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STUDIES ON THE ESTIMATION OF INORGANIC PHOSPHORUS IN PLANT AND ANIMAL SUBSTANCES

By E. B. FORBES, F. M. BEEGLE AND A. F. D. WUSSOW

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STUDIES ON THE ESTIMATION OF INORGANIC PHOSPHORUS IN PLANT AND ANIMAL SUBSTANCES

BY E. B. FORBES, F. M. BEEGLE AND A. F. D. WUSSOW

In connection with studies of the metabolism of plants and animals it is frequently desired to distinguish between simple inorganic phosphates and phosphorus in combination with organic groups. For the purpose of making such separate estimations there were published from this laboratory, by Forbes, Lehmann, Collison and Whittier, as Ohio Agr. Exp. Station Bul. 215, two different methods of inorganic phosphorus determination, one recommended for use with plant substances and the other for use with products of animal origin. Since issuing this publication, five years ago, we have come to realize that the problem was more difficult than we had known it to be, and that further evidence was required for judgment as to the correctness of our analytical procedures. In this spirit we have done further work on these methods, the result being the improvement and establishment of our method for animal substances, and the demonstration of the imperfection of our method for vegetable products. In spite of the considerable measure of failure attending our efforts to determine inorganic phosphorus in vegetable substances, illumination of the problem was accomplished, and on this account we report the results of this study. That portion of this paper which deals with plant substances will be found on pages 3 to 23, and that portion having to do with animal products on pages 23 to 40.

THE ESTIMATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

The essential points of the method of Forbes and associates, as published, were (1) extraction of a 10-gram sample in 300 c. c. of 0.2 percent hydrochloric acid for three hours, (2) filtration, and precipitation of a 250 c. c. portion of the extract, with magnesia mixture and ammonia, (3) washing the precipitate with 2.5 percent ammonia and with 95 percent alcohol, the precipitate being then allowed to dry, (4) the separation of the inorganic phosphates from the remainder of the precipitate by breaking up the paper and precipitate in 100 c. c. of 0.2 percent nitric acid in 95 percent alcohol, (5) filtration of the extract, pipetting out 75 c. c. of the filtrate, (6) evaporation of the alcohol, taking up with 0.2 percent aqueous nitric acid, and estimation of phosphorus in this solution by taking through the molybdate and magnesia mixture precipitates, and estimation as the pyrophosphate in the usual way. The result represents 6.25 grams out of the original 10 grams of sample.

The evidence upon which this method was based was submitted in the original publication. In the further study of this method observations were made as will be discussed. Our bases for judgment as to the correctness of the method were the agreement of triplicates, the completeness of recovery of added phosphates, and the completeness of extraction during the three-hour period specified, as determined by subjection of the residue to further extraction.

In the further study of this method especial attention was given to (1) the use of phenol in the extractive reagent, (2) the completeness of the 3-hour extraction, (3) improvement of the method of filtration, and other details of technique.

In the original publication of the method the use of phenol in the extractive reagent was suggested in cases where the activity of enzymes seemed to affect results. This was considered especially likely to be the case in estimations involving difficult and therefore protracted filtration, since it was found that the slower the filtration the greater would be the amount of inorganic phosphorus found.

In the working out of the details of the method as published, the duration of the extraction period received some consideration, and the 15-minute extraction of Hart and Andrews, from whose method the extractive reagent was adopted, was in our method lengthened to three hours; but our fixing upon this duration of extraction was largely arbitrary, and further evidence was deemed desirable to establish this point in a consistent manner.

Aqueous extracts of vegetable substances are often exceedingly difficult to filter. The method as published involved much difficult filtration, and with some vegetable substances was, on this account, practically unworkable. In the studies here reported these difficulties of filtration were removed.

These points and others were considered in a series of analytical determinations, as reported in the following tables, the work usually including, as a check, a test of the completeness of recovery of added inorganic phosphorus. The method, as used in this work, was as follows:

ACID-ALCOHOL METHOD OF FORBES AND ASSOCIATES FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

Pour exactly 300 c. c. of 0.2 percent hydrochloric acid (4.6 c. c. concentrated hydrochloric acid, sp. gr. 1.18-1.19, per liter) onto 10 grams of sample in a dry 400 c. c. Florence flask. Close with rubber stopper, and shake at intervals of 5 minutes for 3 hours. Filter the extract by suction into dry flasks through S. & S. No. 589 "Blue Ribbon" papers, in a Witt filtering apparatus, or a Büchner funnel.

Measure out a 250 c. c. portion of this filtered extract, and precipitate in a 400 c. c. beaker with 10 c. c. magnesia mixture and 20 c. c. ammonia, sp. gr. 0.9. Allow to stand over night, and filter through double S. & S. No. 589 "White Ribbon" papers, taking care to decant as long as possible without pouring out the precipitate. Then complete the transfer of the precipitate to the paper.

Wash three times with 2.5 percent ammonia, and then three times with 95 percent alcohol. Allow the 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 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.

The result obtained as above represents 6.25 grams out of the original 10 grams of material, and so to reduce to a 1-gram basis multiply by 0.16.

In the analyses reported in the following group of tables (pages 7 to 10) the above standard method was modified, in certain parts of the work, as indicated below:

AQUEOUS HYDROCHLORIC ACID EXTRACTION, PLUS PHOSPHATE

Proceed as on p. 5 except that in place of 300 c. c. of 0.2 percent hydrochloric acid add 250 c. c. of the same and 50 c. c. of phosphate solution containing disodium phosphate equivalent to approximately 25 mg. magnesium pyrophosphate per 50 c. c. Make up this phosphate solution with 0.2 percent hydrochloric acid.

AQUEOUS HYDROCHLORIC ACID-PHENOL EXTRACTION

Proceed as on p. 5 except that in place of 300 c. c. of 0.2 percent hydrochloric acid add 300 c. c. of 0.2 percent hydrochloric acid solution containing 50 gm. phenol per liter.

AQUEOUS HYDROCHLORIC ACID-PHENOL EXTRACTION, PLUS PHOSPHATE

Proceed as in the paragraph above except that in place of 300 c. c. add 250 c. c. of 0.2 percent hydrochloric acid containing 50 gm. phenol per liter, and 50 c. c. of phosphate solution containing disodium phosphate equivalent to approximately 25 mg. magnesium pyrophosphate per 50 c. c. Make up this phosphate solution with 0.2 percent hydrochloric acid containing 50 gm. phenol per liter.

PHOSPHORUS ESTIMATIONS ON REAGENTS AND PHOSPHATE SOLUTIONS USED IN WORK REPORTED IN TABLE I, PAGE 7

	Magnesium pyrophosphate Grams
Blank 1 Aq.-HCl solutions	0.0002
2 Aq.-HCl solutions	0.0002
3 Aq.-HCl solutions	0.0002
Average	0.0002
Blank 1 Aq.-HCl-phenol solutions	0.0002
2 Aq.-HCl-phenol solutions	0.0002
3 Aq.-HCl-phenol solutions	0.0002
Average	0.0002
Phosphate solution (Aq.-HCl) 50 cc. 1	0.0250
Phosphate solution (Aq.-HCl) 50 cc. 2	0.0248
Phosphate solution (Aq.-ACl) 50 cc. 3	0.0248
Average	0.0249
Phosphate solution (Aq.-HCl-phenol) 50 cc. 1	0.0249
Phosphate solution (Aq.-HCl-phenol) 50 cc. 2	0.0249
Phosphate solution (Aq.-HCl-phenol) 50 cc. 3	0.0249
Average	0.0249

TABLE I: TEST OF THE ACID-ALCOHOL METHOD OF FORBES AND ASSOCIATES FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

Sample	Number and treatment	Magnesium pyrophosphate Grams	Inorganic phosphorus Percent	Phosphorus added (magnesium pyrophosphate) Grams	Added phosphorus recovered (magnesium pyrophosphate) Grams	Added phosphorus recovered Percent
Alfalfa	A_1 Aq.-HCl extraction	0.0189				
	A_2 Aq.-HCl extraction	0.0170				
	Average	0.0180	0.0803			
	B_1 Aq.-HCl extr. plus phosphate	0.0188				
	B_2 Aq.-HCl extr. plus phosphate	0.0222				
	B_3 Aq.-HCl extr. plus phosphate	0.0225				
	Average	0.0212		0.0156	0.0032	20.5
	C_1 Aq.-HCl-phenol extraction	0.0182				
	C_2 Aq.-HCl-phenol extraction	0.0168				
	C_3 Aq.-HCl-phenol extraction	0.0175				
	Average	0.0175	0.0780			
	D_1 Aq.-HCl-phenol extr. plus phosphate	0.0210				
	D_2 Aq.-HCl-phenol extr. plus phosphate	0.0203				
	D_3 Aq.-HCl-phenol extr. plus phosphate	0.0229				
	Average	0.0213		0.0156	0.0038	24.3
Blue Grass	A_1 Aq.-HCl extraction	0.0228				
	A_2 Aq.-HCl extraction	0.0225				
	A_3 Aq.-HCl extraction	0.0224				
	Average	0.0226	0.1008			
	B_1 Aq.-HCl extr. plus phosphate	0.0283				
	B_2 Aq.-HCl extr. plus phosphate	0.0230				
	B_3 Aq.-HCl extr. plus phosphate	0.0255				
	Average	0.0256		0.0156	0.0030	19.2
	C_1 Aq.-HCl-phenol extraction	0.0365				
	C_2 Aq.-HCl-phenol extraction	0.0355				
	C_3 Aq.-HCl-phenol extraction	0.0347				
	Average	0.0356	0.1588			
	D_1 Aq.-HCl-phenol extr. plus phosphate	0.0493				
	D_2 Aq.-HCl-phenol extr. plus phosphate	0.0458				
	D_3 Aq.-HCl-phenol extr. plus phosphate	0.0498				
	Average	0.0483		0.0156	0.0127	81.4
Brewer's Grains	A_1 Aq.-HCl extraction	0.0025				
	A_2 Aq.-HCl extraction	0.0021				
	A_3 Aq.-HCl extraction	0.0023				
	Average	0.0023	0.0103			
	B_1 Aq.-HCl extr. plus phosphate	0.0160				
	B_2 Aq.-HCl extr. plus phosphate	0.0148				
	B_3 Aq.-HCl extr. plus phosphate	0.0161				
	Average	0.0156		0.0156	0.0133	85.2
	C_1 Aq.-HCl-phenol extraction	0.0012				
	C_2 Aq.-HCl-phenol extraction	0.0010				
	C_3 Aq.-HCl-phenol extraction	0.0013				
	Average	0.0012	0.0053			
	D_1 Aq.-HCl-phenol extr. plus phosphate	0.0162				
	D_2 Aq.-HCl-phenol extr. plus phosphate	0.0158				
	D_3 Aq.-HCl-phenol extr. plus phosphate	0.0160				
	Average	0.0160		0.0156	0.0148	94.9
Rice Polish	A_1 Aq.-HCl extraction	0.0038				
	A_2 Aq.-HCl extraction	0.0038				
	A_3 Aq.-HCl extraction	0.0035				
	Average	0.0037	0.0165			
	B_1 Aq.-HCl extr. plus phosphate	0.0119				
	B_2 Aq.-HCl extr. plus phosphate	0.0110				
	B_3 Aq.-HCl extr. plus phosphate	0.0111				
	Average	0.0113		0.0156	0.0076	48.7
	C_1 Aq.-HCl-phenol extraction	0.0027				
	C_2 Aq.-HCl-phenol extraction	0.0017				
	C_3 Aq.-HCl-phenol extraction	0.0024				
	Average	0.0023	0.0102			
	D_1 Aq.-HCl-phenol extr. plus phosphate	0.0052				
	D_2 Aq.-HCl-phenol extr. plus phosphate	0.0093				
	D_3 Aq.-HCl-phenol extr. plus phosphate	0.0138				
	Average	0.0094		0.0156	0.0071	45.5

TABLE II: TEST OF THE ACID-ALCOHOL METHOD OF FORBES AND ASSOCIATES FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

Sample	Number and treatment	Magnesium pyrophosphate	Inorganic phosphorus	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered (magnesium pyrophosphate)	Added phosphorus recovered
		Grams	Percent	Grams	Grams	Percent
Alfalfa[1]	A_1 Aq.-HCl extraction	0.0219				
	A_2 Aq.-HCl extraction	0.0184				
	A_3 Aq.-HCl extraction	0.0160				
	Average	0.0188	0.0838			
	B_1 Aq.-HCl extr. plus phosphate	0.0237				
	B_3 Aq.-HCl extr. plus phosphate	0.0235				
	Average	0.0236		0.0156	0.0048	30.8
	C_1 Aq.-HCl-phenol extraction	0.0140				
	C_2 Aq.-HCl-phenol extraction	0.0189				
	C_3 Aq.-HCl-phenol extraction	0.0149				
	Average	0.0159	0.0709			
	D_1 Aq.-HCl-phenol extr. plus phosphate	0.0232				
	D_2 Aq.-HCl-phenol extr. plus phosphate	0.0241				
	Average	0.0236		0.0156	0.0077	49.3
Blue Grass[2]	A_1 Aq.-HCl extraction	0.0380				
	A_2 Aq.-HCl extraction	0.0379				
	A_3 Aq.-HCl extraction	0.0335				
	Average	0.0365	0.1628			
	B_1 Aq.-HCl extr. plus phosphate	0.0321				
	B_2 Aq.-HCl extr. plus phosphate	0.0298				
	B_3 Aq.-HCl extr. plus phosphate	0.0320				
	Average	0.0313		0.0156	−0.0052	−33.3
	C_1 Aq.-HCl-phenol extraction	0.0397				
	C_2 Aq.-HCl-phenol extraction	0.0409				
	C_3 Aq.-HCl-phenol extraction	0.0408				
	Average	0.0404	0.1802			
	D_1 Aq.-HCl-phenol extr. plus phosphate	0.0554				
	D_2 Aq.-HCl-phenol extr. plus phosphate	0.0548				
	D_3 Aq.-HCl-phenol extr. plus phosphate	0.0545				
	Average	0.0549		0.0156	0.0145	92.9
Rice Polish[3]	A_1 Aq.-HCl extraction	0.0036				
	A_2 Aq.-HCl extraction	0.0026				
	A_3 Aq.-HCl extraction	0.0027				
	Average	0.0030	0.0134			
	B_1 Aq.-HCl extr. plus phosphate	0.0076				
	B_2 Aq.-HCl extr. plus phosphate	0.0075				
	B_3 Aq.-HCl extr. plus phosphate	0.0079				
	Average	0.0077		0.0156	0.0047	30.1
	C_1 Aq.-HCl-phenol extraction	0.0020				
	C_2 Aq.-HCl-phenol extraction	0.0020				
	C_3 Aq.-HCl-phenol extraction	0.0020				
	Average	0.0020	0.0089			
	D_1 Aq.-HCl-phenol extr. plus phosphate	0.0064				
	D_2 Aq.-HCl-phenol extr. plus phosphate	0.0082				
	Average	0.0073		0.0156	0.0053	34.

(1) Second set of determinations; first magnesium precipitates allowed to stand an extra day before filtering, and, after filtering, an extra day in acid alcohol. With samples A-1 and A-3 only 200 c. c. of aqueous-HCl extract was used, but the figures given represent 250 c. c. as usual.

(2) With samples A-1, A-2 and A-3 only 200 c. c. of the aqueous HCl extract was used, but weights given for magnesium pyrophosphate represent 250 c. c.

(3) First magnesium precipitate broken up in acid alcohol with stirring rod before filtering off 75 c. c. aliquot.

TABLE III: TEST OF COMPLETENESS OF EXTRACTION AND INFLUENCE OF PHENOL IN THE DETERMINATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

Sample, treatment and sample number		First extraction		Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered (magnesium pyrophosphate)	Second extraction	
		Magnesium pyrophosphate	Inorganic phosphorus			Magnesium pyrophosphate	Excess phosphorus extracted (magnesium pyrophosphate)
		Grams	Percent	Grams	Grams	Grams	Grams
Timothy: Aq.-HCl extraction	A_1	0.0064				0.0012	
	A_2	0.0069				0.0010	
	A_3	0.0053				0.0012	
	Av.	0 0062	0.0276			0.0011	+0.0001
Timothy: Aq.-HCl extr. plus phosphate	A_4	0.0253				0.0032	
	A_5	0.0250				0.0033	
	A_6	0.0256				0.0032	
	Av.	0.0253		0.0153	0.0191	0.0032	−0.0010
Timothy: Aq.-HCl-phenol extraction	B_1	0.0099				0.0006	
	B_2	0.0099				0.0005	
	B_3	0.0096				0.0029[1]	
	Av.	0.0098	0.0437			0.0005	−0.0011
Timothy: Aq.-HCl-phenol extr. plus phosphate	B_4	0.0249				0.0029	
	B_5	0.0246				0.0030	
	B_6	0.0252				0.0006[1]	
	Av.	0.0249		0.0153	0.0151	0.0029	−0.0012
Rice Polish: Aq.-HCl extraction plus phosphate	A_1	0.0186				0.0026	
	A_2	0.0192				0.0032	
	A_3	0.0182				0.0034	
	Av.	0.0187		0.0111	0.0089	0.0031	0.0000
Rice Polish: Aq.-HCl extraction	A_4	0.0098				0.0012	
	A_5	0.0096				0.0012	
	A_6	0.0098				0.0012	
	A_7	0.0098				0.0012	
	Av.	0.0098	0.0434			0.0012	−0.0004
Rice Polish: Aq.-HCl-phenol extr. plus phosphate	B_1	0.0150				0.0017	
	B_2	0.0144				0.0018	
	B_3	0.0150					
	Av.	0.0148		0.0111	0.0110	0.0017	−0.0008
Rice Polish: Aq.-HCl-phenol extraction	B_4	0.0036					
	B_5	0.0040				0.0014	+0.0007
	B_6	0.0040					
	B_7	0.0036					
	Av.	0.0038	0.0169				

[1]Not included in average.

TABLE IV: TEST OF COMPLETENESS OF EXTRACTION AND INFLUENCE OF PHENOL IN THE DETERMINATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

Sample	Treatment and sample number		First extraction		Second extraction	
			Magnesium pyrophosphate	Inorganic phosphorus	Magnesium pyrophosphate	Excess phosphorus extracted (magnesium pyrophosphate)
			Grams	Percent	Grams	Grams
Gluten feed	Aq.-HCl extraction	A_1	0.0211		0.0032	
	Aq.-HCl extraction	A_2	0.0210		0.0032	
	Aq.-HCl extraction	A_3	0.0209			
		Av.	0.0210	0.0936	0.0032	-0.0003
	Aq.-HCl-phenol extraction	B_1	0.0212		0.0011	
	Aq.-HCl-phenol extraction	B_2	0.0207		0.0009	
	Aq.-HCl-phenol extraction	B_3	0.0200		0.0011	
		Av.	0.0206	0.0919	0.0010	—0.0024
Brewer's grains	Aq.-HCl extraction	A_1	0.0026		0.0010	
	Aq.-HCl extraction	A_2	0.0028		0.0010	
	Aq.-HCl extraction	A_3	0.0028		0.0010	
		Av.	0.0027	0.0120	0.0010	0.0007
	Aq.-HCl-phenol extraction	B_1	0.0012[1]		0.0010	
	Aq.-HCl-phenol extraction	B_2	0.0022		0.0010	
	Aq.-HCl-phenol extraction	B_3	0.0026		0.0010	
		Av.	0.0024	0.0107	0.0010	0.0006
Timothy	Aq.-HCl extraction	A_1	0.0062		0.0019	
	Aq.-HCl extraction	A_2	0.0040		0.0016	
	Aq.-HCl extraction	A_3	0.0025		0.0018	
		Av.	0.0042	0.0187	0.0017	0.0011
	Aq.-HCl-phenol extraction	B_1	0.0105		0.0003	
	Aq.-HCl-phenol extraction	B_2	0.0097		0.0006	
	Aq.-HCl-phenol extraction	B_3	0.0106			
		Av.	0.0103	0.0459	0.0004	—0.0010
Wheat	Aq.-HCl extraction	A_1	0.0092			
	Aq.-HCl extraction	A_2	0.0096			
	Aq.-HCl extraction	A_3	0.0092			
		Av.	0.0093	0.0415		
	Aq.-HCl-phenol extraction	B_1	0.0040			
	Aq.-HCl-phenol extraction	B_2	0.0049			
	Aq.-HCl-phenol extraction	B_3	0.0048			
		Av.	0.0046	0.0205		
Wheat bran	Aq.-HCl extraction	A_1	0.0143			
	Aq.-HCl extraction	A_2	0.0134			
	Aq.-HCl extraction	A_3	0.0138			
		Av.	0.0138	0.0615		
	Aq.-HCl-phenol extraction	B_1	0.0145			
	Aq.-HCl-phenol extraction	B_2	0.0157			
	Aq.-HCl-phenol extraction	B_3	0.0140			
		Av.	0.0147	0.0655		

[1]Not included in average.

The recovery of added phosphorus was usually incomplete, the method proving unsatisfactory, as judged by this measure. The recovery varied from a minus quantity in one case, with blue grass (Table II, p. 8) to 100 percent in one case, with rice polish (Table III, p. 9). The variability of results with the same products, in the different sets of estimations, when considered in connection with the difficulties experienced in filtration, suggested to us that at least a part of the incompleteness of recovery of added phosphates was due to the gelatinous character of the magnesia mixture precipitate, notably so in the case of alfalfa, as shown in Tables I and II, pp. 7 and 8. In the work reported in Table II the precipitates remained an extra day in acid-alcohol, and the recovery of added phosphate was more nearly complete than in the work reported in Table I. A further consideration of this factor is reported in Tables VIII to X on pp. 18 to 20.

The presence of phenol gave lower results for inorganic phosphorus with alfalfa, brewer's grains, rice polish, gluten feed and wheat; and higher results with blue grass, timothy and wheat bran. The recovery of added phosphate was usually incomplete; it was almost always higher with phenol than without, and was practically complete with brewer's grains (Table I, p. 7), timothy hay and rice polish (Table III, p. 9). With timothy hay the percent of inorganic phosphate found with phenol was higher than without; the recovery of added phosphate was complete with phenol, and apparently more than complete without phenol.

It is not clear, from these results, what is the nature of the effect of phenol in this estimation. Since the effect of phenol is usually to lower the inorganic phosphorus, though sometimes to increase the same, we might suppose that in the former cases cleavage predominated, except as suppressed by phenol, while in the latter cases the inhibited processes were in the direction of synthesis. We have found phenol, as used in this work, to be without effect on the precipitation of magnesium ammonium phosphate from pure solutions. Its efficiency to prevent enzymatic cleavage was not experimentally demonstrated.

The test of completeness of extraction of inorganic phosphates by 0.2 percent hydrochloric acid in three hours was made with gluten feed, brewer's grains, timothy hay, and rice polish, the results being given in Tables III and IV on pp. 9 and 10. In considering the significance of the weight of pyrophosphate obtained from the second extraction one should bear in mind the fact that this is due largely to dissolved phosphate from the first extraction

remaining adherent to the foodstuff. After making the necessary correction of this weight by subtracting such amount of pyrophosphate as corresponds to the inorganic phosphorus in the liquid retained by the sample, the results are very small, and are more often minus quantities than not, showing that with these four foodstuffs the 3-hour extraction is practically complete. Further tests of the completeness of the 3-hour extraction are reported in Table VI, p. 16.

TEST OF THE METHOD OF R. C. COLLISON

As a possible improvement upon the method thus far considered the similar procedure of R. C. Collison* was studied. Collison's method depends, as does that of Forbes and associates, from which it was derived, on an acid-alcohol separation of inorganic phosphorus from phytin, but this separation takes the form of a direct acid-alcohol extraction of the substance to be analyzed. The two methods differ in certain other details. Collison's method has the advantage of being much more easily workable. Unfortunately, however, as first noted by Grindley and Ross, and later substantiated in this laboratory, results from the use of this method are unsatisfactory in that the 3-hour extraction is either insufficient or causes a cleavage of inorganic from organic phosphorus compounds. A second and even a third 3-hour extraction with acid-alcohol yields considerable amounts of inorganic phosphorus.

The details of the method as used are as follows:

METHOD OF R. C. COLLISON FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

Weigh out 10-gram portions of the samples in triplicate, and place in 400 c. c. Florence flasks, to which add exactly 300 c. c. of 94-96 percent phosphorus-free alcohol, containing 0.2 percent of hydrochloric acid (0.2 percent actual HCl), and close with rubber stopper.

Shake the flasks at intervals of 5 minutes for 3 hours, and filter through dry double filters into dry flasks.

Measure out 250 c. c. aliquots of the filtrates into 400 c. c. beakers; make just alkaline to litmus with ammonia, and allow to stand for 8-12 hours, or over night.

Filter through double filters, and wash with slightly ammoniacal 94-96 percent alcohol. In case a small portion of the precipitate resists transfer from the beaker by the usual means the last traces may be dissolved in 5 drops of hydrochloric acid, with the assistance of a rubber-capped rod. To this acid solution add 10 c. c. of alcohol; make slightly alkaline with ammonia, and then transfer to the filter.

*Jour. Ind. and Eng. Chem., IV, p. 606, 1912.

Wash several times with ammoniacal alcohol; then spread out the inner papers with the precipitate and allow to dry completely. Transfer papers and precipitates to Erlenmeyer flasks containing exactly 100 c. c. of 0.5 percent aqueous solution of nitric acid (0.5 percent actual HNO_3). Close the flasks with rubber stoppers; shake until the precipitates are thoroughly broken up, and let stand over night.

Filter through dry double filters into dry beakers; pipette out 75 c. c. of each filtrate and determine phosphorus in the usual way, precipitating first with acid molybdate solution, then with magnesia mixture, and weigh as the pyrophosphate.

If the final solutions are highly colored, dissolve the pyrophosphates and reprecipitate.

Analytical data from our test of Collison's method are set forth in the following table:

TABLE V: TEST OF METHOD OF R. C. COLLISON FOR THE ESTIMATION OF INORGANIC PHOSPHORUS IN VEGETABLE SUBSTANCES

Sample		Magnesium pyrophosphate	Inorganic phosphorus	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered		Second extraction		
					Magnesium pyrophosphate	Percent	Magnesium pyrophosphate	Excess phosphorus extracted	
								Magnesium pyrophosphate	Percent of sample
		Grams	Percent	Grams	Grams		Grams	Grams	
Corn germ	1	0.0066	0.0294				0.0026	0.0015	0.0067
Corn germ	2	0.0068	0.0303				0.0024	0.0013	0.0058
Corn germ	3	0.0065	0.0290				0.0025	0 0014	0.0062
Same plus phosphate	4	0.0176		0.0116	0.0110		0.0042	0.0013	
Same plus phosphate	5			0.0116			0.0042		
Same plus phosphate	6	0.0178		0.0116	0.0112		0.0043	0.0013	
	Averages		0.0299		0.0111	95.7			0.0062
Wheat germ	1	0.0090	0.0401				0.0040	0.0025	0 0111
Wheat germ	2	0.0091	0.0406				0.0037	0.0022	0.0098
Wheat germ	3	0.0090	0.0401				0.0048	0.0033	0 0147
Same plus phosphate	4	0.0204		0 0116	0.0114		0.0071	0.0037	
Same plus phosphate	5	0.0206		0.0116	0 0116		0.0070	0.0036	
Same plus phosphate	6	0.0204		0.0116	0.0114		0.0068	0.0034	
	Averages		0.0403		0.0115	99.1			0.0119
Rice polish	1	0.0047	0.0210				0.0020	0.0012	0.0053
Rice polish	2	0.0048	0.0214				0.0018	0.0010	0.0045
Rice polish	3	0 0048	0.0214				0 0022	0 0014	0.0062
Same plus phosphate	4	0 0154		0.0116	0.0106		0.0038	0.0012	
Same plus phosphate	5	0.0158		0 0116	0.0110				
Same plus phosphate	6	0.0160		0 0116	0.0112		0 0042	0.0015	
	Averages		0.0213		0 0109	94.0			0.0053
Wheat bran	1	0 0088	0.0392				0.0040	0.0025	0.0111
Wheat bran	2	0.0084	0.0375				0.0042	0.0028	0.0125
Wheat bran	3	0.0084	0.0375				0.0042	0.0028	0.0125
Same plus phosphate	4	0.0182		0.0116	0 0097		0.0064	0 0034	
Same plus phosphate	5	0.0189		0.0116	0.0104		0.0069	0.0038	
Same plus phosphate	6	0.0184		0.0116	0.0099		0.0060	0.0030	
	Averages		0.0381		0.0100	86.2			0.0120

FURTHER CONSIDERATION OF THE METHOD OF FORBES AND ASSOCIATES

Since Collison's method proved unsatisfactory, further work was done, at a later date, in attempts to improve upon the method of Forbes and associates (p. 5). In this work we gave attention to the following points:

(a) The completeness of extraction, (b) the effect of using much larger amounts of magnesia mixture in the precipitation, (c) the allowing of more time for the precipitation with magnesia mixture, (d) the facilitating of filtration by the use of the centrifuge, and (e) the use of mechanical means to break up the precipitate in acid-alcohol to insure the complete solution of the phosphate.

The following tabular data set forth the results of this study, the general method being that of the recovery of known amounts of phosphate introduced into the estimation. The details were as specified on p. 5, though phenol was used only in the tests reported in Table IX, p. 19. Throughout this work the centrifuge was used to facilitate filtration of the 0.2 percent hydrochloric acid extracts. The advantage derived from this treatment was very great. Extracts which it was impossible otherwise to filter within a reasonable time were, with the aid of the centrifuge, filtered without difficulty or delay.

Other constant conditions in this work were the use of an extreme amount (50 c. c.) of magnesia mixture in the preliminary precipitation (instead of 10 c. c. as usual); and three days' time were allowed in all cases for the completion of this precipitation.

Table VI, p.16, reports results from a test of the Acid-Alcohol Method of Forbes and associates (for details see p. 5; modified as above), with alfalfa hay, by the method of recovery of added phosphates, and a test of the completeness of extraction. Alfalfa was selected for this test as that substance which, in our previous experience, had given us the most trouble and the poorest results. Samples 4, 5 and 6 as compared with 1, 2 and 3, show that the recovery of added phosphates was incomplete, except in the case of sample 5, in which case, as explained in the footnote below the table, on account of the accidental breaking of the first filter paper, the precipitation was finally made in the presence of the pulp from this paper. In this case the recovery was complete. This accidental result sustained our hypothesis that our difficulty in recovering added phosphates was due to the physical character of the first magnesia mixture precipitate, its gummy character rendering impossible the complete separation, in acid-alcohol, of the inorganic phosphates from the phytin and other substances present. This point was given further study.

Determinations 7a, 8a and 9a were made in the same way as 1, 2 and 3. Determinations 7b, 8b and 9b were second extractions of the residues from determinations 7a, 8a and 9a. The results from the second extraction equalled only the residual amount of phosphate clinging to the sample, from the first extraction; that is, no more inorganic phosphate was dissolved in a three-hour extraction, following the three-hour extraction regularly prescribed in the method. The extraction was complete at the end of the first three-hour treatment.

TABLE VI: TEST OF INORGANIC PHOSPHORUS ESTIMATION ON VEGETABLE SUBSTANCES BY THE ACID-ALCOHOL METHOD (WITHOUT PHENOL)

Substance	Sample No.	Treatment	Magnesium pyrophosphate Grams	Percent inorganic phosphorus	Added phosphorus (magnesium pyrophosphate) Grams	Added phosphorus recovered: Magnesium pyrophosphate Grams	Added phosphorus recovered: Percent
Alfalfa hay	1	Filtrate precip. with 50 c. c. magnesia mixture + 25 c. c ammonia; without added phosphate	.0176				
	2		.0181				
	3		.0178				
	Average		.0178	.0992			
Alfalfa hay	4	Same, with added phosphate	.0425		.0299	.0246	82.27
	5*		.0477		.0299	.0299	100.00
	6		.0404		.0299	.0226	75.58
	Average		.0435				85.95
Alfalfa hay	7a	Without added phosphate; same as 1, 2 and 3	.0182				
	8a		0181				
	9a		.0181				
	Average		.0181	.1008			
Alfalfa hay	7b	Second extraction of samples 7a, 8a and 9a	.0062		.0060***		
	8b**		.0060		.0060		
	9b		.0058		.0060		
	Average		.0060				

Weight of samples 10 grams; results represent one-half of this amount.

*During the filtration of the first magnesia mixture precipitate the filter paper broke. This paper was then added to the beaker containing the precipitate, was beaten up into a pulp, and the filtration continued through a new paper.

**Samples 7b, 8b and 9b are the extraction residues from 7a, 8a and 9a, with filter paper added, and also enough 0.2 percent hydrochloric acid solution to make the original volume of 300 c. c. This set was extracted for three hours to test the completeness of the previous extraction.

***Magnesium pyrophosphate equivalent to phosphorus in solution, from previous extraction, remaining in the sample.

Considering the data in Table VII, below, with samples 1-9 the first magnesia mixture precipitates were extracted with 200 c. c. of 0.2 percent nitric acid in alcohol instead of 100 c. c. as usual, in order to test the sufficiency of the latter amount to neutralize the ammonia remaining in the precipitates, and to dissolve the phosphates. With samples 10-12 the usual 100 c. c. of acid alcohol were used. The comparison shows that 100 c. c. of acid was probably sufficient, though the result from sample 11, for some unknown reason was low.

The second and third sets of triplicates, samples 4-9, contrast results from the addition of phosphates (to be recovered) after the extraction (immediately before precipitation) and previous to the three-hour extraction. We see here no evidence of a retention of added phosphates by the solid substance of the sample.

TABLE VII: TEST OF INORGANIC PHOSPHORUS ESTIMATION ON VEGETABLE SUBSTANCES BY THE ACID-ALCOHOL METHOD (WITHOUT PHENOL)

Substance	Sample No.	Treatment	Magnesium pyrophosphate	Added phosphorus (magnesium pyrophosphate)	Added phosphorus recovered	
					Magnesium pyrophosphate	
			Gm.	Gm.	Gm.	Percent
Alfalfa hay	1	Filtrate precipitated with 50 c. c. mag. mixture and 20 c. c. ammonia, 200 c. c. acid alcohol used to extract magnesia mixture precipitate.	.0183	.0000		
	2		.0180			
	3		.0184			
	Ave.		.0182			
Alfalfa hay	4	Same as above + 25 c. c. phosphate solution just before precipitation.	.0603	.04485		
	5		.0606			
	6		.0604			
	Ave.		.0604		.0429	94.1
Alfalfa hay	7	Same as 1, 2 and 3 except that 25 c. c. phosphate solution added, shaken for 3 hrs., filtered and filtrate precip. as 1, 2 and 3.	.0466	0299	.0284	94.98
	8		.0466	"	.0284	
	9		.0472	"	.0290	96.99
	Ave.					95.98
Alfalfa hay	10	Same as 7, 8 and 9, except that only 100 c. c. acid alcohol were used in extraction of magnesia mixture precipitate.	.0467		.0285	95.32
	11		.0447		.0265	88.63
	12		.0464		.0282	94.31
	Ave.					92.75
Alfalfa hay	13	25 c. c. phosphate solution put in flasks+175 c. c. .2% nitric acid in alcohol+2 filter papers, shaken, filtered and aliquot taken for precipitation.	.0404	.03987	.0404	101.1
	14		.0404		.0404	
	15		.0402		.0402	
	Ave.					

Weight of samples, 10 grams; results represent 0.5 of this amount.

Determinations 13-15 were made to ascertain whether or not we could get a complete recovery of phosphates from filter paper pulp. It was possible completely to recover the phosphates. This test has a bearing on work to follow, and shows that the incomplete recovery of added phosphates could not be due to the presence of filter paper pulp.

The recovery of added phosphates in these estimations on alfalfa was fairly satisfactory.

TABLE VIII: TEST OF INORGANIC PHOSPHORUS ESTIMATION ON VEGETABLE SUBSTANCES BY THE ACID-ALCOHOL METHOD (WITHOUT PHENOL)

Substance	Sample No.	Treatment	Magnesium pyrophosphate Grams	Percent inorganic phosphorus	Added phosphorus (magnesium pyrophosphate) Grams	Added phosphorus recovered: Magnesium pyrophosphate Grams	Added phosphorus recovered: Percent
Blue grass	1	Filtrate precip. with 50 c. c. magnesia mixture+25 c. c ammonia, plus paper pulp	.0373	.2078			
	2		.0371	.2067			
	3		.0386	.2151			
	Average			.2099			
Blue grass	4	Same +25 c. c. phosphate solution	.0624		.0250	.0251	100.04
	5		.0623		.0250	.0252	100.08
	6		.0633		.0250	.0247	98.80
	Average						99.97
Rice polish	7	Same as 1, 2 and 3	.0070				
	8		.0070				
	9						
	Average		.0070	.0390			
Rice polish	10	Same+25 c. c phosphate solution	.0295		.0250	.0225	
	11		.0295		.0250	.0225	
	12						
	Average					.0225	90.00

Weight of samples 10 grams; results represent 0.5 of this amount

TABLE IX: TEST OF INORGANIC PHOSPHORUS ESTIMATION ON VEGETABLE SUBSTANCES BY THE ACID-ALCOHOL METHOD (WITH PHENOL)

Substance	Sample No.	Treatment	Magnesium pyrophosphate Gm.	Percent inorganic phosphorus	Added phosphorus (magnesium pyrophosphate) Gm.	Added phosphorus recovered	
						Magnesium pyrophosphate Gm.	Percent
Rice polish	1	Extracted with 0.2% hydrochloric acid + 50 gm. phenol per liter; filtrate precipitated with magnesia mixture + 25 c. c. ammonia.	.0080	.0446			
	2		.0072	.0401			
	3						
	Ave.			.0424			
Rice polish + phosphate	4	Same	.0543		.0768	.0467	60.81
	5		.0461		.0768	.0385	50.13
	6		.0443		.0768	.0367	47.79
	Ave.						52.91
Middlings	7	Same	.0132				
	8		.0133				
	9		.0129				
	Ave.		.0131	.0724			
Middlings + phosphate	10	Same	.0830		.0768	.0699	91.01
	11		.0864		.0768	.0733	95.44
	12		.0847		.0768	.0716	93.23
	Ave.						93.23
Soy beans	13	Same	.0102				
	14		.0105				
	15		.0102				
	Ave.		.0103	.0574			
Soy beans + phosphate	16	Same	.0790		.0768	.0687	89.45
	17		.0801		.0768	.0698	90.89
	18		.0823		.0768	.0720	93.75
	Ave.		.0805				91.36

Weight of samples 10 grams; results represent 0.5 of this amount.

TABLE X: TEST OF INORGANIC PHOSPHORUS ESTIMATION ON VEGETABLE SUBSTANCES BY THE ACID-ALCOHOL METHOD (WITHOUT PHENOL)

Substance	Sample No.	Treatment	Magnesium pyrophosphate (Gram)	Percent inorganic phosphorus	Added phosphorus (magnesium pyrophosphate) (Gram)	Added phosphorus recovered: Magnesium pyrophosphate (Gram)	Added phosphorus recovered: Percent
Soy beans	1	Usual method, plus filter paper pulp; without phosphate	.0102				
	2		.0103				
	3		.0101				
	Average		.0102	.0568			
Soy beans	1	Same, plus phosphate	.0336		.0261	.0234	90.03
	2		.0345		.0261	.0243	93.10
	3		.0347		.0261	.0245	93.87
	Average						92.33
Middlings	1	Same, without phosphate	.0135				
	2		.0131				
	3		.0139				
	Average		.0135	.0752			
Middlings	1	Same, with phosphate	.0432		.0261	.0297	113.79
	2		.0430		.0261	.0295	113.02
	3		.0418		.0261	.0283	108.42
	Average						111.74
Oat straw	1	Same, without phosphate	.0062				
	2		.0058				
	3		.0061				
	Average		.0060	.0334			
Oat straw	1	Same, with phosphate	.0312		.0261	.0252	96.55
	2		.0319		.0261	.0259	99.23
	3		.0318		.0261	.0258	98.85
	Average						98.21

Weight of samples 10 grams; results represent 0.5 of this amount.

Table VIII, p. 18, sets forth results of estimations on blue grass and rice polish, with and without added phosphate, with filter paper pulp added, in the first precipitation, to maintain a readily permeable condition in the precipitate.

With blue grass the results may be considered perfect. With rice polish the recovery of added phosphates was 90 percent efficient. The loss amounted to 0.0025 gm. magnesium pyrophosphate per determination.

Table IX, p. 19, is a record of determinations on rice polish, wheat middlings and soy beans, with and without added phosphate, with phenol added to the extractive reagent to prevent possible enzyme action involving phosphorus compounds, and with filter paper pulp added to facilitate solution of the phosphates in the precipitate. The recovery of added phosphates was unsatisfactory; the effect of the phenol on the physical condition of the first magnesia mixture precipitate being of such a nature as to hinder the dissolving out of the included phosphates.

Table X, p. 20, sets forth results from determinations on soy beans, wheat middlings and oat straw, with and without added phosphate, with filter paper pulp, and without phenol added to the extracting sample. With oat straw the results were satisfactory. With soy beans the recovery of added phosphates was incomplete, while with wheat middlings we seem to have recovered 3 milligrams more phosphate than was added. In this case we can not ascribe the imperfection of the result, as usual, to the physical condition of the magnesia mixture precipitate. Here, it seems, that there must have been a cleavage of inorganic from organic phosphorus, either enzymatic or as a result of the extractive treatment. Anderson* has shown that 0.2 percent hydrochloric acid is not sufficiently concentrated to prevent enzymatic hydrolysis of the phytin of wheat bran, and, since middlings contains bran, this observation must apply to middlings as well. How general may be enzymatic hydrolysis of organic phosphorus compounds in our 0.2 percent hydrochloric acid extractive reagent we are unable to say. In the case of wheat middlings only has the extent of the hydrolysis been shown, in our work, to be sufficient more than to offset the various factors tending to give low results for inorganic phosphorus. Our second three-hour extractions of timothy, rice polish, gluten feed and brewer's grains showed no considerable additional inorganic phosphorus resulting either from solution or hydrolytic decomposition of organic compounds.

CONCLUSIONS FROM WORK ON INORGANIC PHOSPHORUS ESTIMATION IN VEGETABLE SUBSTANCES

(1) A three-hour extraction with 0.2 percent hydrochloric acid in water appears to accomplish practically complete solution of the inorganic phosphates of finely ground vegetable substances, but in the case of wheat middlings was shown to allow enzymatic hydrolysis of organic phosphorus, with the liberation of inorganic phosphate.

*Anderson, R. J.: Journ. Biol. Chem. XX (1915) 483-491.

(2) The introduction of filter paper pulp into such an extract materially assists in the maintenance of an easily penetrable condition in a magnesia mixture precipitate from the same.

(3) It was found possible completely to recover phosphates from filter paper pulp alone as used in this work.

(4) The use of the centrifuge very greatly facilitates the filtration of dilute aqueous-acid extracts of vegetable substances.

(5) There has appeared no reason to doubt the completeness of the precipitation of the inorganic phosphates from the 0.2 percent hydrochloric acid solution, through the use of magnesia mixture and ammonia.

(6) The separation of the inorganic phosphates from the phytin and other constituents of the magnesia mixture precipitate, through the agency of 0.2 percent nitric acid in 95 percent alcohol, is attended by difficulties which have not yet been overcome. That these difficulties are largely physical, as determined by the bulky and often gummy nature of the magnesia mixture precipitate, seems to be a fact. That they are in part of a chemical nature, and due to the cleavage of phytin or other organic phosphorus compounds of the magnesia mixture precipitate through the agency of enzymes appears also to be true.

(7) The use of phenol (50 gm. per liter) in the extractive reagent was shown not to affect the precipitation and estimation of phosphates in pure solutions. In the estimation of inorganic phosphorus in extracts of vegetable substances the presence of phenol appeared to favor the recovery of added phosphates. Phenol, when used in this way, sometimes increased but more commonly decreased the inorganic phosphorus. In extracts of certain vegetable products the presence of phenol increased the difficulty, rather commonly experienced, in breaking up the magnesia mixture precipitate in acid alcohol.

(8) Modification of the acid-alcohol method of Forbes and associates by the introduction of filter paper pulp into the extract from which the phosphates are to be precipitated, the use of excessive amounts of magnesia mixture in this first precipitation, and allowing unusual duration of time for this precipitation gave apparently perfect results, as judged by recovery of added phosphates, in certain cases, but unsatisfactory results in others.

(9) Incompleteness of recovery of added phosphates was shown not to be due to retention of phosphates by the solid substance of the sample.

(10) We are unable to recommend this method, or any other, as reliable for the estimation of inorganic phosphorus in vegetable substances generally.

(11) The acid-alcohol extraction of the method of R. C. Collison is either incomplete, in three hours, or else causes a cleavage of organic compounds of phosphorus, with the liberation of inorganic phosphate.

THE ESTIMATION OF WATER-SOLUBLE INORGANIC PHOSPHORUS IN ANIMAL SUBSTANCES

The method of determination of inorganic phosphorus in animal substances, as published from this laboratory, is an adaptation of the usual magnesia mixture method for phosphorus estimation to the conditions of work with water-extracts of animal products. The original points, therefore, in our procedures, as specified for various animal tissues, are mechanical and chemical details of extraction, filtration and precipitation. Considerable care has been bestowed upon the quantitative proof of the correctness of these details, especially as providing for complete extraction, rapid filtration, and prevention of the cleavage of the organic phosphorus compounds from which the inorganic are to be separated. These studies are in the nature of comparisons of the neutral molybdate method of Emmett and Grindley, the barium chlorid method of Siegfried and Singewald and the magnesia mixture method of Forbes and associates, usually checked by the recovery of known amounts of added phosphate; and they appear to result in the establishment of the last-named method as reliable and workable with a wide range of animal tissues.

In the use of this method one should recognize the fact that it seeks to estimate the *water-soluble* inorganic phosphates only. This figure would be at least practically the same as *total* inorganic phosphates for most animal tissues and products, but in the case of tissues such as bone, which contain large amounts of inorganic phosphates which are insoluble in water, this method is inapplicable.

The work on animal tissues was done in three series of determinations, one each in the years 1912, 1913 and 1914. The remainder of this paper sets forth the details of this work. The discussion of the results of the work of 1912-1913 will be found on pages 33 and 34, and of 1914 on pages 38 to 40.

THE WORK OF 1912

The work of 1912 consisted of a comparison of the methods of Emmett and Grindley, Siegfried and Singewald, and Forbes and associates with muscle, blood and brain. The muscle used was a

vacuum-dried product prepared in the laboratory of Dr. H. S. Grindley, of the University of Illinois. The following schedule shows methods of precipitation, amounts of extracts used, number of repeats, and amount of standard phosphate solution added to certain of these determinations:

	A Neutral Molybdate Precipitation (Emmett and Grindley)	A-1 500 c. c. extract A-2 500 c. c. extract A-3 250 c. c. extract + 25 c. c. phosphate solution A-4 250 c. c. extract + 25 c. c. phosphate solution
1. Muscle 2. Blood 3. Brain	B Barium Chlorid Precipitation (Siegfried and Singewald)	B-1 500 c. c. extract B-2 500 c. c. extract B-3 250 c. c. extract + 25 c. c. phosphate solution B-4 250 c. c. extract + 25 c. c. phosphate solution
	C Magnesia Mixture Precipitation (Forbes, *et al*)	C-1 500 c. c. extract C-2 500 c. c. extract C-3 250 c. c. extract + 25 c. c. phosphate solution C-4 250 c. c. extract + 25 c. c. phosphate solution

The work with muscle was performed on a cold water extract prepared as specified below:

OUTLINE FOR THE PREPARATION OF A COLD WATER EXTRACT OF DESICCATED FLESH FOR THE DETERMINATION OF INORGANIC PHOSPHORUS

Weigh out about 45 grams of the vacuum-dried meat, and divide it among sixteen 150 c. c. beakers. To each beaker with its contents add about 3-5 c. c. of distilled water. Break up any lumps and stir well with a glass rod until the mass forms a thick paste. Add 50 c. c. of distilled water to each beaker and stir thoroughly for 15 minutes. Allow the insoluble portion to settle for a few minutes (3-5) and decant the supernatant liquid through wet 11 cm. filters. Collect the filtrates in 250 c. c. Florence flasks. Take care that the funnels touch the sides of the necks of the flasks. Drain the residues thoroughly, keeping as much of them in the beakers as possible. Treat these residues with 25 c. c. of distilled water, stirring for 5-7 minutes, and then allowing 3-5 minutes for the solid particles to settle before filtering. Decant, etc., as described above. Repeat this last treatment until the filtrate measures about 220 c. c. Then transfer the entire residue to the filter and wash twice with about 8-10 c. c. of distilled water. Allow all the liquid to pass through the filter before adding the next extract. Whenever the major portion of the residue has become mechanically transferred to the filter, return it to the beaker, using great care not to break the filter paper. Take the sixteen filtrates of about 250 c. c. each and transfer all of them to a measuring flask. Wash out each Florence flask twice, using about 5-8 c. c. of distilled water each time. Make the extract up to 5000 c. c. and mix it thoroughly without too much mechanical agitation.

With blood, the comparisons were made on an extract prepared with the aid of ammonium sulphate, the details of procedure being as specified below:

METHOD FOR THE PREPARATION OF HOT WATER-AMMONIUM SULPHATE EXTRACT OF BLOOD

Weigh about 50 grams of fresh blood, or its equivalent of oxalated blood, into each of six 400 c. c. beakers. To each beaker add a few c. c. of distilled water and work up the blood and water with a glass rod. Make up to about 200 c. c. with boiling distilled water. Place over a flame and gradually bring to boiling, with constant stirring. When boiling begins add to each beaker 20 c. c. of 20 percent ammonium sulphate solution. Boil with constant stirring for about 10 minutes. Decant onto sand on linen. When the liquid is through lift the coagulum off from the sand and transfer to a mortar. Grind the coagulum to a smooth paste and transfer from mortar to beaker with boiling distilled water. Make up to about 80 c. c. with the same. Stir for 8 minutes and pour contents again onto the sand filter. After the extract is through return the coagulum to the mortar and grind a second time, transferring to the beaker as before with boiling distilled water. Repeat this process of 8-minute extractions of the coagulum in hot water and filtration as above directed, without further grinding, until the filtrates measure about 750 c. c. each. Wash out each beaker twice with 8-10 c. c. hot distilled water, completing the transfer of the coagulum and extract to the sand. Wash the coagulum on the sand twice with boiling water from a wash bottle. At all times allow the filter to drain well between additions of extract or wash water. Combine the six filtrates of about 800 c. c. each, washing out the containers of each twice with distilled water. Make the extract up to 5000 c. c. and mix.

Still a different method of manipulation, with the aid of ammonium sulphate, was necessary for the extraction of brain. The details are as specified below:

DIRECTIONS FOR THE PREPARATION OF HOT WATER-AMMONIUM SULPHATE EXTRACT OF BRAIN

Weigh out about 10 grams of brain into each of ten 250 c. c. beakers. To each beaker add a few c. c. of distilled water and work up the brain and water with a glass rod. Make up to about 100 c. c. with boiling water. Place over a flame and gradually bring to boiling, with constant stirring. After boiling has begun add to each beaker 20 c. c. of 20 percent ammonium sulphate solution. Boil for about 10 minutes. Allow to settle for a moment and decant liquid onto sand on linen. In case the extracts do not filter readily, carefully push the coagulum to one side or return to the beakers. Add to the beakers containing the coagulum 50 c. c. of 0.1 percent ammonium sulphate solution; stir for one minute and decant the liquid onto the filter. Repeat this process of one-minute extractions of the coagulum in 0.1 percent ammonium sulphate solution, and filtration as above directed, until the filtrates measure about 450 c. c. Wash out each beaker twice with 8-10 c. c. of hot 0.1 percent ammonium sulphate solution, completing the transfer of the coagulum and extract to the sand.

Wash the coagulum twice with the above wash solution from a wash bottle. At all times allow the filter to drain well between additions of extract or wash solution. Combine the 10 filtrates, washing out the container of each of the filtrates twice with 5-8 c. c. of distilled water. Make the extract up to 5000 c. c. and mix.

The three methods compared, for the precipitation of inorganic phosphorus in the extracts prepared as above, and for the final estimation of the phosphorus, were as specified below:

EMMETT AND GRINDLEY NEUTRAL AMMONIUM MOLYBDATE METHOD FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN WATER EXTRACTS OF FLESH

Measure out the number and volumes of extracts indicated in the schedule on p. 24. Evaporate, with frequent stirring, on the water or steam bath to approximately 20 to 25 c. c. While hot, filter into 300 c. c. beakers, using doubled 11 cm. No. 589 (Blue Ribbon brand) S. and S. papers. Wash beakers, precipitates, and filters thoroughly with hot water. The volume of the resulting filtrate and washings should be about 125 c. c. Add 10 grams of ammonium nitrate and heat upon the water bath to 60° C. Then add 10 c. c. of nitric acid (sp. gr. 1.20); stir, and add 125 c. c. of clear ammonium molybdate solution. (Neutral ammonium molybdate is prepared by adding ammonia to the ordinary molybdate solution, using litmus paper as an indicator. This work should be done very carefully, and both red and blue litmus paper used). Reheat, bringing temperature to 60° C. Keep at this temperature for 15 minutes. Stir vigorously every few minutes during this time. Remove from the bath and allow the solutions to stand 2 hours in a warm place. Decant the clear supernatant liquid through doubled 11 cm. No. 589 (Blue Ribbon brand) S. and S. filters. Transfer the remaining liquid and precipitate to the filters, using a 10 percent ammonium nitrate solution. Wash precipitates and beaker four or five times with small volumes of the ammonium nitrate solution. Dissolve the yellow precipitate upon the filter, and that in the precipitating beaker, with dilute ammonium hydroxid (2.5 percent) and hot water, collecting the filtrate in a 250 c. c. beaker. Wash thoroughly; neutralize the solution with nitric acid (1.20 sp. gr.), and make up to approximately 150 c. c. Add 5 grams of ammonium nitrate; heat upon the water bath to 60° C. and then carefully add, while stirring, 5 c. c. of concentrated nitric acid and 50 c. c. of clear acid molybdate solution. Digest at 60° C. for 15 minutes, stirring occasionally. From here on continue the determination of phosphorus as usual, weighing the phosphorus as magnesium pyrophosphate.

SIEGFRIED AND SINGEWALD METHOD, AS USED BY EMMETT AND GRINDLEY, FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN WATER EXTRACTS OF ANIMAL SUBSTANCES

Measure out the number and volumes of extracts specified in the schedule on p. 24. To each portion add 50 c. c. of a 10 percent barium chlorid solution and 10 c. c. of 10 percent ammonium hydroxid. Stir the solutions every 15 minutes for a period of one hour, allow to stand undisturbed for at least 12 hours, and then filter (decanting at first, as much as possible) through

double quantitative filters. Wash the beakers, precipitates and filters repeatedly, with small quantities of wash water containing 10 c. c. of the barium chlorid solution and 10 c. c. of the dilute ammonium hydroxid per liter. Place the upper filters containing the precipitates in the beakers in which the precipitation occurred, and digest at room temperature with 35 c. c. of dilute nitric acid (sp. gr. 1.20) with frequent stirring. Filter the acid solution through the second filter, which was not removed from the funnel, and wash the beakers and filters thoroughly with hot water. Neutralize the filtrates with ammonia, slightly acidify with nitric acid; add 10 grams of ammonium nitrate, dilute to about 125 c. c. and heat upon the water-bath to 60° C. Add 100 c. c. of acid ammonium molybdate and continue the phosphorus determination from here on as usual.

MAGNESIA MIXTURE METHOD OF FORBES AND ASSOCIATES FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN EXTRACTS OF ANIMAL TISSUES

Measure out the number and volumes of extracts specified in the schedule on p. 24. Add 10 c. c. magnesia mixture, stirring freely; allow to stand 15 minutes, and add 25 c. c. of ammonia, sp. gr. 0.90; 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 at 60° for two hours. Then continue in the usual way for the gravimetric estimation of phosphorus as the pyrophosphate.

The numerical results of this comparative study of methods are reported in the following table, these data being discussed, together with those from the work of 1913, on pages 33-34.

TABLE XI: COMPARISON OF METHODS OF ESTIMATION OF INORGANIC PHOSPHORUS IN ANIMAL PRODUCTS

Sample	Method, and sample number		Volume of extract used	Phosphorus added (magnesium pyrophosphate)	Magnesium pyrophosphate obtained	Inorganic phosphorus	Added phosphorus recovered	
							Magnesium pyrophosphate	Percent
			c. c.	Grams	Grams	Percent	Grams	
Muscle	Neutral Molybdate Method (Emmett and Grindley)	A_1	500		0.0824	0.5018		
		A_2	500		0 0814	0.4956		
		A_3	250	0.0419	0.0814		0.0405	
		A_4	250	0.0419	0.0811		0.0402	
	Average					0.4987	0.0403	96.2
Muscle	Barium Chlorid Method (Siegfried and Singewald)	B_1	500		0.0836	0.5090		
		B_2	500		0.0826	0.5029		
		B_3	250	0.0419	0.0850		0.0435	
		B_4	250	0.0419	0.0848		0.0433	
	Average					0.5059	0.0434	103.6
Muscle	Magnesia Mixture Method (Forbes and Associates)	C_1	500		0.0792	0.4822		
		C_2	500		0.0794	0.4834		
		C_3	250	0.0419	0.0798		0.0402	
		C_4	250	0.0419	0.0806		0 0410	
	Average					0.4828	0.0406	96.9
Blood	Neutral Molybdate Method (Emmett and Grindley)	A_1	500		0.0380	0.0281		
		A_2	500		0.0394	0.0292		
		A_3	250	0.0419	0.0568		0.0375	
		A_4	250	0.0419	0.0584		0.0391	
	Average					0.0286	0.0383	91.4
Blood	Barium Chlorid Method (Siegfried and Singewald)	B_1	500		0.0088	0.0065		
		B_2	500		0.0094	0.0070		
		B_3	250	0.0419	0.0166		0.0121	
		B_4	250	0.0419	0.0164		0.0119	
	Average					0.0067	0.0120	28.6
Blood	Magnesia Mixture Method (Forbes and Associates)	C_1	500		0.0210	0.0156		
		C_2	500		0.0212	0.0157		
		C_3	250	0.0419	0.0514		0.0409	
		C_4	250	0.0419	0.0518		0.0413	
	Average					0 0156	0.0411	98.1
Brain	Neutral Molybdate Method (Emmett and Grindley)	A_1	500		0.0280	0 0776		
		A_2	500		0.0327	0.0907		
		A_3	250	0.0419	0.0540		0.0237	
		A_4	250	0.0419	0.0533		0.0230	
	Average					0.0841	0.0233	55.6
Brain	Barium Chlorid Method (Siegfried and Singewald)	B_1	500		0.0034	0.0094		
		B_2	500		0.0038	0.0105		
		B_3	250	0.0419	0.0158		0.0140	
		B_4	250	0.0419	0.0170		0.0152	
	Average					0.0099	0.0146	34.6
Brain	Magnesia Mixture Method (Forbes and Associates)	C_1	500			0.0599		
		C_2	500			0.0582		
		C_3	250	0.0419	0.0216		0.0392	
		C_4	250	0.0419	0.0210		0.0401	
					0.0498			
	Average				0.0507	0.0590	0.0396	94.5

Notes: Weights of samples used: muscle (vacuum dried sample)—45.7822 grams; blood (fresh pig's blood)—376.2846 grams; brain (fresh calf brain)—100.5238 grams; extracts made up to 5000 c. c.

THE WORK OF 1913

The work of 1913 consisted of a further comparison of the methods of Emmett and Grindley, and of Forbes and associates, with muscle, blood and brain, the consideration of the method of Siegfried and Singewald being discontinued. The following schedule indicates the estimations made.

1. Muscle 2. Blood 3. Brain	A Extract of samples as weighed	A_1	Neutral molybdate precipitation
		A_2	" " "
		A_3	" " "
		A_4	Magnesia mixture precipitation
		A_5	" " "
		A_6	" " "
	B Extract of samples as weighed plus 25 c. c. phosphate solution	B_1	Neutral molybdate precipitation
		B_2	" " "
		B_3	" " "
		B_4	Magnesia mixture precipitation
		B_5	" " "
		B_6	" " "

Phosphorus was also determined by both methods of precipitation in the phosphate solution used; and blank determinations were made, in triplicate, on the reagents.

DIRECTIONS FOR THE PREPARATION OF COLD WATER EXTRACT OF MUSCLE

A Weigh out 10-12 grams of fresh muscle and divide as nearly equally as possible between two small beakers. Moisten the samples with a few c. c. of distilled water, and break up lumps with a glass rod. Add 50 c. c. of water to each beaker and stir contents for 15 minutes. Allow insoluble residue to settle for 3-5 minutes; then decant the liquid from each beaker through filters into beakers; allow to drain, and add 25 c. c. of water. Stir for 7-8 minutes, and after allowing to settle, decant onto the same filter. Continue this treatment, using each time 25 c. c. of water, until the filtrates measure about 230 c. c. each. Allow the filters to drain completely between extractions. Whenever the major portion of the residue has become mechanically transferred to the filter return it to the beaker, using care not to break the filter paper. After the last extraction throw the entire contents of each beaker onto the filter, and, when drained, wash twice with small quantities of distilled water. Combine the two extracts, and use for the precipitation of the phosphates under **A**.

B Weigh out same quantity of flesh as specified above, and divide as nearly equally as possible between two small beakers; work up with a few c. c. of distilled water; add 25 c. c. of aqueous solution of disodium phosphate, equivalent to about 40 mg. magnesium pyrophosphate, dividing as nearly equally as possible between the two beakers, and proceed as directed under **A**. The extract thus obtained is ready for precipitation as under **B**.

METHOD FOR THE PREPARATION OF HOT WATER-AMMONIUM SULPHATE EXTRACT OF BLOOD

A Weigh out 30-35 grams of fresh blood, or the equivalent of oxalated blood, into a 400 c. c. beaker. Add a few c. c. of distilled water, and work up the blood and water with a glass rod. Make up to about 150 c. c. with boiling distilled water. Place over a flame, and gradually bring to boiling, with constant stirring. When boiling begins add 20 c. c. of 20 percent ammonium sulphate solution. Boil, with constant stirring, for about ten minutes. Decant onto a filter of sand on linen, receiving the filtrate in an 800 c. c. beaker. When the liquid is through, lift the coagulum from the sand, and transfer it to a mortar. Grind to a smooth paste and transfer from mortar to beaker with boiling distilled water. Make up to about 50 c. c. with the same; stir for 8 minutes and pour contents again onto the sand filter. After the extract is through, return the coagulum to the mortar, and grind a second time, transferring to the beaker as before with boiling distilled water. Repeat this process of 8-minute extractions of the coagulum in hot water, and filtration as above directed, without further grinding, until the filtrate measures about 450 c. c. Wash out each beaker twice with 8-10 c. c. of hot water, completing the transfer of the coagulum and extract to the sand. Wash the coagulum on the sand twice with boiling water from a wash bottle. At all times allow the filter to drain well between additions of extract or wash water. This extract of about 500 c. c. is ready for precipitation as under A.

B Weigh out same quantity of blood as specified above. Work up with a few c. c. of distilled water; add 25 c. c. of an aqueous solution of disodium phosphate equivalent to about 40 mg. magnesium pyrophosphate, and proceed as directed under A. The extract thus obtained is ready for precipitation under B.

METHOD FOR THE PREPARATION OF HOT WATER-AMMONIUM SULPHATE EXTRACT OF BRAIN

A Weigh out about ten grams of brain into a 250 c. c. beaker. Add a few c. c. of distilled water, and work up the brain and water with a glass rod. Make up to about 100 c. c. with boiling water; place over a flame, and gradually bring to boiling, with constant stirring. After boiling has begun add 20 c. c. of 20 percent ammonium sulphate solution; boil gently for about ten minutes; allow to settle for a moment, and decant liquid slowly onto a filter of sand on linen,* receiving the extract in an 800 c. c. beaker. Add to the beaker containing the coagulum 50 c. c. of 0.1 percent ammonium sulphate solution. Stir for one minute, keeping over flame and at the boiling point; decant the liquid onto the filter. Repeat this process of one-minute extractions of the coagulum in 0.1 percent ammonium sulphate solution, and filtration as above directed, until the filtrate measures about 450 c. c. Wash out the beaker twice with 8-10 c. c. of hot 0.1 percent ammonium sulphate solution, completing the transfer of the coagulum and extract to the sand. Wash the coagulum twice with the above wash solution from a wash bottle. At all times allow the filter to drain well between additions of extract or wash solution. This extract of about 500 c. c. is ready for precipitation under A.

*It is desirable to prevent the extract or coagulum from coming in contact with the linen before passing through the sand. To this end pour extract slowly onto center of sand or into a cup-shaped depression.

B Weigh out same quantity of brain as specified above; work up with a few c. c. of distilled water; add 25 c. c. of an aqueous solution of disodium phosphate equivalent to about 40 mg. of magnesium pyrophosphate, and proceed as directed under A. The extract thus obtained is ready for precipitation under B.

NEUTRAL AMMONIUM MOLYBDATE METHOD OF EMMETT AND GRINDLEY FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN WATER EXTRACTS OF FLESH

Treat 3 of the extracts prepared according to the directions on the preceding pages under A, and 3 of those prepared according to B as follows: Evaporate, with frequent stirring, on the water or steam bath, to approximately 20-25 c. c. While hot, filter into 300 c. c. beakers, using doubled 11 cm. No. 589 (Blue Ribbon brand) S. and S. papers. Wash beakers, precipitates, and filters thoroughly with hot water. The volume of the resulting filtrate and washings should be about 125 c. c. Add 10 grams of ammonium nitrate and heat upon the water bath to 60° C. Then add 10 c. c. of nitric acid (sp. gr. 1.20); stir, and add 125 c. c. of clear neutral molybdic solution. (Neutral ammonium molybdate is prepared by adding ammonia to the ordinary molybdic solution, using litmus paper as an indicator. This work should be done very carefully, and both red and blue litmus paper used). Reheat, bringing temperature to 60° C. Keep at this temperature for 15 minutes. Stir vigorously every few minutes during this time. Remove from the bath and allow the solutions to stand 2 hours in a warm place. Decant the clear supernatant liquid through doubled 11 cm. No. 589 (Blue Ribbon brand) S. and S. filters. Transfer the remaining liquid and precipitate to the filters, using a 10 percent ammonium nitrate solution. Wash precipitates and beakers four or five times with small volumes of the ammonium nitrate solution. Dissolve the yellow precipitate upon the filter and that in the precipitating beaker with dilute ammonia (2.5 percent) and hot water, collecting the filtrate in a 250 c. c. beaker. Wash thoroughly. Neutralize the solution with nitric acid (1.20 sp. gr.), and make up to approximately 150 c. c. Add 5 grams of ammonium nitrate; heat upon the water bath to 60° C. and then carefully add, while stirring, 5 c. c. of concentrated nitric acid and 50 c. c. of clear acid molybdic solution. Digest at 60° C. for 15 minutes, stirring occasionally. From this point continue the determination of phosphorus as usual, weighing the phosphorus as magnesium pyrophosphate.

MAGNESIA MIXTURE METHOD OF FORBES AND ASSOCIATES FOR THE DETERMINATION OF INORGANIC PHOSPHORUS IN EXTRACTS OF ANIMAL TISSUES

Treat three of the extracts prepared according to the directions on the preceding pages under A, and three of those prepared according to B as follows:

Add 10 c. c. magnesia mixture, stirring freely. Allow to stand 15 minutes; add 25 c. c. ammonia, sp. gr. 0.90; 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 and that remaining in the beaker in which the precipitation was made with dilute nitric acid (1:1) and hot water, receiving the solution in 400 c. c. beakers. Neutralize the nitric acid with ammonia; make slightly acid with nitric acid; add 5 grams ammonium nitrate, and precipitate in the usual way with molybdate solution. Continue in the usual way for the gravimetric estimation of phosphorus as the pyrophosphate.

The numerical results of the work of 1913 are reported in the following group of tables, these data being discussed, together with those from the work of 1912, on pages 33 and 34.

TABLE XII: COMPARISON OF METHODS OF ESTIMATION OF INORGANIC PHOSPHORUS IN ANIMAL PRODUCTS

Sample	Treatment, and sample number	Weight of sample	Magnesium pyrophosphate	Inorganic phosphorus	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered (magnesium pyrophosphate)	Added phosphorus recovered	Blank estimations on reagents
		Grams	Grams	Percent	Grams	Grams	Percent	Grams
Muscle	Neutral Molybdate A_1	13.5485	0.0276	0.0568				
	Neutral Molybdate A_2	11.5760	0.0240	0.0578				
	Neutral Molybdate A_3	13.3025	0.0259	0.0543				
	Average			0.0563				
	Same plus phosphate B_1	11.9395	0.0648			0.0407		
	Same plus phosphate B_2	11.5370	0.0636			0.0403		
	Same plus phosphate B_3	12.6880	0.0654			0.0398		
	Average				0.0417	0.0403	96.6	
	Magnesia Mixture A_4	14.5075	0.0303	0.0582				
	Magnesia Mixture A_5	10.0850	0.0219	0.0605				
	Magnesia Mixture A_6	11.4130	0.0229	0.0559				
	Average			0.0582				
	Same plus phosphate B_4	11.7065	0.0617			0.0373		
	Same plus phosphate B_5	11.3530	0.0606			0.0369		
	Same plus phosphate B_6	12.1045	0.0621			0.0368		
	Average				0.0417	0.0370	88.7	
Blood	Neutral Molybdate A_1	29.1950	0.0324[1]	0.0309				0.0020
	Neutral Molybdate A_2	35.0955	0.0359[1]	0.0285				0.0016
	Neutral Molybdate A_3	28.4170	0.0257[1]	0.0252				0.0018
	Average			0.0282				0.0018
	Same plus phosphate B_1	36.5820	0.0718[1]			0.0348		
	Same plus phosphate B_2	27.3368	0.0662[1]			0.0385		
	Same plus phosphate B_3	32.8275	0.0699[1]			0.0367		
	Average				0.0417	0.0367	88.0	
	Magnesia Mixture A_4	30.9601	0.0157[1]	0.0141				0.0030
	Magnesia Mixture A_5	32.4669	0.0146[1]	0.0125				0.0037
	Magnesia Mixture A_6	29.2820	0.0151[1]	0.0144				0.0038
	Average			0.0137				0.0035
	Same plus phosphate B_4	35.6872	0.0545[1]			0.0370		
	Same plus phosphate B_5	35.2757	0.0565[1]			0.0392		
	Same plus phosphate B_6	36.1812	0.0532[1]			0.0354		
	Average				0.0417	0.0372	89.2	
Brain	Magnesia Mixture A_4	10.1650	0.0246[1]	0.0675				0.0010
	Magnesia Mixture A_5	10.3040	0.0251[1]	0.0679				0.0010
	Magnesia Mixture A_6	10.9550	0.0267[1]	0.0679				0.0006
	Average			0.0678				0.0009
	Same plus phosphate B_4	10.5095	0.0512[1]			0.0257		
	Same plus phosphate B_5	9.2880	0.0483[1]			0.0257		
	Same plus phosphate B_6	10.5465	0.0514[1]			0.0258		
	Average				0.0417	0.0257	96.6	
	Magnesia Mixture $2A_4$	7.7011	0.0176[1]	0.0662				0.0008
	Magnesia Mixture $2A_5$	9.2368	0.0209[1]	0.0652				0.0006
	Average							0.0007
	Same plus phosphate $2B_4$	10.7215	0.0507[1]			0.0263		
	Same plus phosphate $2B_5$	9.2277	0.0474[1]			0.0264		
	Same plus phosphate $2B_6$	10.5182	0.0502[1]			0.0263		
	Average				0.0267	0.0263	98.9	

[1]Blanks deducted.

TABLE XIII: TEST OF EFFECTS OF HEAT AND AMMONIUM SULPHATE IN THE ESTIMATION OF INORGANIC PHOSPHORUS IN BLOOD

Blood of Pig Used; Extracted with 3.33 Percent Ammonium Sulphate Solution; Each Extract About 500 c. c. in Volume

No.	Weight of sample Grams	Treatment	Magnesium pyrophosphate Grams	Inorganic phosphorus Percent
1	33.0990	Extract evaporated; boiled; filtered	0.0217	0.0183
2	34.4230	" " " "	0.0222	0.0180
Ave.				0.0181
3	33.3960	Extract evaporated; filtered	0.0216	0.0180
4	31.3340	" " "	0.0218	0.0194
Ave.				0.0187
5	33.7026	Extract precipitated directly	0.0108	0.0089
6	33.2960	" " "	0.0108	0.0090
Ave.				0.0089

CONCLUSIONS FROM WORK OF 1912 AND 1913 ON INORGANIC PHOSPHORUS ESTIMATION IN ANIMAL SUBSTANCES

The neutral molybdate method of Emmett and Grindley, the barium chlorid method of Siegfried and Singewald, (provided a sufficient excess of barium chlorid is used) and the magnesia mixture method of Forbes and associates all gave satisfactory results, which were practically identical, on vacuum-dried muscle.

The barium chlorid method was found inapplicable in the presence of ammonium sulphate, and hence was not useful on extracts of blood and brain prepared with the aid of this reagent.

The neutral molybdate method gave results on blood which were apparently too high, a decomposition of organic phosphorus seeming to result from the heat used during the concentration of the extract. Some difficulty was experienced in the recovery of inorganic phosphorus added to blood. The recovery was slightly greater with the magnesia mixture than with the neutral molybdate method.

As compared with the magnesia mixture method the neutral molybdate method gave, on extract of brain, prepared with the aid of ammonium sulphate, higher results for inorganic phosphorus, with lower recovery of added phosphates, (Table XI, p. 28). The difficulties of filtration are greater with the neutral molybdate than with the magnesia mixture method.

Readily filterable extracts of brain may be prepared by the use of 3.33 percent ammonium sulphate solution in place of 0.1 percent ammonium sulphate in each place where the latter is specified in the

published magnesia mixture method (see p. 25); and the hindering effect of the added amount of ammonium sulphate on the precipitation of phosphorus by magnesia mixture may be overcome by the substitution of 50 c. c. of magnesia mixture for the 10 c. c. as specified, and allowing the precipitate to stand 3 days before filtering. With these modifications the magnesia mixture method is readily workable on brain; concordant results are obtained, and added phosphate is all recovered. The work on brain reported in Table XII, p. 32, was done by this modified method.

In the trial reported as Samples A4, 5 and 6, and B4, 5 and 6, Table XII, p. 32, the phosphate was all recovered but 0.0009 gm. magnesium pyrophosphate. In the trial reported as Samples 2A4, 5 and 6, and 2B4, 5 and 6 the loss was 0.0004 gm. magnesium pyrophosphate. This set was precipitated in a refrigerated room.

The neutral molybdate method can not be used satisfactorily with extracts prepared as suggested above, with the aid of ammonium sulphate, and it is not practicable to prepare cold water extracts of brain as in the neutral molybdate method, which has been used principally with flesh.

The test of the influence of heat on inorganic phosphorus estimation in blood, as set forth in Table XIII, p. 33, shows that the high results on blood obtained by the neutral molybdate method must be due to the cleavage of organic phosphorus by the heat used in the evaporation of the extract. While the duration of heating used in this method is much greater than in the preparation of hot water-ammonium sulphate extracts of tissues as in the magnesia mixture method, this test raises the question of the existence and magnitude of such cleavage. This point has received further consideration.

The recovery of added phosphates from the extract of muscle by the magnesia mixture method is usually practically complete (see Table XI, p. 28). In the last analyses, however, (Table XII, p. 32), the recovery of added phosphate was appreciably incomplete, though the determination without the added phosphate was higher than by the neutral molybdate method, where the recovery of added phosphate was practically complete. The low recovery of added phosphate from both blood and muscle, as reported in this table, suggests that the conditions were not perfect for the precipitation of this amount of magnesium ammonium phosphate. These imperfections were eliminated in the later work. With brain the recovery of added phosphate was complete, since special measures (added amounts of magnesia mixture, and increased time allowed for precipitation) were taken to insure complete precipitation.

THE WORK OF 1914

In the previous work on inorganic phosphorus estimation on animal tissues three methods have been compared, namely, the Neutral Molybdate Method of Emmett and Grindley, the modified Barium Chlorid Method of Siegfried and Singewald, and the Magnesia Mixture Method of Forbes and associates. Satisfactory comparisons of these methods have been made on muscle, the results, with this tissue, being practically identical; and certain important limitations to the applicability of the two methods first mentioned, to tissues other than muscle, have been established. It was now desired to test, by the method of recovery of added phosphatés, the accuracy of the Magnesia Mixture Method, in its latest form, with animal tissues of diverse character, and also to study individually, a number of details of this method, namely (1) the influence of heat, as specified, (2) the method of filtration, (3) the completeness of extraction, (4) the influence of ammonium sulphate, as specified, (5) the effects of varying amounts of ammonium sulphate and (6) the effects of different methods of use of ammonium sulphate.

In the test of the accuracy of the Magnesia Mixture Method, determinations were made, in triplicate, on blood, brain, flesh and liver, with and without the addition of known amounts of inorganic phosphate. The detailed directions followed in this test are on pages 35-37, and the results are set forth in Tables XIV and XV on pages 41 and 42. The results of the further analytical proving of the details of this method were all made on blood. The data are to be found in Tables XVI-XXI on pages 43 to 48.

WATER-SOLUBLE INORGANIC PHOSPHORUS IN ANIMAL TISSUES

From samples of finely divided tissue prepare extracts as specified under **A, B, C** or **D**, and determine inorganic phosphorus as specified under **E**.

A. PREPARATION OF COLD WATER EXTRACT OF FLESH

Weigh out 10-12 grams of fresh muscle, and divide as nearly equally as possible between two small beakers. Moisten the samples with a few c. c. of distilled water, and break up lumps with a glass rod. Add 50 c. c. of water to each beaker and stir contents for 15 minutes. Allow insoluble residue to settle for 3-5 minutes; then decant the liquid from each beaker through filters into beakers; and add 25 c. c. of distilled water to the residue in the beakers. Stir for 7-8 minutes, and after allowing to settle, decant onto the same filter. Continue this treatment, using each time 25 c. c. of water, until the filtrates measure about 230 c. c. each. Allow the filters to drain completely between extractions. Whenever the major portion of the residue has become mechanically transferred to the filter return it to the beaker, using care not to break

the filter paper. After the last extraction throw the entire contents of each beaker onto the filter, and, when drained, wash twice with small quantities of distilled water. Combine the two extracts, and determine inorganic phosphorus as under E.

B. PREPARATION OF HOT WATER-AMMONIUM SULPHATE EXTRACT OF BLOOD

Weigh out 30-35 grams of fresh blood, (entire portions as caught from the animal) into a porcelain mortar. Grind and transfer to a 400 c. c. beaker with hot distilled water. Make up to about 150 c. c. with boiling distilled water. Place over a flame, and gradually bring to boiling, with constant stirring. When boiling begins add 20 c. c. of 20 percent ammonium sulphate solution. Boil, with constant stirring, for about ten minutes. Decant onto an 18 cm. filter paper, receiving the filtrate in an 800 c. c. beaker. When the liquid is through, lift the coagulum from the paper, being very careful not to break the paper filter, and transfer it, along with that remaining in the beaker, to the mortar. Grind to a smooth paste and transfer from mortar to beaker with boiling 3.33 percent ammonium sulphate solution. Make up to about 50 c. c. with the same, stir for 8 minutes, and pour contents again onto the filter paper. After the extract is through, return the coagulum to the mortar and grind a second time, transferring to the beaker as before with boiling 3.33 percent ammonium sulphate solution. Repeat this process of 8-minute extractions of the coagulum in hot 3.33 percent ammonium sulphate solution, and filtration as above directed, without further grinding, until the filtrate measures about 450 c. c. Wash out each beaker twice with 8-10 c. c. of hot 3.33 percent ammonium sulphate solution, completing the transfer of the coagulum and extract to the filter paper. Wash the coagulum on the paper twice with boiling 3.33 percent ammonium sulphate solution from a wash bottle. At all times allow the filter to drain well between additions of extract or wash solution. This extract of about 500 c. c. is ready for precipitation as described under E.

C. PREPARATION OF HOT WATER-AMMONIUM SULPHATE EXTRACT OF LIVER

Weigh by difference from closed weighing bottles 15-20 gram portions of finely ground liver into 400 c. c. beakers. Add a few c. c. of cold distilled water, and beat up with a stirring rod to separate the particles of tissue. Add enough boiling distilled water to make the volume about 150 c. c.; place over a flame and bring to boiling. Add 10 c. c. of 20 percent ammonium sulphate solution, 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. paper filters. Add 50 c. c. of boiling water and stir for 8 minutes, without further heating over a flame, and decant onto the filter again. Repeat this addition of 50 c. c. of hot distilled water, stirring, and decanting eight times, returning the coagulum to the beaker as soon as any considerable amount collects upon the filter. With the eighth portion of water throw the entire contents of the beaker onto the filter and wash twice with hot water from a wash bottle. At all times allow the filter to drain well between additions of extract or wash water. This extract of about 600 c. c. is now ready for precipitation as described under E.

D. PREPARATION OF HOT WATER-AMMONIUM SULPHATE EXTRACT OF BRAIN

Weigh out about ten grams of brain into a 250 c. c. beaker. Add a few cubic centimeters of distilled water, and work up the brain and water with a glass rod. Make up to about 100 c. c. with boiling water; place over a flame, and gradually bring to boiling, with constant stirring. While boiling vigorously (not before) add 20 c. c. of 20 percent ammonium sulphate solution; boil gently for about 10 minutes; allow to settle for a moment, and decant liquid slowly onto a filter of acid-washed glass maker's sand on linen, receiving the extract in an 800 c. c. beaker. Add to the beaker containing the coagulum 50 c. c. of a hot 3.33 percent ammonium sulphate solution. Stir for one minute, keeping over flame and at the boiling point; decant the liquid onto the filter. Repeat this process of one-minute extractions of the coagulum in hot 3.33 percent ammonium sulphate solution, and filtration as above directed, until the filtrate measures about 450 c. c. Wash out the beaker twice with 8-10 c. c. of hot 3.33 percent ammonium sulphate solution, completing the transfer of the coagulum and extract to the sand. Wash the coagulum twice with the above wash solution from a wash bottle. At all times allow the filter to drain well between additions of extract or wash solution.

This extract of about 500 c. c. is ready for precipitation as directed under **E.**

PRECAUTIONS

In making extracts of brain it is desirable that the analyst give careful attention to the handling of the sample. The coagulum is very soft. It should be stirred only enough to keep it in motion. If roughly handled in returning from the sand filter to the beaker it becomes too much broken up and holds onto a great deal of liquid. To prevent the extract or the coagulum from coming into contact with the linen before passing through the sand pour the extract slowly into a slight depression in the center of the sand, or, better yet, onto a thin film of absorbent cotton 1½ inches in diameter, laid over a depression in the sand. The coagulum remains on the cotton, and its return to the beaker is thereby facilitated. If the cotton is not broken up by needless stirring it can be taken out of the beaker with a glass rod and returned to the sand each time a partial extract is to be filtered. Care is necessary to prevent loss through bumping, on account of sand in the beakers during the last extractions. Each partial extract should be boiling hot at the time filtration begins.

E. MAGNESIA MIXTURE METHOD FOR THE DETERMINATION OF WATER-SOLUBLE INORGANIC PHOSPHORUS IN EXTRACTS OF ANIMAL TISSUES

To the extracts prepared according to the preceding directions add 50 c. c. magnesia mixture, stirring freely. Allow to stand 15 minutes; add 25 c. c. ammonium hydroxid, sp. gr. 0.90; cover, and allow to stand three days. Filter, and wash the precipitate with 2.5 percent ammonia water. Dissolve the precipitate on the filter paper and that remaining in the beaker in which the precipitation was made with dilute nitric acid (1:1) and hot water, receiving the solution in 400 c. c. beakers. Neutralize the nitric acid with ammonium hydroxid; make slightly acid with nitric acid. Add 5 grams ammonium nitrate, and precipitate in the usual way with molybdate solution. Continue in the usual way for the gravimetric estimation of phosphorus as the pyrophosphate.

DISCUSSION OF RESULTS OF WORK OF 1914

The data in Tables XIV and XV, pages 41 and 42, show that as tested by the recovery of added phosphates, the Magnesia Mixture Method, in the form stated on pages 35-37, gives results apparently characterized by a high degree of accuracy. The recovery of added phosphates was 96 percent efficient with liver, 97 percent with flesh, 99 percent with brain and 100 percent with blood.

In consideration of the close agreement of triplicates, the high percentage of recovery of added phosphates, and the amounts of coagulum from which the phosphates were recovered, these results are considered a satisfactory demonstration of the reliability of the method.

In the further scrutiny and analysis of the method, however, it was deemed advisable to test individually certain of its details. Blood was selected for this work, since the ready decomposition of its phosphocarnic acid was considered likely to reveal possible improprieties of procedure. The results of these studies on blood are set forth in Tables XVI to XXI on pages 43-48.

Table XVI, page 43, gives results from a study of the effects of heat and ammonium sulphate in this estimation. A cold-water extract of steer blood was used. This extract was obtained through the use of a centrifuge.

In sets A and C the phosphates were precipitated direct, with magnesia mixture, with and without ammonium sulphate added (in the cold) before precipitation. The results were practically identical, and show that, in the cold, ammonium sulphate does not affect inorganic phosphate determination in blood.

Sets B and D were boiled containing different amounts of ammonium sulphate. The boiling and precipitation of inorganic phosphates in 1.25 percent solution of ammonium sulphate (20 c. c. of 20 percent ammonium sulphate, as specified) gave weights of magnesium pyrophosphate half of a milligram greater than those obtained from boiling and precipitation in a 3.33 percent solution of ammonium sulphate.

These results were, in both cases, appreciably lower than those obtained from A and C, with and without ammonium sulphate, but without boiling. These results show, therefore, that ammonium sulphate, in the cold, is without influence on the inorganic phosphorus estimation, but that boiling and ammonium sulphate together, as used, not only do not split off inorganic from organic phosphorus compounds, but, as shown by the lower results obtained, cause a coagulation and precipitation of organic phosphorus in the

water extract which when not so precipitated remains in solution until precipitated by the magnesia mixture, after which it may be hydrolyzed by nitric acid in the later steps of the phosphorus estimation.

Considering the possibility that the lower results above noted as obtained in the presence of ammonium sulphate might be due to the mechanical inclusion of phosphates in the coagulum, another set of determinations was made, as reported in Table XVII, p. 44. The grinding of the coagulum with sand, to allow of more complete extraction and washing, gave exactly the same result as did the washing of the coagulum by decantation, in the usual way. Therefore, the extraction, as usually carried out, is complete, and coagulation by boiling and ammonium sulphate does not lock up inorganic phosphate by mechanical inclusion. Further, as in the previous set of analyses, lower results were obtained with boiling and ammonium sulphate than with direct precipitation in the cold, though the recovery of added phosphates was perfect in both cases. This reinforces our previous observation as to the precipitation of organic phosphorus from cold-water extracts of blood, along with the inorganic phosphates. Thus, boiling and ammonium sulphate are needed to coagulate a certain water-soluble organic phosphorus fraction of blood in the estimation of inorganic phosphorus by the Magnesia Mixture Method.

The results in Table XVIII, p. 45, show that acid alcohol (0.2 percent nitric acid) will dissolve the organic phosphorus which is precipitated, along with the phosphates, by magnesia mixture alone, in cold-water extracts of blood; a separation of the organic from the inorganic phosphorus in this precipitate, by the use of this reagent, therefore, is not possible.

In Table XIX, p. 46, we have results from tests made to determine (1) whether hot water or ammonium sulphate should be used in the completion of the extraction of the coagulum from the boiling with ammonium sulphate, and (2) whether, in the extraction of blood, the partial extracts should be filtered through sand on linen or through filter paper.

Lower results (and, therefore, in the light of the previous evidence, more nearly correct results) were obtained when a 3.33 percent solution of ammonium sulphate, rather than hot water, was used in the completion of the extraction of the coagulum. The recovery of added phosphates was also higher under these circumstances. Filtration of the blood extracts through paper was found preferable to filtration through sand on linen.

Table XX, p. 47, reports a further test of the desirability of using ammonium sulphate in the completion of the extraction of the coagulum from the preliminary boiling with ammonium sulphate. As in the previous work the results obtained favored the use of the 3.33 percent solution, since this procedure led to lower results for inorganic phosphorus and more nearly perfect recovery of added phosphates.

Table XXI, p. 48, sets forth results from a comparison of the use of different amounts of ammonium sulphate in the coagulation and extraction of blood. No advantage could be demonstrated as due to the use of solutions of ammonium sulphate more concentrated than the 3.33 percent solution used in the preceding tests; that is, the use of 3.33 percent solutions gave lower, and apparently more nearly correct results than were obtained with a 1.25 percent solution, while further increase of the concentration of the ammonium sulphate solution did not lead to further decrease in inorganic phosphate.

CONCLUSIONS FROM WORK OF 1914* ON INORGANIC PHOSPHORUS ESTIMATION IN ANIMAL SUBSTANCES

(1) The Magnesia Mixture Method gives satisfactorily agreeing results on blood, brain, liver, and flesh, with a recovery of 96-100 percent of added phosphates.

(2) Neither ammonium sulphate, nor boiling and ammonium sulphate together, as used in the Magnesia Mixture Method, were found to cause a splitting off of inorganic from organic phosphorus in blood.

(3) The use of heat and ammonium sulphate, as in the Magnesia Mixture Method, gives lower results than are obtained without heat and ammonium sulphate, though the recovery of added phosphates is perfect; and evidence was obtained that these lower results were due not to inclusion of phosphates in the coagulum obtained by the use of heat and ammonium sulphate, but to the precipitation of water-soluble organic phosphorus compounds which, without the use of heat and ammonium sulphate, yield up their phosphorus as inorganic phosphate, under the influence of the nitric acid used in the subsequent steps of the inorganic phosphorus estimation.

(4) It was found advisable to wash the coagulum with 3.33 percent ammonium sulphate rather than with hot water. A more concentrated solution was shown not to be necessary.

(5) In the case of blood, the filtration of the extract through paper was found preferable to the filtration through sand on linen, which is necessary in the case of brain.

*For conclusions from the work of 1912 and 1913 see pages 33 and 34.

(6) The methods of determination of inorganic phosphorus in blood, brain, flesh and liver as outlined on pages 35 to 37, including the details of extraction as well as of actual estimation of phosphates, were provisionally adopted in 1914 as official methods of the Association of Official Agricultural Chemists.

For conclusions from the work on vegetable substances see page 21.

TABLE XIV: TEST OF MAGNESIA MIXTURE METHOD FOR INORGANIC PHOSPHORUS IN ANIMAL TISSUES BY RECOVERY OF ADDED PHOSPHATES

A=Without Phosphates B=With Added Phosphates

Sample and determination No.	Weight of sample	Magnesium pyrophosphate	Inorganic phosphorus	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered	
					Magnesium pyrophosphate	Percent
	Grams	Grams*	Percent	Grams	Grams	
Blood A_1	31.30	0.0069	0.00614			
A_2	30.00	0.0069	0.00641			
A_3	25.00	0.0051	0.00568			
Average			0.00607			
B_1	26.10	0.0558			0.0501	
B_2	28.20	0.0563			0.0501	
B_3	30.50	0.0572			0.0505	
Average				0.0497	0.0502	101.00
A_1	33.70	0.0064	0.00529			
A_2	33.60	0.0060	0.00505			
A_3	31.20	0.0060	0.00544			
Average			0.00526			
B_1	30.40	0.0550			0.0493	
B_2	32.20	0.0557			0.0496	
B_3	35.80	0.0568			0.0500	
Average				0.0496	0.0496	100.00

*All blanks deducted.

TABLE XV: TEST OF MAGNESIA MIXTURE METHOD FOR INORGANIC PHOSPHORUS IN ANIMAL TISSUES BY RECOVERY OF ADDED PHOSPHATES

A=Without Phosphates B=With Added Phosphates

Sample and determination No.	Weight of sample	Magnesium pyrophosphate	Inorganic phosphorus	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered	
					Magnesium pyrophosphate	Percent
	Grams	Grams*	Percent	Grams	Grams	
Brain A_1	8.5600	Lost				
A_2	7.7011	0.0176	0.0636			
A_3	9.2368	0.0209	0.0630			
Average			0.0633			
B_1	10.7215	0.0507			0.0263	
B_2	9.2277	0.0474			0.0264	
B_3	10.5182	0.0502			0.0263	
Average				0.0266	0.0263	98.87
Flesh A_1	13.4653	0.0272	0.0562			
A_2	10.3444	0.0207	0.0557			
A_3	11.1769	0.0223	0.0555			
Average			0.0558			
B_1	10.9638	0.0694			0.0474	
B_2	11.7942	0.0725			0.0488	
B_3	11.3154	0.0708			0.0481	
Average				0.0496	0.0481	96.97
Liver A_1	16.2155	0.0627	0.1077			
A_2	13.8000	0.0552	0.1144			
A_3	14.4309	0.0519	0.1002			
Average			0.1064			
B_1	14.9094	0.1045			0.0475	
B_2	14.9658	0.1054			0.0482	
B_3	16.2232	0.1094			0 0474	
Average				0.0496	0.0477	96.16

*All blanks deducted.

TABLE XVI: TEST OF EFFECTS OF BOILING AND VARYING AMOUNTS OF AMMONIUM SULPHATE IN THE ESTIMATION OF INORGANIC PHOSPHORUS IN STEER BLOOD BY THE MAGNESIA MIXTURE METHOD—COLD WATER EXTRACTS

Sample No.	Treatment	Volume extract c. c.	Magnesium pyrophosphate Gms.	Phosphorus Mgs.
A_1	Extract precipitated direct with magnesia mixture	300	0.0091	2.535
A_2		300	0.0087	2.424
A_3		300	0.0087	2.424
Average				2.461
B_1	Extract brought to boiling; ammonium sulphate added to make 1.25 percent solution; then boiled for ten minutes	300	0.0079	2.201
B_2		300	0.0078	2.173
B_3		300	0.0081	2.257
Average				2.210
C_1	Same as A, with ammonium sulphate to make 1.25 percent solution added before precipitation	300	0.0085	2.368
C_2		300	0.0085	2.368
C_3		300	0.0086	2.396
Average				2.374
D_1	Same as B, with ammonium sulphate added to make 3.33 percent solution	300	0.0075	2.090
D_2		300	0.0074	2.062
D_3		300	0.0073	2.034
Average				2.062

All of the above extracts and filtrates were precipitated by adding 50 c. c. of magnesia mixture to the cool solution, and then, after standing a short period, 25 c. c. of ammonia (sp. gr. .96).

TABLE XVII: TEST OF COMPLETENESS OF EXTRACTION AND EFFECTS OF BOILING AND AMMONIUM SULPHATE IN THE ESTIMATION OF INORGANIC PHOSPHORUS IN CALF BLOOD BY THE MAGNESIA MIXTURE METHOD— COLD WATER EXTRACTS

Sample No.	Treatment	Volume of extract	Magnesium pyrophosphate	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered	
					Magnesium pyrophosphate	Percent
		c. c.	Grams	Grams	Grams	
A_1	Extract precipitated direct with magnesia mixture	300	0.0107			
A_2		300	0.0105			
A_3		300	0.0103			
Average			0.0105			
A_4	Same as $A_{1, 2, 3}$ + 25 c. c. of phosphate solution	300	0.0619			
A_5		300	0.0618			
A_6		300	0.0620			
Average			0.0619	0.0517	0.0514	98.42
B_1	Extract brought to boiling; 20 c. c. of 20% ammonium sulphate added, and boiled for 10 minutes, filtered and washed by decantation	300	0.0083			
B_2		300	0.0086			
B_3		300	0.0091			
Average			0.0087			
B_4	Same as $B_{1, 2, 3}$ but coagulum ground with fine sand for more complete extraction and washing	300	0.0090			
B_5		300	0.0084			
B_6		300	0.0088			
Average			0.0087			
C_1	Same as $B_{1, 2, 3}$ + 25 c. c. phosphate solution	300	0.0603			
C_2		300	0 0601			
C_3		300	0.0604			
Average			0.0603	0.0517	0.0516	99.86
C_4	Same as $B_{4, 5, 6}$ + 25 c. c. phosphate solution	300	0.0601			
C_5		300	0.0609			
C_6		300	0.0602			
Average			0.0604	0.0517	0.0517	100.00

All of the above extracts and filtrates were precipitated by adding 50 c. c. of magnesia mixture to the cool solution, and then, after standing a short period, 25 c. c. of ammonia (sp. gr. .96).

TABLE XVIII: TEST OF EFFECTS OF BOILING AND AMMONIUM SULPHATE IN THE ESTIMATION OF INORGANIC PHOSPHORUS IN STEER BLOOD BY THE MAGNESIA MIXTURE METHOD—COLD WATER EXTRACTS

Sample No.	Treatment	Volume of extract	Magnesium pyrophosphate	Phosphorus
		c. c.	Grams	Mg.
A_1	Extract precipitated direct with magnesia mixture	200	0.0075	2.089
A_2		200	0.0070	1.950
A_3		200	0.0070	1.950
Average			0.0072	1.996
A_4	Extract precipitated as $A_{1,2,3}$ and precipitate dissolved in acid alcohol (.2% nitric acid) and phosphorus determined in aliquots of this solution	200	0.0076	2.117
A_5		200	0.0073	2.034
A_6		200	0.0065	1.811
Average			0.0071	1 987
B_1	Extract boiled for 20 minutes with 20 c. c. of 20% ammonium sulphate, filtered and precipitated direct	200	0.0062	1.727
B_2		200	0.0058	1.616
B_3		200	0.0096*	
Average			0.0060	1.671

*Precipitate fused during ignition; not included in average.

All of the above extracts and filtrates were precipitated by adding 50 c. c. of magnesia mixture to the cool solution, and then, after standing a short period, 25 c. c. of ammonia (sp. gr. .96).

TABLE XIX: TEST OF METHODS OF USE OF AMMONIUM SULPHATE AND OF METHODS OF FILTRATION IN THE ESTIMATION OF INORGANIC PHOSPHORUS IN STEER BLOOD BY THE MAGNESIA MIXTURE METHOD—HOT WATER-AMMONIUM SULPHATE EXTRACTS

Sample No.	Treatment	Weight of sample	Magnesium pyrophosphate	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered	
					Magnesium pyrophosphate	Percent
		Grams	Grams	Grams	Grams	
1	Sample extracted in usual way with 20 c. c. of 20% ammonium sulphate; filtered through sand on linen	34.2	0.0079			
2		26.6	0.0061			
3		30.7	0.0064			
Average		30.5	0.0068			
		1.0	0.000223			
4	Same as 1, 2, 3 + 25 c. c. phosphate solution	30.7	0.0583	0.0537	0.0515	
5		33.1	0.0582	0.0537	0.0508	
6		38.2	0.0590	0.0537	0.0505	
Average					0.0509	94.78
7	Sample extracted as usual; then subsequent extractions made with 3.33% hot ammonium sulphate; filtered through sand on linen	28.4	0.0040			
8		34.2	0.0060			
9		39.1	0.0069			
Average		33.9	0.0056			
		1.0	0.000165			
10	Same as 7, 8, 9+25 c. c. of phosphate solution	37.6	0.0590	0.0537	0.0528	
11		40.9	0.0590	0.0537	0.0522	
12		32.4	0.0576	0.0537	0.0522	
Average					0.0524	97.57
13	Sample extracted usual way, + 25 c. c. phosphate solution; filtered through paper instead of sand on linen (4, 5 and 6)	32.6	0.0583	0.0537	0.0510	
14		33.9	0.0596	0.0537	0.0520	
Average					0.0515	95.90
15	Sample extracted usual way + 25 c. c. phosphate solution; subsequent extraction with 3.33% ammonium sulphate; filtration through paper instead of sand on linen (10, 11, 12)	31.5	0.0600	0.0537	0.0548	
16		37.4	0.0603	0.0537	0.0541	
Average					0.0544	101.303

The above filtrates were precipitated by adding 50 c. c. of magnesia mixture to the cool solution, and then, after standing a short period, 25 c. c. of ammonia (sp. gr. .96).

TABLE XX: TEST OF METHODS OF USE OF AMMONIUM SULPHATE IN THE ESTIMATION OF INORGANIC PHOSPHORUS IN STEER BLOOD BY THE MAGNESIA MIXTURE METHOD—HOT WATER-AMMONIUM SULPHATE EXTRACTS

Sample No.	Treatment	Weight of sample	Magnesium pyrophosphate	Phosphorus added (magnesium pyrophosphate	Added phosphorus recovered	
					Magnesium pyrophosphate	Percent
		Grams	Grams	Grams	Grams	
A_1	Sample extracted with 20 c. c. of 20% ammonium sulphate as usual; all subsequent extractions made with hot water	32.0	0.0078			
A_2		32.8	0.0080			
A_3		34.6	0.0083			
Average		33.1	0.0080			
		1.0	0.00024			
A_4	Same as $A_{1, 2, 3}$+25 c. c. phosphate solution	33.5	0.0573			
A_5		34.1	0.0569			
A_6		26.3	0.0556			
Average		31.3	0.0566	0.0496	0.0491	98.99
B_1	Same as $A_{1, 2, 3}$ only all subsequent extractions made with 3.33% ammonium sulphate instead of water	33.7	0.0064			
B_2		33.6	0.0060			
B_3		31.2	0.0060			
Average		32.8	0.0061			
		1.0	0.00019			
B_4	Same as $B_{1, 2, 3}$+25 c. c. phosphate solution	30.4	0.0550			
B_5		32.2	0.0556			
B_6		35.8	0.0568			
Average		32.8	0.0558	0.0496	0.0497	100.20
Blank 1			0.0005			
2			0.0005			
3			0.0004			

*All blanks deducted.

All of the above filtrates were precipitated by adding 50 c. c. magnesia mixture to the cool solution, and then, after standing a short period, 25 c. c. of ammonia (sp. gr. .96).

TABLE XXI: TEST OF VARYING AMOUNTS OF AMMONIUM SULPHATE IN THE ESTIMATION OF INORGANIC PHOSPHORUS IN STEER BLOOD BY THE MAGNESIA MIXTURE METHOD—HOT WATER-AMMONIUM SULPHATE EXTRACTS

Sample No.	Treatment	Weight of sample	Magnesium pyrophosphate*	Phosphorus added (magnesium pyrophosphate)	Added phosphorus recovered	
					Magnesium pyrophosphate	Percent
		Grams	Grams	Grams	Grams	
A_1	Sample extracted with 3.33% ammonium sulphate throughout	31.3	0.0069			
A_2		30.0	0.0069			
A_3		25.0	0.0051			
Average		28.76	0.0063			
		1.0	·0.00022			
A_4	Same as $A_{1, 2, 3}$ + 25 c. c. phosphate solution	26.1	0.0558			
A_5		28.2	0.0563			
A_6		30.5	0.0572			
Average		28.3	0.0564	0.0497	0.0502	101.00
B_1	Sample extracted with 4% ammonium sulphate throughout	27.8	0.0071			
B_2		27.0	0.0069			
B_3		30.8	0.0074			
Average		28.5	0.0071			
		1.0	0.00025			
B_4	Same as $B_{1, 2, 3}$ + 25 c. c. phosphate solution	31.4	0.0584			
B_5		31.2	0.0579			
B_6		41.3	0.0576			
Average		34.6	0.0580	0.0497	0.0493	99.20
C_1	Sample extracted with 5% ammonium sulphate throughout	28.1	0.0065			
C_2		28.2	0.0070			
C_3		30.5	0.0067			
Average		28.9	0.0067			
		1.0	0.00023			
C_4	Same as $C_{1, 2, 3}$ + 25 c. c. phosphate solution	30.5	0.0582			
C_5		26.9	0.0575			
C_6		45.5	0.0589			
Average		34.3	0.0582	0.0497	0.0503	101.21
C_7	500 c. c. of 5% ammonium sulphate + 25 c. c. phosphate solution		0.0498			
C_8			0.0496			
Average			0.0497	0.0497		100.00

All of the above filtrates precipitated by adding 50 c. c. magnesia mixture to the cool solution, and then, after standing for a short period, 25 c. c. of ammonia (sp. gr. .96).

*All blanks deducted.