CLXXII. DETERMINATION OF CALCIUM IN RAT URINE

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(Received 17 June 1938)

THE assay of parathyroid hormone by the method of Dyer [1937] requires the mass determination of Ca in small amounts of rat's urine. The methods given in the literature apply chiefly to human urine, of which large amounts are readily available, and involve incineration, which greatly limits their usefulness for mass analysis.

The chief objection to precipitating Ca oxalate directly from urine appears to have been the danger of simultaneous adsorption or precipitation of uric acid [cf. McCrudden, 1911-12]. This is not very probable in the case of rat or mouse urine, in which uric acid is replaced largely by allantoin, and could probably be avoided in any case by varying the concentration and pH of urine and the duration and temperature of precipitation. In the case of rat or mouse urine a further disturbing factor enters, namely, the invariable presence of protein in these urines, and this has, as will be seen later, a small but definite interfering effect.

The object of the present research was to determine whether, and under what conditions, the microtitration method of Halverson & Bergeim [1917] is applicable to urine, deproteinized or not, the results being compared with those obtained for ashed urine.

EXPERIMENTAL

(1) *Materials*

Rat and mouse urines were collected in glass metabolism cages of the type described by Dyer [1937], with groups of 5 rats or 10 mice in each cage; the daily output was made up with diluted acetic acid to 100 ml. Human urine was taken from healthy adults.

(2) *Methods*

The Halverson-Bergeim technique, as modified by Van Slyke & Sendroy [1929], was applied in all cases to the final determination of Ca. The following minor modifications were found desirable. The precipitate often has a tendency to adhere to the sides of the tube and to float on the surface of the liquid. This can be obviated by adding oxalate to the solution at 80° and allowing it to cool to room temperature; large crystals of Ca oxalate are thus obtained which readily settle. Three washings with 1:50 ammonia are preferable to two, as recommended by Van Slyke.

The filtered acidified urines were deproteinized by mixing 10 ml. of 20% trichloroacetic acid with 25 ml. of diluted urine (containing 0.4-0.8 mg. Ca), and centrifuging after 30 min.

In view of the destructive action of phosphates on platinum vessels, dry incineration is not a desirable procedure. Wet ashing with $\text{H}_2\text{SO}_4\text{-(NH}_4)_2\text{S}_2\text{O}_8$, by the method of Shohl & Pedley [1922], or with $\text{H}_2\text{SO}_4\text{-KHSO}_4$ gave much lower results than those obtained by direct precipitation, both for urine and for standard CaCl_2 solution. Since the only difference in the final solutions was the presence of Na_2SO_4 , this was added in amounts of 0.1 g. to 10 ml. of standard solution containing 0.1235 mg. Ca, and Ca was determined as above. The amount recovered fell rapidly as the amount of $\text{SO}_4^{=}$ added exceeded 0.1 g., being only 0.06 mg., or 50% of the actual value, with 0.9 g. $\text{SO}_4^{=}$. It follows that microdetermination of Ca is not possible under the given conditions of incineration.

Ashing with HNO_3 , by the method of Dyer, gave results which were substantially identical with the theoretical results. Thus the Ca content of a standard solution was 0.3169 mg. by direct precipitation, and 0.3189 mg. after incineration. Applied to urine the method also gave satisfactory results, and the values so obtained were taken as representing the true Ca content of the urines.

Apart from protein, uric acid has been supposed to interfere with direct precipitation of Ca. The precipitates obtained from amounts of urine (rat, mouse or human) containing 0.15–2 mg. Ca were tested by the Folin-Denis reaction for uric acid, but in no case was a positive reaction obtained, even after standing for 24 hr.

A necessary preliminary to the study of conditions of precipitation from urine was to determine the effect of varying the conditions under which Ca was determined in standard solutions, namely, duration, temperature and pH of the precipitation, time of centrifuging and number of washings. The results, given in Table I, show that the degree of accuracy is the same whether precipitation takes place at 3° or at 37°, and that precipitation is complete after 3 hr. Varying the pH from 3 to 6 does not significantly affect the results and the same is the case when the time of centrifuging is varied from 5 to 15 min.

Finally, it appears from the last column that the precipitates should be washed with three 3 ml. portions of 1:50 ammonia. It is evident that the

Table I. *Effect of varying duration, temperature and pH of precipitation, time of centrifuging and number of washings. All tubes contain 0.1235 mg. Ca. The figures in parentheses refer to the number of determinations, of which the mean is given*

Temperature at which		Mg. Ca found with various intervals elapsing before centrifuging						Time of centri- fuging min. at 3000 r.p.m.	pH	No. of wash- ings
Oxa- late added	Systems kept be- fore centri- fuging									
		40 min.	1 hr.	2 hr.	3 hr.	4 hr.	24 hr.			
19-21°	19-21°	0.1188 (5)	0.1196 (5)	0.1154 (3)	0.1188 (5)	0.1202 (3)	0.1181 (3)	15	4.5	3
19-21°	3°	0.1147 (2)	0.1198 (2)	—	0.1238 (2)	—	—	15	4.5	3
19-21°	37°	0.1188 (2)	0.1188 (2)	—	0.1268 (2)	—	—	15	4.5	3
80°	20°	—	—	—	—	0.1188 (2)	—	15	3	3
80°	20°	—	—	—	—	0.1233 (2)	—	15	3-4	3
80°	20°	—	—	—	—	0.1263 (4)	—	15	4-4.5	3
80°	20°	—	—	—	—	0.1239 (6)	—	15	4.5-5	3
80°	20°	—	—	—	—	0.1223 (2)	—	15	5-6	3
80°	20°	—	—	—	—	0.1256 (3)	—	5	4.5	3
80°	20°	—	—	—	—	0.1288 (2)	—	10	4.5	3
80°	20°	—	—	—	—	0.1225 (3)	—	15	4.5	3
80°	20°	—	—	—	—	0.1369 (2)	—	15	4.5	1
80°	20°	—	—	—	—	0.1268 (3)	—	15	4.5	2
80°	20°	—	—	—	—	0.1241 (3)	—	15	4.5	3

method is applicable over a wide range of conditions, which might be so selected as to exclude the interfering action of urinary constituents.

An analogous study was made of the optimum conditions for the determination of Ca in deproteinized rat urine. On the basis of these results the following method was adopted. The metabolism cage is rinsed with 5 % acetic acid and the urine + washings are diluted to 100 ml. 10 ml. of 20 % trichloroacetic acid are mixed with 25 ml. of filtered diluted urine and the solution is centrifuged after 30 min. (15 min. at 3000 r.p.m.). 5 ml. of the clear solution are diluted to 10 ml. in a calibrated conical centrifuge tube, and 1 ml. of 20 % Na acetate is added, followed by 6 drops of bromocresol green indicator. 1:1 ammonia is then added drop by drop, with constant stirring, to a yellowish-green colour, indicating a pH of 4.5–5. The tubes are then placed in a water bath at 80°, and 1 ml. of 4 % ammonium oxalate is added. After 5 min. the tubes are removed from the water bath, and are maintained at room temperature for < 4 hr., after which they are centrifuged (5 min. at 2500–3000 r.p.m.). The supernatant fluid is removed by suction, leaving 0.2 ml. of solution, the precipitate is dissolved in 2 ml. of $N H_2SO_4$ (5 min. at 100°) and the solution is titrated at 70° with 0.01 $N KMnO_4$.

Table II. *Effect of varying duration and temperature of precipitation in deproteinized rat urine*

Temp. before centrifuging	Mg. Ca found in 5 ml. urine, with a precipitation period of		
	1 hr.	2 hr.	3 hr.
3°	0.0714	0.0704	0.0734
21°	0.0765	0.0805	0.0754
37°	0.0775	0.0815	0.0805

The effect of varying the duration and temperature of precipitation is shown in Table II; the values given represent the means of two determinations. It appears that higher values are obtained at 37° than at 3°, but that with a duration of precipitation of 3 hr. the difference is not significant. Over a pH range of 3.5–6.5 (Table III) the highest values were obtained at pH 4.25–4.55. At pH 6.5 phosphate crystals (probably $MgNH_4PO_4 \cdot 6H_2O$) were formed in some cases, and these interfered with the collection and washing of the Ca oxalate precipitate. As in the case of standard $CaCl_2$ solutions, 3 washings of the precipitate were found to be essential. Varying the duration of centrifuging from 5 to 15 min. did not affect the results. Ca was determined in 2–10 ml. of deproteinized urine; the results, calculated for 5 ml. of urine, were 0.1157, 0.1110, 0.1082, 0.1128, 0.1131 and 0.1107, for 2, 3, 4, 5, 8 and 10 ml. of urine respectively, corresponding with maximum deviations from the mean of ± 0.012 mg.; this difference does not exceed the maximum error of the titration.

Table III. *Completeness of precipitation of Ca from deproteinized rat urine at varying pH*

pH	3.55	3.75	4.25	4.40	4.55	5.90	6.50
Mg. Ca	0.0735	0.0754	0.0886	0.0835	0.0825	0.0815	0.0775

A comparison of the results obtained with and without deproteinization (Table IV) shows that whilst the mean results do not in any case differ by more than the permissible error of titration, yet the differences between the individual determinations are greater in presence of protein, and the mean results are consistently lower.

Table IV. *Results of determining Ca in untreated and deproteinized rat urines. All values refer to 5 ml. of urine*

No. of urine	Mg. Ca found in		Difference
	Untreated urine	Deproteinized urine	
1	0.087 0.080 0.077 } 0.081	0.092 0.088 0.088 } 0.089	0.008
2	0.118 0.110 0.112 } 0.113	0.118 0.117 0.124 } 0.119	0.006
3	0.166 0.160 0.153 } 0.159	0.163 0.165 0.165 } 0.164	0.005
4	0.086 0.087 } 0.087	0.098 0.102 } 0.100	0.013

A comparison of the results obtained for deproteinized and ashed rat urines (24 samples) gave a mean difference of 0.009 mg. in 5 ml. of urine in favour of the ashed urines. The difference is not significant, and it was hence concluded that incineration is not essential.

The results for mouse urine were not so satisfactory (Table V), as the differences between deproteinized and ashed urines in some cases exceeded 0.03 mg. In the case of human urines even greater differences were found, the values for ashed urine being from 0.015 to 0.060 mg. higher than for untreated urine. It may perhaps be concluded from these results that part of the Ca of human urine is not precipitable by oxalate and that under our conditions uric acid is not an interfering factor.

Table V. *Determination of Ca in mouse and human urines. The values given are the means of 3 determinations*

No. of urine	Source of urine	Mg. Ca found in 5 ml. of				
		Untreated urine <i>a</i>	Deproteinized urine <i>b</i>	Difference <i>a-b</i>	Ashed urine <i>c</i>	Difference <i>b-c</i>
1	Mouse	0.267	0.303	- 0.036	0.270	+ 0.033
2	"	0.272	0.265	+ 0.007	0.262	+ 0.003
3	"	0.158	0.196	- 0.038	0.189	+ 0.007
4	"	0.235	0.247	- 0.012	0.266	- 0.019
5	"	0.287	0.289	- 0.002	0.307	- 0.018
6	"	0.262	0.280	- 0.018	0.293	- 0.013
7	"	0.315	0.349	- 0.034	0.374	- 0.025
						<i>a-c</i>
1	Human	0.325	—	—	0.356	- 0.031
2	"	0.435	—	—	0.495	- 0.060
3	"	0.728	—	—	0.772	- 0.044
4	"	0.497	—	—	0.539	- 0.042
5	"	1.683	—	—	1.713	- 0.030
6	"	0.304	—	—	0.319	- 0.015

SUMMARY

1. Calcium can be determined with satisfactory accuracy in small amounts of deproteinized rat urine by a modification of the method of Halverson & Bergeim. Incineration of the urine is not essential, but deproteinization is desirable.

2. Uric acid is not precipitated together with Ca oxalate, nor is it adsorbed by the precipitate. Higher values are given by incinerated than by untreated human urine.

3. Precipitation of Ca oxalate is inhibited by $\text{SO}_4^{=}$ in concentrations of over 1 %. Methods of ashing involving the addition of H_2SO_4 are not applicable to the micro-determination of Ca in urine.

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