CCXXVII. THE DETERMINATION OF INORGANIC PHOSPHATE IN THE PRESENCE OF ARSENIC.

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For the study of the effects of the arsenate ion on the activity of phosphatases an analytical procedure is necessary which will eliminate the usual interference of arsenate with the colorimetric determination of phosphate. The close chemical relationship of these ions and the need of avoiding hydrolysis of the residual phosphoric esters are factors which present serious difficulties in the solution of the problem in hand. None of the procedures described in the literature was found to be dependable, particularly with higher arsenate concentrations. A method has been evolved, therefore, which permits the accurate colorimetric determination of inorganic phosphate even in the presence of M arsenate.

EXPERIMENTAL.

Method of subtracting a correction for arsenate alone.

In the few reported studies of the effect of arsenate on phosphatases [Neuberg and Leibowitz, 1927; Mayer, 1928; Macfarlane, 1930; Lohmann, 1930; Braunschtein, 1931; Morgulis and Pinto, 1932] the methods employed usually involved the use of a correction, though in some instances it is not clearly indicated how the arsenate effect was avoided. This correction is applied by subtracting from the arsenate-phosphate colour value, a value obtained for the same arsenate concentration alone.

Under the conditions in this laboratory the use of such a correction proved satisfactory only in the presence of small amounts of arsenate, and provided also that the time of colour development in the arsenate solution was, at the time of reading, precisely the same as with the solution containing arsenate and phosphate. With concentrations of arsenate higher than $M/1000$ this correction no longer gave reliable results, as shown in Table I.

Table I. *Showing the amount of "phosphorus" found in solutions of known arsenate and phosphate concentrations by subtracting a correction for the colour developed by arsenate alone. 0.1 mg. P was present in each case.*

Arsenate conc. in original solution	mg. P found after colour developing		
	5 mins.	60 mins.	120 mins.
$M/10$	0.460	0.296	0.171
$M/100$	0.264	0.200	0.115
$M/250$	0.181	0.150	0.108
$M/500$	0.138	0.108	0.104
$M/750$	0.121	0.109	0.103
$M/1000$	0.099	0.101	0.099
$M/1250$	0.101	0.102	0.101

The unreliability of the correction in higher arsenate concentrations appears even when using Lohmann and Jendrassik's modification [1926] of the Fiske and Subbarow method [1925] which, it has been claimed [Braunstein, 1931], gives dependable results owing to rapid development of the blue colour at 37°. The arsenomolybdate blue colour takes much longer to reach a stable intensity and to become proportional to the amount of arsenic present than is usually realised. Furthermore the mixture of arsenate and phosphate develops a blue colour at a rate even faster than phosphate alone. However, Table I shows that if the colours are permitted to reach a stable state, a condition which may require 2 hours or more, the correction can be more accurately applied. There remains, of course, the great disadvantage of having in some cases to apply a correction equivalent to 20 or more times the amount of phosphorus present.

Reduction of arsenate to arsenite.

At this point it was observed that arsenite, in contrast with arsenate, does not give a blue colour with phosphorus reagents, using either the method of Fiske and Subbarow [1925] or the Briggs [1922] modification of Bell and Doisy's method. This suggested reducing the arsenate to arsenite before determining the phosphate; details of a method for doing this have been elaborated.

Since this method was devised somewhat similar ideas have been found in two recent papers. Tananaeff and Potschinok [1932] suggested a procedure using Na_2SO_3 and HNO_3 before adding the regular reagents. A trial of the method as outlined, proved it to be satisfactory for amounts of arsenate up to about $M/500$, but not more. Tschopp and Tschopp [1932] in a valuable discussion of the formation of various "molybdenum-blue" compounds indicated that the addition of 1 cc. of 20 % NaHSO_3 just before adding the phosphorus reagents would prevent the formation of the arsenomolybdate. It is probable, however, that unless some time were allowed for reduction this procedure would not be very effective.

The reduction of larger amounts of arsenate requires appreciable time, but is accelerated by higher temperatures. Temperatures from 20° to 60° were tried, and 50° was selected as the most suitable for rapid reduction, having due regard to possible hydrolysis of residual phosphoric esters and to the removal of sulphur dioxide from the solution.

Effect of extra bisulphite on the phosphate determination.

The presence of sodium bisulphite in the usual phosphorus reagents had suggested its use for reducing the arsenate, rather than that of something which would have to be removed, such as H_2S ; but it was necessary to determine the effects of extra bisulphite and of added acid on the accuracy of the determination. Table II shows the results obtained in the presence of varying amounts of sulphuric acid and of sodium bisulphite, using Fiske and Subbarow's method. Similar flasks were left at 50° for various times, as indicated, and the phosphate determinations were then made. Each 25 cc. volumetric flask had received 1 cc. of standard phosphate solution containing 0.1 mg. phosphorus, then the acid and the bisulphite; water was added to make a volume of about 10 cc. At the stated time the flasks were removed from the bath, the reagents were added, and the solution was diluted to the mark as usual. The conditions of the experiment permitted immediate reading of the colour intensity.

Comparing numbers 4 and 6 in Table II it is noted that an increase in the acid diminishes the recovery of phosphorus; a more striking effect is observed

Table II. *Showing the effect on the phosphate determination of varying amounts of 3N sulphuric acid and of sodium bisulphite, acting for different times at 50° before the addition of phosphorus reagents. 0.1 mg. P was present in each case.*

	cc. H ₂ SO ₄	g. NaHSO ₃	mg. P found		
			20 mins.	40 mins.	60 mins.
1	0	0	0.098	0.102	0.099
2	0.5	0.5	0.096	0.100	0.099
3	0.5	1.0	0.091	0.094	0.095
4	1.0	0.5	0.097	0.101	0.099
5	1.0	1.0	0.092	0.095	0.094
6	2.0	0.5	0.090	0.092	0.092
7	2.0	1.0	0.087	0.088	0.088

as the bisulphite is increased. It is therefore necessary to avoid large amounts of bisulphite. By calculation 0.1 g. of NaHSO₃ can produce enough SO₂ to reduce 20 cc. of *M*/10 arsenate. It was therefore decided that about 0.4 to 0.5 g. of bisulphite and 0.5 cc. of acid per flask would be safe to use and give a sufficient excess of SO₂. These quantities leave the bisulphite in excess because 0.5 cc. of 3N H₂SO₄ is equivalent to only 0.3 g. NaHSO₃; if, however, trichloroacetic acid is used to precipitate proteins it supplies the needed extra acidity. 1 cc. of 5 % trichloroacetic acid is equivalent to 0.03 g. NaHSO₃. Moreover, it is safe to increase the sulphuric acid to 1 cc. if desired.

The treatment with 0.5 cc. of 3N H₂SO₄ and 0.4 g. NaHSO₃ at 50° for 1 hour has no appreciable hydrolytic effect on 10 cc. of 2.5 % sodium β-glycerophosphate, and only very slight hydrolysis occurs with 10 cc. of 1.2 % sodium hexosediphosphate.

Table III shows the times necessary for the reduction of various concentrations of arsenate at 20° and at 50°, all in the presence of 0.1 mg. of phosphorus. When the recovered value approximates to 0.1 mg. of phosphorus, reduction is considered to be complete.

Table III. *Showing the colour, expressed as mg. of P, developed by 0.1 mg. P in the form of inorganic phosphate and varying amounts of arsenate after reduction had progressed for different periods of time, at 20° and at 50°. 0.1 mg. P was present in each case.*

Arsenate concentration in original solution	20°				50°			
	15 mins.	30 mins.	45 mins.	60 mins.	15 mins.	30 mins.	45 mins.	60 mins.
<i>M</i> /10	0.337	0.263	0.190	0.130	0.253	0.177	0.126	0.101
<i>M</i> /100	0.227	0.182	0.135	0.109	0.156	0.109	0.099	0.100
<i>M</i> /250	0.147	0.114	0.104	0.100	0.116	0.100	0.100	0.100
<i>M</i> /500	0.134	0.104	0.100	0.100	0.104	0.100	0.100	0.098
<i>M</i> /1000	0.112	0.100	0.101	0.099	0.098	0.099	0.100	0.098
<i>M</i> /2000	0.102	0.100	0.098	0.100	0.100	0.100	0.099	0.100
None	0.098	0.098	0.099	0.098	0.097	0.098	0.100	0.099

From Table III it will be noted that heating at 50° is distinctly advantageous, *M*/10 arsenate being reduced in 60 minutes; *M* arsenate was found to be completely reduced in 70 minutes. It is also noteworthy that the accuracy is not impaired by leaving the flasks at 50° for an hour, even though reduction may be complete in less time. The experiment shown in the table was performed with the 0.1 mg. phosphorus present in 2 cc. of the arsenate solution, but the

same result is obtained in about the same time if the phosphorus is present in 10 cc. of arsenate solution of the concentration indicated. Dilution up to 15 cc. does not noticeably affect the reduction.

Procedure.

The procedure used is, therefore, as follows. All or part of the trichloroacetic acid filtrate of the sample from an enzyme digest is transferred accurately to a 25 cc. volumetric flask as usual. To each flask are added 0.5 cc. of 3N H_2SO_4 and about 0.4 g. of NaHSO_3 . The necessary bisulphite can be estimated with sufficient accuracy without weighing, after a little practice, and is introduced into the flask through a funnel. The flasks are then left in a water-bath at $50^\circ (\pm 1^\circ)$, usually for one hour, after which they are removed, and the reagents are added for determining phosphate. The elevated temperature causes a more rapid development of the blue colour than usual, and readings may be made at once with Fiske and Subbarow's method, or in 15 minutes with Briggs's modification of Bell and Doisy's method. The standards should be developed for at least 3 minutes at 50° in order to ensure a stable colour intensity.

Using the procedure outlined, attempts were made to determine known quantities of inorganic phosphorus, each in the presence of different amounts of arsenate. Typical results are given in Table IV, where it is seen that the accuracy of the method falls within the usual limits of error for the determination of inorganic phosphate in the absence of arsenate or other interfering ions.

Table IV. *Showing the amounts of phosphorus found in mixtures of various amounts of arsenate and of phosphate, after reduction of the arsenate at 50° .*

mg. P present	mg. P found				
	After 30 minutes' reduction of arsenate				After 60 minutes' reduction of arsenate
	M/1000 As	M/500 As	M/250 As	M/100 As	M/10 As
0.050	0.049	0.048	0.048	0.050	0.050
0.200	0.199	0.198	0.197	0.200	0.201
0.300	0.296	0.296	0.300	0.300	0.306
0.400	0.400	0.400	0.400	0.400	0.396

With the aid of this method an investigation of the effects of arsenate on the activity of the phosphatases of bacteria, yeast, taka-diastase and mammalian tissue has been commenced.

SUMMARY.

In colorimetric phosphate determinations arsenate interferes by developing a blue colour with the ordinary reagents. In order to study the effects of arsenate on phosphatase activity this interference must be prevented, without, at the same time, hydrolysing the residual phosphoric esters. No method previously described was found to be sufficiently accurate in the presence of the higher concentrations of arsenate. A satisfactory procedure has been devised involving reduction of the arsenate to arsenite, a form of arsenic which does not interfere with the phosphate determination.

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