method was therefore given a careful trial. The points which we sought to determine were (1) whether or not the use of heat and ammonium sulphate gave the same inorganic phosphorus content in the extract as Grindley's cold-water extraction, and (2) whether or not the direct precipitation with magnesia mixture effected a complete precipitation of the inorganic phosphates without bringing down other phosphorus compounds to contaminate the pyrophosphate.

### COMPARISON OF METHODS FOR THE ESTIMATION OF INORGANIC PHOSPHORUS IN MEAT

<table>
<thead>
<tr>
<th>Grams magnesium pyrophosphate per gram fresh substance</th>
<th>Emmet and Grindley's Method</th>
<th>Magnesia Method</th>
<th>Acid-Alcohol Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precipitated (1) with neutral molybdate and minimum of acid, (2) with official acid molybdate, (3) with magnesia mixture, and (4) with magnesia mixture</td>
<td>Precipitated (1) with magnesia mixture, (2) with official acid molybdate, and (5) with magnesia mixture</td>
<td>Precipitated (1) with magnesia mixture, and dissolved in acid alcohol, (2) with magnesia mixture, (3) with official acid molybdate, and (4) with magnesia mixture</td>
</tr>
<tr>
<td>Muscle sample No. 1 Cold-water extract</td>
<td>.0049</td>
<td>.0031</td>
<td>.0048</td>
</tr>
<tr>
<td></td>
<td>.0048</td>
<td>.0031</td>
<td>.0048</td>
</tr>
<tr>
<td>Muscle sample No. 1 Boiled with ammonium sulphate</td>
<td>.0050</td>
<td>.0050</td>
<td>.0049</td>
</tr>
<tr>
<td></td>
<td>.0051</td>
<td>.0050</td>
<td>.0049</td>
</tr>
<tr>
<td>Muscle sample No. 2 Cold-water extract</td>
<td>.0049</td>
<td>.0053</td>
<td>.0051</td>
</tr>
<tr>
<td>Muscle sample No. 2 Hot-water extract</td>
<td>.0053</td>
<td>.0056</td>
<td>.0033</td>
</tr>
<tr>
<td>Muscle sample No. 2 Boiled with ammonium sulphate</td>
<td>.0056</td>
<td>.0056</td>
<td>.0034</td>
</tr>
<tr>
<td>Muscle sample No. 2 Boiled with ammonium sulphate</td>
<td>.0054</td>
<td>.0055</td>
<td>.0054</td>
</tr>
<tr>
<td>Liver sample No. 1 Hot-water extract; ammonium sulphate added, then concentrated by boiling</td>
<td>.0049</td>
<td>.0050</td>
<td>.0049</td>
</tr>
<tr>
<td>Liver sample No. 1 Boiled with ammonium sulphate</td>
<td>.0049</td>
<td>.0050</td>
<td>.0049</td>
</tr>
</tbody>
</table>
The above table sets forth our results in this work. The figures represent the weight of pyrophosphate from the inorganic phosphorus in one gram of meat.

In making these determinations the amount of meat represented by each precipitate was about three grams.

With a cold-water extract of muscle sample No. 1, we compared Emmet and Grindley's method, with our magnesia method, and also with our acid-alcohol method, which we have adopted for vegetable substances.

The triplicates agree almost exactly, and the determinations by the different methods also agree, the differences being well within the limit of legitimate error of work. This excellent agreement in results by three different methods, shows that the inorganic phosphorus of flesh is a definite part thereof, and that it is susceptible of accurate estimation.

The agreement three times each by three well-considered methods also shows that they are all probably correct.

The acid-alcohol method is as long as the Emmet and Grindley method, and was included in this trial because its agreement with the other methods would not only strengthen them, but would also tend to sustain our method for inorganic phosphorus on vegetable substances. The results fulfill these desired conditions.

The magnesia method is decidedly the best of the three, however, because of the aforementioned advantages of brevity, purity of precipitates, and ease and certainty of manipulation.

A second extract of muscle sample No. 1 was prepared by boiling with ammonium sulphate, for the purpose of ascertaining what effect, if any, this salt might have in our estimation of inorganic phosphorus in other tissues, especially in kidney and brain, where we have found ammonium sulphate especially useful in the preparation of the extracts.

The close agreement of our triplicates and the very close agreement of the results by each of the three methods, and the agreement of determinations on this hot-water-ammonium sulphate extract with the equally satisfactory results on the cold-water extract show that ammonium sulphate is without effect on the estimation of inorganic phosphorus, due regard being given, of course, in the Emmet and Grindley method, to the sulphuric acid radical which this salt introduces into the solution.

With a second sample of muscle we compared the magnesia method and the acid-alcohol method on a cold-water extract, with the magnesia method on a hot-water extract, and with the Emmet and Grindley, and the magnesia methods on a hot-water-ammonium sulphate extract.
The three methods again checked remarkably well, and there appears to be no difference in the inorganic phosphorus contents of the cold-water, the hot-water, and the hot-water-ammonium sulphate extracts.

With a sample of hog liver we compared two methods of use of ammonium sulphate. In one case the tissue was boiled with the sulphate, as in the preparation of the extract of brain and kidney, while in the other the sulphate was added to the filtered hot-water extract, and was present only during the concentration of the extract by boiling.

The magnesia method once more gave identical results with those from the acid-alcohol method, and the presence of ammonium sulphate was again found to be without demonstrable effect, either on the extraction or the estimation of the inorganic phosphorus.

In making a final comparison of the three methods, it may be said that the amounts of phosphorus found by the three are practically identical, but with slightly higher results, just perceptible, and well within the limit of error of work, in favor of the magnesia method.

The details of our magnesia method for the estimation of inorganic phosphorus in muscle, liver, kidney and brain are as follows:

**PREPARATION OF SAMPLES**

The animals whose tissues are to be compared must be killed in the same way, and should be quickly and thoroughly bled in order that the blood content of the tissues may be the result of natural conditions, rather than differences in method of killing, such as varying lengths of time between stunning and bleeding.

In order to get a definite product for analysis, the brain should be freed from its vascular investing coats by carefully stripping these away with forceps.

The gall bladder should be removed from the liver, and also such tough connective tissues at the base of the organ as would interfere with the free grinding of the functional liver tissue.

The kidneys are removed from their capsules; the fat, blood-vessels and ureter are removed with scissors from the hilus, and the interior of the organ is opened and freed from contents.

The muscular tissue is freed from superficial fat and from tendons.

All work is done as promptly as possible, with all possible precautions to prevent evaporation.
The tissues are prepared for sampling by grinding with an "Eclipse" grinder. An electric motor attachment is a great saver of labor, and by facilitating the work, helps to minimize loss of moisture by evaporation.

The materials are ground three times, with careful mixing between grindings, and samples are placed in glass jars which have screw tops lined with rubber. The bottles of ground meat are then frozen up as quickly as possible, and kept in this condition until the determination can be started.

PREPARATION OF EXTRACTS

Weigh by difference from closed weighing bottles, 20-gram portions of liver, kidney or muscle into 400 c. c. beakers. In the case of brain, weigh out likewise about 20 grams, but divide it between two 250 c. c. beakers.

Beat up in a little cold water, to separate the particles of meat; add 200 c. c. of boiling water for muscle, liver and kidney, and 100 c. c. of the same for each half of the brain sample, and bring to boiling. Add ammonium sulphate in solution equivalent to 1 gram for each 10 grams of muscle, liver and kidney, and to 4 grams of the same for each 10 grams of brain, and continue to boil for 10 minutes.

Remove from the flame; allow to settle for a moment, and decant the boiling-hot liquid onto 18 cm. folded paper filters, for muscle liver and kidney, and onto sand in an 18 cm. linen filter in the case of brain.

Add 100 c. c. of boiling water to the liver, kidney and muscle remaining in the beakers, and in the case of brain 50 c. c. of 0.1 percent boiling ammonium sulphate solution to each half of the sample.

Stir for about 8 minutes in the case of liver, kidney and muscle, and for about one minute with brain, and decant the liquid onto the filter. Repeat this addition of 100 c. c. portions of water, (50 c. c. of 0.1 percent ammonium sulphate with brain), stirring and decanting five times; repeat three times more with 50 c. c. portions of boiling water with liver, kidney and muscle, and with 25 c. c. portions of 0.1 percent ammonium sulphate solution with brain. With the eighth portion of water, (or 0.1 percent ammonium sulphate), throw the entire contents of the beaker onto the filter, and wash with hot water from a wash bottle, with liver, kidney and muscle, and with hot 0.1 percent ammonium sulphate solution for brain. The two portions of brain extract are combined. The extracts of all the tissues are then made up to 1000 c. c.
It is especially important that the brain extracts be kept and handled boiling-hot. If the sand filter clogs during the filtration, this process may be greatly facilitated by lightly scratching over the surface of the sand with a stirring rod, taking care not to touch the linen.

It is not advisable to attempt to handle more than 10 grams of brain in one beaker, on account of the necessity of completing the filtration promptly while the solution is hot.

**DETERMINATION OF INORGANIC PHOSPHORUS**

Measure out 250 c. c. portions of the extract into 400 c. c. beakers. Evaporate to 50 c. c. by gentle boiling; filter while hot, and wash with boiling water. Cool, and add 10 c. c. magnesia mixture, stirring freely; allow to stand 15 minutes, and add 10 c. c. of ammonia, specific gravity .90. Cover, and allow to stand over night.

On the next morning filter, and wash the precipitate with 2.5 percent ammonia water. Dissolve the precipitate on the filter paper with dilute nitric acid into the same beaker in which the first precipitation was made, and wash the papers thoroughly with hot water.

Render the resulting solutions nearly neutral; add 5 grams of ammonium nitrate; heat to 65° C.; add 50 c. c. of official acid molybdate solution, and keep contents at 60°, stirring frequently, for 2 hours.

Then continue in the usual way for the gravimetric estimation of phosphorus as the pyrophosphate.*

Each 250 c. c. of the extract will, of course, represent one-fourth of the original sample, and therefore will contain the inorganic phosphorus from about 5 grams of fresh substance.

**SUMMARY**

Inorganic phosphorus in plant and animal tissues appears to be susceptible of practically as accurate quantitative estimation as in ordinary inorganic analysis.

The principle of our method of inorganic phosphorus determination in plant substances is (1) extraction of the inorganic phosphorus with .2 percent hydrochloric acid, (2) precipitation with magnesia mixture, nucleinic acid being removed in solution, (3) dissolving the inorganic phosphorus out of this precipitate by the use of acid-alcohol, phytin remaining behind with the paper, (4) evaporation of the alcohol from the acid-alcohol solution of the inorganic phosphates, followed by solution of the phosphates in acid and a gravimetric estimation of phosphorus in the usual way by

precipitation, (5) first with official acid molybdate solution and then, (6) with magnesia mixture, and finally (7) burning to pyrophosphate.

Where necessary to prevent enzyme action phenol may be used in the extractive reagent.

This method is somewhat tedious but is clean-cut, workable and exact.

The method for animal substances is in brief (1) extraction with boiling ammonium sulphate solution, (2) filtration, concentration by boiling, and precipitation with magnesia mixture, (3) a gravimetric estimation of phosphorus by precipitation, first with official molybdate solution, then (4) with magnesia mixture, and finally (5) burning to the pyrophosphate.

This method is equally applicable to muscle, liver, kidney and brain.