CLXXIV. THE QUANTITATIVE SEPARATION OF THE SKELETON OF SMALL ANIMALS

By V. SUBRAHMANYAN, J. DUCKWORTH AND W. GODDEN

From the Rowett Institute, Aberdeen

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STUDIES of the effects of dietary changes on the inorganic composition of the skeleton and on the rate of deposition of substances in the skeleton have been impeded by the difficulties involved in its complete separation from the soft tissues. Although dissection can always be employed as a final resort, the procedure is tedious and time-consuming and the danger of loss of small bones or bone fragments is always present. While it is sufficient simply to analyse the leg bones in certain types of experiments, other investigations require information of a different kind and to such cases the present technique has been found well suited. Comparisons are generally between litter mates of the same sex. The method can also be advantageously used in cases where pathological calcification of the soft tissues occurs, in which case total body analyses are very misleading.

Methods are on record which depend upon hydrolysing the flesh with boiling alkali or glycerol-alkali. It was felt that such methods are open to criticism since there is the danger that calcium, magnesium and phosphorus from the soft tissues may precipitate under such conditions and lead to erroneous results. This is of even greater importance in experiments where pathological calcification of the soft tissues is found. It seemed advisable to resort to less drastic treatment.

The present communication describes the conditions under which the skeleton can be obtained free from other tissue by means of proteolytic enzymes. The method has been applied only to the rat and all details given refer to that animal.

Analytical methods. The methods of analysis applied to aliquots of the dilute HCl ash extracts were:

Calcium ...	...	Clark & Collip [1925]
Magnesium ...	...	Godden & Duckworth [1935]
Phosphorus ...	...	Fiske & Subbarow [1925]

METHOD

The method consists of incubating the partially cleaned skeleton with either papain or trypsin. Both enzymes have given satisfactory results, papain being somewhat more rapid and thorough in action than trypsin. However, we have been unable to obtain papain samples which were not highly contaminated with Ca and, although this did not appear to affect the values we obtained, we have felt that the use of the much purer trypsin was probably preferable.

The value of buffers in maintaining a fairly constant pH was investigated, but this added refinement was abandoned. The use of phosphate buffers was obviously excluded and such others as were tested showed a tendency (more or

less marked according to the buffer used) to remove inorganic matter from the skeleton. Moreover, very little, if any, advantage was gained by using buffers.

In cases where papain was employed, marked acceleration of the action was observed after activation of the preparation by a few drops of 3% HCN.

The technique finally adopted is as follows: after killing the animal by gas, the hide, viscera and intestinal tract are removed and the carcass placed in boiling distilled water for 2 min. Such portions of the flesh as can then be easily dissected are removed and the skeleton is then placed in a beaker and 100–200 ml. of 0.5% trypsin are added. The beaker and contents are then placed in an incubator and digested for 4–6 hr. at 37° with hourly stirring.

In cases where papain is used, better results are obtained if incubation is carried out at 50°. At the end of the period of incubation the liquid is poured off the skeleton and the bones are washed several times with hot distilled water in order to remove small fragments of tissue. Care must be exercised to ensure that none of the smaller bones are lost during decanting. Disarticulation is fairly complete except in the case of the spine; the spinal cord is still intact and must be dissected out. The brain also requires separate removal.

RESULTS

For the purpose of testing the method, it was decided to use the left and right femora of rats. A series of such comparisons was first made in which both of the bones were cleaned by mechanical dissection in the usual way. The results are given in Table I and, except in the case of one animal (rat no. 4), show a very close agreement between the two sides. The values for rat no. 4 show a marked variation for the weight of ash as well as for the weights of Ca and P in the ash. An occasional animal showing such differences has been found in similar tests in other experiments.

Table I. *Comparison of the composition of the left and right femora after mechanical cleaning*

Rat no.	Side	Wt. of dry fat- free femur mg.	Wt. of ash mg.	Ca in ash mg.	P in ash mg.
1	Left	86.6	44.7	17.1	8.4
	Right	87.7	45.1	17.3	8.4
2	Left	136.6	78.4	30.4	14.3
	Right	138.0	77.8	30.4	14.2
3	Left	136.1	79.8	31.0	14.3
	Right	138.2	79.7	31.2	14.5
4*	Left	239.7	140.4	54.8	25.3
	Right	271.1	158.2	60.6	28.0
5	Left	246.5	146.6	55.6	26.3
	Right	250.2	145.5	55.2	25.7
6	Left	282.1	171.8	65.2	30.7
	Right	278.8	170.6	65.6	30.1

* It will be noted that there was a large difference between the two femora of this rat. This difference was quite obvious at the time of dissection.

Table II gives the results for a similar comparison in which the left femur was cleaned by mechanical dissection and the right femur by digestion with papain. Table III shows similar data for a series in which the right femur was cleaned by digestion with trypsin. The agreement between the data for the two sides for

Table II. *Comparison of the methods of cleaning the femora; left femur, mechanically cleaned; right femur, digested by papain*

Rat no.	Side	Wt. of dry fat- free femur mg.	Wt. of ash mg.	Ca in ash mg.	P in ash mg.
7	Left	93.6	46.2	17.0	8.7
	Right	86.5	44.8	16.8	8.5
8	Left	87.2	43.3	16.0	8.0
	Right	74.3	41.7	15.7	8.0
9	Left	149.6	77.2	28.6	14.2
	Right	129.8	74.9	28.2	14.2
10	Left	149.2	78.3	29.2	14.4
	Right	131.2	75.6	28.2	14.1
11	Left	191.0	118.4	45.0	21.2
	Right	189.3	118.3	45.4	21.2
12	Left	214.3	128.3	48.4	22.8
	Right	203.0	128.4	49.6	22.4

Table III. *Comparison of the methods of cleaning the femora; left femur, mechanically cleaned; right femur, digested by trypsin*

Rat no.	Side	Wt. of dry fat- free femur mg.	Wt. of ash mg.	Ca in ash mg.	P in ash mg.
13	Left	105.3	57.3	21.4	10.6
	Right	94.4	54.9	20.8	10.0
14	Left	100.0	56.2	21.0	10.1
	Right	93.7	56.0	21.1	10.1
15	Left	149.0	86.0	32.6	15.6
	Right	139.5	85.1	32.6	15.6
16	Left	152.3	88.2	33.8	15.8
	Right	145.6	88.7	34.2	15.8
17	Left	303.5	183.7	70.0	33.3
	Right	288.1	182.6	70.0	33.1
18	Left	313.2	187.9	71.2	33.6
	Right	304.1	187.0	71.6	33.6

Table IV. *Ca and P losses from bone during digestion*

Wt. of bone mg.	Ca mg.	P mg.
1571	0.57	0.20
1607	0.56	0.20

individual rats is remarkably close. The root mean square of the differences between the left and right ash values in the case of mechanical cleaning is 0.8, in the case of papain treatment 1.7 and in the case of trypsin treatment 1.2.

As a further check, the fore and hind leg bones of two rats were mechanically cleaned, air-dried and weighed and then incubated for 6 hr. with 0.5% trypsin solution. The amounts of Ca and P passing into solution were then determined and the amounts, as shown in Table IV, were found to be practically negligible.

The combined data show that in all cases the differences and the slight losses obtained are not significant in terms of the total ash or total amount of each element in the skeleton and fall within the range of analytical error.

It is clear that larger differences arise in the weights of the dry fat-free femora as a result of the enzyme treatment. This is undoubtedly due to the

proteolytic action of the enzyme on the organic matrix of the bone. Data obtained by this method for organic residue would, therefore, be false. This does not, however, apply to the inorganic constituents investigated, which are not appreciably affected.

Since the above studies were completed, it became necessary, for the purpose of another investigation, to determine the Mg content of the entire skeleton of rats. As a check, the skeleton and the right femur were cleaned by the use of trypsin and the left femur by mechanical dissection. The root mean square of the differences between the weights of Mg found in the two legs was 0.06 mg., the average total value being 2.06 mg.

SUMMARY

A proteolytic method of separating the entire skeleton of the rat for the estimation of Ca, Mg and P is described.

REFERENCES

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