Micro-Determination of Soluble Silica in Urine

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Silicon may be determined in solution by the spectrophotometric method of Dienert & Wandenbulche (1923), as modified by King & Stantial (1933). This involves the formation of the yellow silicomolybdate and the reduction of this complex to molybdenum blue by 1-amino-2-naphthol-4-sulphonic acid reagent. Measurement of this colour provides a means of determining silicon.

A disadvantage of this method of determining silicon is that phosphate interferes by forming phosphomolybdate, which is similarly reduced to molybdenum blue. Various methods have been used for preventing phosphate interference during silicon determination. Khaler (1941) used mineral acids to prevent the formation of phosphomolybdate. Complex-forming agents such as oxalic acid (Schwartz, 1942; De Sesa & Rogers, 1954) or citric acid (Case, 1944; De Sesa & Rogers, 1954) were used to bleach the interfering phosphomolybdate while leaving the silicomolybdate unaffected. Paul & Pover (1960*a*) have developed a method of selectively removing phosphomolybdate by ethyl acetate. In their experiments, silicon was determined in the aqueous phase after reduction of the silicomolybdate to molybdenum blue.

These methods of eliminating phosphate during the determination of silicon are successful when only small amounts of phosphate are present to interfere. However, in the presence of high concentrations of phosphate, such as are found in rat and human urines, these methods are not applicable unless the urines are diluted considerably. This is undesirable, for when silica is present in trace quantities it might escape determination.

When phosphate is present in high concentrations, it can be removed by precipitation. Thayer (1930) claimed that phosphate was completely removed by precipitation with calcium chloride and ammonia at about pH 8.0 without simultaneous removal of any silicate. King & Stantial (1933) also reported precipitation of phosphate by calcium chloride and ammonia during silica determination in urine. However, none of these workers investigated the effect of pH on silicate and phosphate

precipitation. As it has been the author's experience that calcium chloride and ammonia cause precipitation of silicate at pH 8.0, a systematic study of the effect of pH on silicate and phosphate precipitation by calcium chloride and ammonia was made. From the results obtained it was possible to develop an accurate and reproducible method of determining silicon in rat and human urines.

EXPERIMENTAL

Materials

When possible, reagents of A.R. grade were used.

Silica standard. Solutions of soluble silica were prepared by fusing 10.0 mg. of finely divided quartz with 0.5 g. of powdered sucrose and 1.5 g. of sodium peroxide in a Parr bomb, and dissolving the fused material in 100 ml. of water in a polythene beaker. Further solutions of silicate were prepared by dilution and used as standards.

Phosphate standard. A stock solution of phosphate was prepared by dissolving 0.5 g. of anhydrous disodium hydrogen phosphate in water and diluting to 100 ml. Standard solutions were prepared from it by dilution.

Ammonium molybdate. The tetrahydrate (5.0 g.) was dissolved in 100 ml. of water.

1-Amino-2-naphthol-4-sulphonic acid reagent. The acid (200 mg.) was dissolved in water with 12.0 g. of sodium metabisulphite and 2.4 g. of sodium sulphite heptahydrate and diluted to 100 ml. After the solution had stood overnight it was filtered into a brown-glass bottle and kept in a cool place.

Calcium chloride. The anhydrous salt (10.0 g.) was dissolved in 100 ml. of water.

Ammonia solution. Aqueous ammonia (sp.gr. 0.880) was diluted to give a 2*N*-solution, which was stored in a polythene bottle. This solution contained a negligible amount of silicate.

Urine samples. Rat and human urines were collected and stored in polythene beakers containing liquid paraffin.

Trimethylhexadecylsilane. This was synthesized by the method of Paul & Pover (1960*b*). The method involved the methylation of trichlorohexadecylsilane (Found: Si 9.39. $(\text{CH}_3)_3\text{SiC}_{16}\text{H}_{33}$ requires Si, 9.40%; the infrared spectrum of trimethylhexadecylsilane showed two bands at 8.025 and 11.8–12.02 μ).

Methods

Precipitation of phosphate in urine. Urine (2.0 ml.) was mixed with 8.0 ml. of water. A portion (4.0 ml.) of the diluted urine was acidified by adding 0.2 ml. of acetic acid

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and the mixture was shaken. Calcium chloride solution (4.0 ml. of 10%, w/v) was added, together with 2.0 ml. of aqueous ammonia (2N). The mixture was then shaken and set aside for 15 min. This solution, which was at pH 7.0, was filtered and the filtrate used for the colorimetric determination. Blanks containing water in place of urine, brought to pH 7.0, were similarly prepared.

Determination of silicon. A portion (2.0 ml.) of the filtrate was pipetted into a 50 ml. separating funnel, followed by the addition, with shaking, of 2.0 ml. of N-hydrochloric acid. Ammonium molybdate solution (4.0 ml. of 5%, w/v) was then added and the solution mixed by shaking for 3 min. This solution had a pH of 1.0. It was then extracted once with 20 ml. of ethyl acetate for 30 sec. The aqueous phase was separated and 4.0 ml. of 10% (w/v) citric acid solution added to it with shaking. The solution was allowed to stand for 2 min., after which 1.0 ml. of the amino-naphtholsulphonic acid reagent was added with shaking, and the resulting solution set aside for 10 min. to allow maximum colour to develop. Any precipitate formed was removed at this stage by centrifuging. The extinction of the blue solution, which was linear to silicon concentration in the range 5–60 $\mu\text{g.}$ of SiO_2 , was then measured against a similarly treated blank at 690 m μ .

Effect of pH on the precipitation of phosphate and silicate. To each of several 4 ml. samples of disodium hydrogen phosphate solution (2.26 mg./ml.) or sodium silicate (0.125 mg. of SiO_2 /ml.) were added 4.0 ml. of 10% calcium chloride solution and 2.0 ml. of 2N-ammonia solution. The mixtures were brought to different pH values in the range 4.0–10.0, by adding acetic acid. After centrifuging, the phosphate, or silicate, was determined in the supernatant.

Determination of phosphate. Phosphate was determined by the method of King (1932).

RESULTS AND DISCUSSION

The results recorded in Table 1 demonstrate that the present method of determining silicon is accurate and reproducible. King & Stantial (1933) reported that when 1-amino-2-naphthol-4-sulphonic acid reagent was used as the reducing agent a persistent 'blank' colour was obtained. The author has found that the addition of citric acid before the reduction eliminated this 'blank' colour without affecting the reduction by the aminonaphtholsulphonic acid reagent.

In Table 2 are recorded results demonstrating the effect of pH on phosphate precipitation. These results were obtained by determining the remaining phosphate concentration in the supernatant, after precipitation and centrifuging. At pH 4.0 no precipitation of phosphate occurred; at pH 5.0, 35.5% of the phosphate was precipitated. At pH 7.2, only 5.4% of the total phosphate was present in the supernatant, 94.6% having been precipitated. Total precipitation of phosphate was obtained between pH 9.0 and 10.0.

If the results of phosphate precipitation are compared with those of Table 3 it can be seen that, unlike phosphate, silicate is not precipitated

Table 1. *Recovery of known amounts of silicon*

Samples of trimethylhexadecylsilane were fused in a Parr bomb and their silicon contents determined by the modified colorimetric method with 1-amino-2-naphthol-4-sulphonic acid reagent in the presence of citric acid, to effect reduction of silicomolybdate (for details see Experimental section).

Wt. of trimethyl-hexadecylsilane fused (mg.)	Silicon present (mg.)	Silicon found (mg.)	Error (%)
15.6	1.46	1.50	2.7
15.0	1.42	1.40	1.4
16.0	1.50	1.45	3.3
14.9	1.40	1.40	0.0
15.7	1.47	1.47	0.0
14.6	1.37	1.41	2.9
28.7	2.71	2.68	1.1
15.1	1.42	1.36	4.2
Mean 1.95 ± 0.38			

Table 2. *Effect of pH on phosphate precipitation*

Solutions of sodium phosphate, each containing 11.30 mg. of anhydrous disodium hydrogen phosphate, were treated with the precipitating reagents and the resulting solutions adjusted to one of the pH values below. After centrifuging, the clear supernatant was examined for phosphate (for details see Experimental section).

pH	Amount of phosphate recovered in supernatant (mg.)	Phosphate recovered in supernatant (%)
4.0	11.30	100.0
5.0	7.29	64.5
6.0	1.16	10.3
7.2	0.605	5.4
8.2	0.467	4.1
9.0	0.275	2.4
10.0	<0.005	<0.05

Table 3. *Effect of pH on silicate precipitation*

Solutions of sodium silicate, each containing 0.500 mg. of SiO_2 , were treated with the precipitating reagents and the resulting solutions adjusted to one of the pH values given below. After centrifuging, the clear supernatant was examined for silicate.

pH	Amount of silicate recovered in supernatant (mg. of SiO_2)	Silicate recovered in supernatant (%)
5.0	0.500	100.0
6.0	0.500	100.0
7.0	0.500	100.0
8.0	0.445	89.0
9.0	0.407	81.4
10.0	0.330	66.0

between pH 5.0 and 7.0. However, silicate precipitation occurred at pH 8.0, 10% of the total silicate being precipitated at this pH. Nevertheless, even at pH 10.0, at which pH all the phosphate was precipitated, only 34% of the silicate was precipitated.

In order to determine whether co-precipitation of silicate occurred during phosphate precipitation, the effect of pH on silicate precipitation in the presence of phosphate was studied. These results reported in Table 4 are in good agreement with those of Table 3, thus demonstrating that co-precipitation of silicate does not occur during phosphate precipitation.

By taking advantage of the difference at pH 7.0 between the precipitation of phosphate and silicate, a method of determining silicate in urine was developed. As only a small amount of phosphate escapes precipitation at pH 7.0, whereas silicate is

not precipitated at this pH, it was possible to precipitate the bulk of the phosphate at neutral pH and then eliminate the remaining phosphate by extracting the phosphomolybdate with ethyl acetate in the manner described by Paul & Pover (1960a). When this technique was applied, accurate and reproducible determinations of small amounts of silicon in rat and human urine were obtained (Table 5). As rat and human urines contain large amounts of phosphate (about 30 mg./ml. in rat urine), the method should be generally useful for determining trace amounts of silicon in very high concentrations of phosphate. In the experiments reported here, silica, in the presence of up to about 100 times its own concentration of phosphate, was accurately determined.

SUMMARY

1. An accurate and reproducible method of determining small amounts of silica in rat and human urine is described. The method involves precipitating the bulk of the phosphate present in urine, by calcium chloride and ammonia at pH 7.0, and removing the remaining phosphate by selective extraction of the phosphomolybdate with ethyl acetate. Silica was then determined in the aqueous phase after reduction of the silicomolybdate by 1-amino-2-naphthol-4-sulphonic acid reagent in the presence of citric acid.

2. The method is capable of general applicability to determinations of trace amounts of silica in high concentrations of phosphate.

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Table 4. *Effect of pH on silicate precipitation in the presence of phosphate*

Solutions of sodium silicate, each containing 0.228 mg. of silica plus 25.0 mg. of disodium hydrogen phosphate, were treated with the precipitating reagents and the resulting solutions adjusted to one of the pH values given below. After centrifuging, the clear supernatant was examined for silicate.

pH	Amount of silicate recovered in supernatant (mg. of SiO ₂)	Silicate recovered in supernatant (%)
5.0	0.228	100.0
6.0	0.228	100.0
7.0	0.228	100.0
8.0	0.210	92.1
9.0	0.189	82.9
10.0	0.156	68.4

Table 5. *Recovery of silica in rat and human urines*

Urine (2 ml.) was made up to 10 ml. with water. A portion (4 ml.) of this solution was then treated with the precipitating reagents. After centrifuging, soluble silica was determined in the supernatant. (For details see text.) To 2 ml. of the same urine, 2 ml. of silicate solution was added and the whole made up to 10 ml. After precipitation and centrifuging as described above, the total silica content of the urine was determined. All values are expressed as milligrams.

Urine	SiO ₂ /2 ml. of urine	SiO ₂ added to 2 ml. of urine	Total SiO ₂ found in 2 ml. of urine	SiO ₂ recovered
Rat	0.110	0.260	0.370	0.260
	0.150	0.300	0.450	0.290
	0.110	0.400	0.510	0.390
	0.140	0.100	0.240	0.09
	0.120	0.07	0.190	0.07
Human	0.020	0.240	0.260	0.250
	0.021	0.400	0.421	0.400
	0.030	0.300	0.330	0.300
	0.020	0.340	0.360	0.330
	0.020	0.100	0.120	0.100

REFERENCES

- Case, O. P. (1944). *Industr. Engng Chem. (Anal.)*, **16**, 309.
De Sesa, M. A. & Rogers, L. B. (1954). *Analyt. Chem.* **26**, 1278.
Dienert, F. & Wandenbulche, F. (1923). *C.R. Acad. Sci., Paris*, **176**, 1478.
Khalel, H. L. (1941). *Industr. Engng Chem. (Anal.)*, **13**, 536.
King, E. J. (1932). *Biochem. J.* **26**, 292.
King, E. J. & Stantial, H. (1933). *Biochem. J.* **27**, 990.
Paul, J. & Pover, W. F. R. (1960a). *Anal. chim. acta*, **22**, 185.
Paul, J. & Pover, W. F. R. (1960b). *Arch. Biochem. Biophys.* **87**, 312.
Schwartz, M. C. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 893.
Thayer, L. A. (1930). *Industr. Engng Chem. (Anal.)*, **2**, 277.

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Amino Acid-Activating Enzymes in Muscle

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The enzymic 'activation' of amino acids (Hoagland, 1955) is recognized and measured by the formation of hydroxamic acids in the presence of amino acids, adenosine triphosphate and hydroxylamine, or by the incorporation of radioactive pyrophosphate into adenosine triphosphate in the presence of amino acids. There is ample evidence (e.g. Kingdon, Webster & Davie, 1958) that activation involves the reaction of the amino acid with adenosine triphosphate to form an amino acid-adenylic acid anhydride and pyrophosphate. Magnesium appears to be essential for this reaction, which is generally considered to be the first step in the synthesis of protein from amino acids. It seems likely that separate activating enzymes for all the individual amino acids exist. Some have been purified, e.g. for methionine (Berg, 1956), tyrosine (Van de Ven, Konigsberger & Overbeek, 1958), valine (Berg & Ofengand, 1958), tryptophan (Davie, Konigsberger & Lipmann, 1956), alanine (Goldstein & Holley, 1960) and serine (Webster & Davie, 1959).

The presence of such enzymes activating various amino acids has been demonstrated in many animal and plant tissues and in micro-organisms (e.g. Nismann, Bergmann & Berg, 1957; Marcus, 1959; McCorquodale & Mueller, 1958; Webster, 1959; Lipmann, 1958; Novelli, 1958; Cole, Coote & Work, 1957). In animal tissues they have been studied largely in the 'soluble' fraction obtained after high-speed centrifuging of tissue homogenates. Recently (Reis, Coote & Work, 1959; Craddock & Simpson, 1960), however, their presence in mitochondria has been demonstrated.

No study of amino acid-activating enzymes in skeletal muscle has yet been reported. This paper

describes such experiments, carried out with mouse and human skeletal muscle. In addition, similar measurements were made with muscle from mice with hereditary muscular dystrophy, as part of a study of muscle-protein synthesis in this disease.

EXPERIMENTAL

Mice. All the mice used were of the inbred strain 129, bred from animals obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A. This strain carries a recessive gene which produces, in the homozygote, a progressive muscular dystrophy (Michelson, Russell & Harman, 1955). The 'normal' mice of this strain may be either homozygous (double dominant) or heterozygous with respect to the gene. The animals were fed without restriction on 'breeding sow cubes' (J. N. Brown and Son, Minden Street, Newcastle upon Tyne) supplemented daily with fresh vegetables and were used when 2-3 months old. All the affected mice which were used had been frequently observed to drag their hind legs behind them.

Human muscle. Human quadriceps muscle was obtained in the course of orthopaedic operations.

Preparation of muscle 'cell-sap' fraction. The mice were killed by dislocation of the neck and the muscle from the hind legs was removed as quickly and completely as possible into a cold (0-2°) solution of 0.02M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) (obtained by dilution of a 2M-solution which had been adjusted to pH 7.4 with HCl) and 0.05M-KCl. Human muscle was placed in a similar solution immediately upon removal. Visible fat was removed and the muscle chopped finely with scissors. It was homogenized in 4-5 vol. of tris-KCl in a Potter-Elvehjem homogenizer with a Perspex pestle; a loosely fitting tube was used first with subsequent treatment in a tighter tube to break up the fibres completely. The homogenate was centrifuged for 1 hr. at 70 000 g in a Spinco model L ultra-centrifuge.