Table 1. *Breakdown of stored samples of barium ATP*
(P values expressed as percentage of acid-labile + free P.)

Preparation	Oxford	Oxford	Cambridge	Commercial
Type of salt	Dibarium	Dibarium	Monobarium	Monobarium
Water content (%)	5.6	5.4	11.7	11.2
Temp. of storage	Room	Room	0°	0°
Age of specimen (yr.)	2.75	1	1.3	>2
Free PO ₄	10	13	12	26
Inorganic pyrophosphate	6	6	5	19

pyrophosphate content is not as high in the Oxford and Cambridge samples as might have been expected from the rate of breakdown of some laboratory and commercial specimens previously investigated. Nevertheless, the amount of inorganic phosphate is very considerable, and in the Cambridge sample, at least, has risen from nil to 12 % of the acid-labile P in less than a year and a half. It was earlier sug-

gested (Bailey, 1949) that the presence of free phosphate in reputable ATP specimens was itself indicative of breakdown, and this statement is still valid. A test for inorganic pyrophosphate also needs to be made, and this has been greatly facilitated by the simple method which Rowles & Stocken have evolved for the preparation of yeast inorganic pyrophosphatase.

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Studies on the Skeletal Tissues

1. THE DEGREE OF CALCIFICATION OF SELECTED MAMMALIAN BONES

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Considerable attention has been paid to the composition of the inorganic part of the skeletal tissues, but little knowledge of the organic matrix upon which these salts are deposited exists. Marked differences between the rate of metabolism of cortical and epiphyseal bone are indicated by the uptake of radioactive tracer elements such as phosphorus and calcium (Manly & Bale, 1939; Hevesy, Levi & Rebbe, 1940; Manly, Hodge & Manly, 1940; Armstrong & Barnum, 1948). It is important to know how far these metabolic differences are paralleled, for example, by variations in degree of calcification of the organic matrix.

Baker, Butterworth & Langley (1946) examined the calcium and nitrogen contents of cortical and cancellous bone from human femur and ribs. These authors paid particular attention to the methods used for cleaning the bones, interpreting earlier results (Booher & Hansmann, 1931; Shear & Kramer, 1928) in the light of their experience. They found that there was no significant difference between the calcium contents of the various adult bones; the

mean value for all types examined was 25.5 % (limiting values 24 and 27 %). The only difference between the nitrogen contents was a small but statistically significant one between the value for the cancellous bone of rib and of femur. The rib bones of babies contained significantly less calcium and more nitrogen than the bones from adults. Strobino & Farr (1949) studied the distribution of nitrogen and ash in the radii, tibiae, humeri and ulnae from five cows and oxen of different ages. They found that a minimum value for nitrogen and a maximum for ash existed at the longitudinal mid-point of the bones. The degree of calcification of all the bones increased with age.

In the work to be described three types of bone from cats, rabbits and rats have been compared, namely cortical and cancellous bone from femur and alveolar bone surrounding the teeth. Calcium, phosphorus and nitrogen contents have been examined. The femoral bones were used so that our results might be compared with those of Baker *et al.* (1946). Alveolar bone has been examined because it

is well known that in pyorrhea alveolaris gross resorption of this structure takes place. We wished to know how far this liability to resorption in the different species of animal might be correlated with chemical composition.

METHODS

Well fed, adult animals were killed either by exsanguination under ether anaesthesia or by a blow on the back of the head; the mandibles and femurs were removed and dissected from soft tissue within 1–2 hr. The femurs were split into pieces with forceps and the mid 3 cm. of the bones reserved as representative of cortical bone. The epiphyses were cut from the ends of the bones and the trabeculae below the epiphysis (i.e. the metaphysis) later used as cancellous bone. The teeth were extracted from the mandibles and these bones split longitudinally for cleaning.

Cleaning the bones. Baker *et al.* (1946) divided the bones into small pieces weighing about 10 mg. and cleaned each with brush and needle under the dissecting microscope. This process is excessively laborious, demanding, as these authors say, several hours' work before sufficient cleaned tissue is obtained for analysis.

The method finally adopted by us was to spray the bones with a strong fine jet of hot water (50–60°) until the pieces, including the cancellous tissue, appeared free from haemoglobin and marrow. It was sometimes necessary to split the larger pieces in order to give the spray free access to the interstices of the trabecular bone. Finally, the tissues were examined under a dissecting microscope. This technique yields glistening white structures from the small animals which we examined, and thorough dissection revealed the absence of soft tissues. The cortical bones were scraped with a scalpel and thoroughly brushed with water before applying the spray treatment. All the bones were lightly brushed with ethanol and ether after treatment. In cleaning the mandibular structures it was found that the soft tissue immediately surrounding the teeth (the periodontal membrane) was not removed by spraying with hot water. The sockets of the teeth were, therefore, scraped with the point of a scalpel under a dissecting microscope until they were clean. The bones were roughly dried at room temperature. The pieces of cortical bone and the trabeculae of the metaphysis were then reduced to a fine powder with a dental burr. Preliminary examination (cf. Table 3) of the alveolar bone showed that considerable difference in chemical composition exists between the exterior (i.e. labial and lingual surfaces) and the interior of the bone. It was finally decided that most useful biological information would come from an examination of that part of the alveolar bone, including trabecular bone, which immediately surrounds the teeth. Consequently the protuberances left after splitting the mandible, representing the interdental alveolar bone, were powdered and examined.

Treatment and analysis of the bone powders. The samples were extracted for 2 hr. in the Soxhlet apparatus with ethyl ether to remove the bulk of the lipids, freed of ether at room temperature and finally dried to constant weight over P_2O_5 at 80° and 14–16 mm. pressure. A further small amount of volatile matter could be removed by lowering the pressure to 0.01–0.02 mm. pressure. The significance of this loss, however, is not understood and the higher pressure was, therefore, used.

The inorganic salts were extracted by the following method. Exact amounts of the dried defatted powders (0.04–0.08 g.) were weighed out and covered with 10 ml. of 20% trichloroacetic acid. The tubes containing the mixture were shaken, corked, and placed in the refrigerator at 0–4° overnight. Water (5 ml.) was then added to each tube which was centrifuged and suitable amounts of the clear supernatants taken for Ca and P estimations. This method was compared with several others which involved incineration of the bone powder and extraction with other strengths of trichloroacetic acid. The results did not differ significantly from those obtained using the method as described above. The Clark-Collip modification of the Kramer-Tisdall method originally devised for Ca estimations in blood was altered for bone work. If calcium oxalate is precipitated from hot slightly acid solutions, interference from calcium phosphate formation, which would otherwise occur, is avoided. Coarse calcium oxalate crystals are obtained which settle quickly and completely. The presence of acid and NH_4Cl diminishes the risk of occlusion of ammonium oxalate in the calcium oxalate precipitate. Ca was, therefore, estimated by the following technique: 2 drops of an aqueous bromocresol green indicator were added to 5.0 ml. of a suitable solution of the trichloroacetic acid extracts in 15 ml. conical centrifuge tubes and the solutions adjusted to approximately pH 5 (greenish blue colour of indicator) by first adding a few drops of 2N- NH_4OH followed by 1–2 drops of N-HCl. The solutions were then warmed to 80° and saturated ammonium oxalate solution (1.0 ml.) added. Heating was continued for 2 hr. by the end of which time the supernatant was clear. Very occasionally, clear supernatants were not obtained, but it was found that the addition of a few drops of 10% 'Teepol' allowed complete centrifugation of the precipitates. The oxalate in the precipitates was estimated by the standard Clark-Collip modification of the Kramer-Tisdall method. P was estimated in the extracts by the Martland & Robison (1926) modification of the Briggs method. N was estimated in the whole-bone powders by the micro-Kjeldahl technique using an 18 hr. period of incineration with concentrated H_2SO_4 and a $CuSeO_3 \cdot K_2SO_4$ catalyst. Two drops of 20 vol. H_2O_2 were added towards the end of the heating period.

RESULTS

The cat, rabbit and rat were selected for the most detailed study, partly because these species were of suitable size and reasonably easy to obtain, and partly because they differ in degree of susceptibility to resorption of the alveolar bone.

In preliminary examinations no difference was found between the composition of the femurs from the left and right leg of individual animals. Therefore with such animals as rats, which have small femurs, the bones from both legs were ground and the powders from two animals pooled.

The figures obtained for the various bones were treated as independent groups for the statistical analyses recorded in Table 2. Too little is known about the differential behaviour of cortical and cancellous bone to justify pairing the results for the two types of bones from the same individual. For example, removal of cancellous bone can occur in

conditions in which no change in the cortical bone is detectable (Bauer, Aub & Albright, 1929).

Three points of importance emerge from the results recorded in Tables 1 and 2. First, the amounts of calcium and phosphorus in the femurs of all three species of animal are about the same. The differences between the results for these three species are not statistically significant for either cortical or cancellous bone. Secondly, the amounts of calcium and phosphorus in the cancellous bone of all three species are very significantly lower than those in the cortical tissue. Thirdly, the calcium content of alveolar bones in the three species bears no constant relation to that either of cortical or cancellous bone.

Thus it seems unlikely that any considerable differences in the composition of bone occur between the individuals of species. The agreement in variance between our results and those of Baker *et al.* (1946) indicates that there was no great dissimilarity, in the freedom from soft tissues, in the bones used by the two sets of workers. The divergence in the results for other animals from those for man may be noted. The mean value for the calcium content of the cortical bone of the cat, rat and rabbit varies from 27.3 to 27.9%, whereas that for the comparable human bone is 25.59%. In the human bones there is no difference between the calcium content of the cortical and the cancellous bone, whereas in the three species of small mammals we have examined

Table 1. *The percentage composition of the cortical and cancellous (metaphyseal) femoral bone and the mandibular molar alveolar bone from cats, rats and rabbits*

	Cortical				Cancellous				Alveolar			
	Ca	P	N	Ca/N	Ca	P	N	Ca/N	Ca	P	N	Ca/N
Rabbit												
Number of results*	17	17	17	17	18	18	16	15	18	18	15	14
Mean value	27.3	12.4	3.36	8.23	25.2	11.4	4.43	5.73	28.0	12.7	2.94	9.79
Standard deviation	0.73	0.30	0.17	0.54	0.84	0.35	0.27	0.38	0.68	0.46	0.34	1.05
Cat												
Number of results*	9	18	17	9	8	16	15	8	10	17	11	6
Mean value	27.3	12.0	4.13	6.66	25.1	10.9	5.00	5.21	25.3	11.0	4.83	5.26
Standard deviation	0.77	0.56	0.24	0.50	0.55	0.36	0.24	0.37	0.92	0.49	0.24	0.30
Rat												
Number of results*	12	12	9	8	8	9	8	8	12	11	—	—
Mean value	27.9	12.6	3.35	7.8	26.2	11.5	3.94	6.67	27.1	11.9	—	—
Standard deviation	0.57	0.50	0.25	0.46	1.01	0.54	0.39	0.74	0.51	0.45	—	—

* Results from individual animals except for rats in which each represents a pool from two animals.

Table 2. *Statistical analysis of the differences in composition of the various bones*

(*n*=Degrees of freedom; *t*=Student's statistic; *P*=Probability that the groups compared are drawn from identical populations.)

	<i>n</i>			<i>t</i>			<i>P</i>		
	Ca	P	N	Ca	P	N	Ca	P	N
Rabbit									
Cortical/cancellous femur	35	35	30	8.1	9.1	12.8	≤0.01	≤0.01	≤0.01
Cortical/alveolar	35	38	31	3.0	2.5	4.4	<0.01	0.02	<0.01
Cat									
Cortical/cancellous femur	23	32	30	7.7	6.8	10.1	≤0.01	≤0.01	≤0.01
Cortical/alveolar	20	33	26	5.5	5.5	7.7	≤0.01	≤0.01	≤0.01
Alveolar/cancellous femur	21	—	—	0.64	—	—	0.05	—	—
Rat									
Cortical/cancellous femur	18	19	13	4.8	3.9	3.7	≤0.01	0.01	0.01
Cortical/alveolar	24	—	—	3.6	—	—	0.01	—	—

The femoral bones. The degree of variation from individual to individual in the calcium content of the cortical part of the femoral bones leads to a coefficient of variation ranging from 2.05 for the rat to 2.85 for the cat, and from 2.18 to 3.62 for the cancellous tissue. These values are quite small and are to be compared with values, calculated from the results, given by Baker *et al.* (1946), of 3.08 for human cortical bone from the femur and 2.53 and 3.08 for femur and rib cancellous bone, respectively.

there is a large and highly significant difference. Our result for the cancellous bone agrees approximately with that obtained by Baker *et al.* (1946) for both cancellous and cortical bone from adult humans.

We have also obtained preliminary results for ferret femurs, and with these animals the concentration of calcium in the two types of bone seems to be very similar to that in the cat, rat and rabbit.

The constancy of the calcium and phosphorus contents of the femoral bones of the three animals

does not seem to extend to other constituents. That this is so is indicated by the higher nitrogen content of the cat bones (Table 1). This result is reminiscent of the smaller but significant difference that Baker *et al.* (1946) found between the nitrogen content of rib and of femur cancellous human bone which was likewise not paralleled by a corresponding difference in calcium content.

The alveolar bones. No results are available from studies of human alveolar bone for comparison to be made between the small animals we have examined and man. We have ourselves examined two pieces of mandibular alveolar bone removed from adult

represent an average for two bones of differing composition then one might expect a greater variation from individual to individual. In fact, the variances for two out of the three species are lower and for the remaining one only very slightly higher than those for the other bones. A further contra-indication to the suggestion that the results for the alveolus are merely characteristic of mixtures is their close agreement with those for one of the other two types of bone in the cat and rat. In the former animal the cancellous bone contains 25.1% calcium and the alveolus 25.3%, in the latter the figures are 27.9% for cortical and 27.1% for alveolar bone.

Table 3. *A comparison of the composition of the 'inner' and 'outer' ('lamina dura') parts of the mandibular molar alveolar bone of rabbits*

	Outer part			Inner part		
	Ca (%)	N (%)	Ca/N	Ca (%)	N (%)	Ca/N
1st rabbit	28.7	2.7	10.6	27.8	3.0	9.3
2nd rabbit	29.5	2.3	12.9	28.1	2.4	11.5
3rd rabbit	28.6	3.0	11.6	27.1	3.3	8.2
Mean values	28.9	2.7	11.7	27.7	2.9	9.7

human beings, and the results obtained agree with the implication of the work of Baker *et al.* (1946) that bone throughout the adult human skeleton is calcified to about the same extent.

Table 1 shows that the calcium content of the alveolus from animals sometimes appears to be similar to that of the cancellous femoral bone (cat), sometimes to that of the cortical bone (rat) and sometimes to be more calcified than even the latter (rabbit). The nitrogen contents vary inversely with the calcium contents as might be expected. As was pointed out under the Methods section considerable analytical differences exist between the outer plates of bone of the alveolus and the internal structures. Typical of the results we have obtained are those illustrated in Table 3. It is not possible to identify this difference with distinct anatomical structures, since the so-called 'lamina dura' covers both the 'outside' (labial and lingual aspects) of the bone and folds over the crest, forming the tooth sockets. By selecting, as we have, the protuberances left on splitting the mandible longitudinally we have included some 'lamina dura' and some of the trabecular bone which lies underneath. A separation between the two was not feasible for this type of work with small animals. Thus one possible interpretation of our results is that the differences between the composition of our so-called alveolar bone in the three species are simply a reflexion of the inclusion of different amounts of the more calcified 'lamina dura'.

An argument which might be set against this suggestion is that if the results we have obtained

DISCUSSION

The interpretation of analytical results for the composition of bone must, as Baker *et al.* (1946) quite rightly point out, depend upon the cleanliness of the bone samples used. Our own criteria have been microscopic inspection and the inability to remove any more nitrogenous substances by washing. Thus our criteria are similar, although our method is less laborious, than that adopted by Baker *et al.* (1946), and may be applicable only to bone from small animals. It seems unlikely that our results, which show a lower degree of calcification of the cancellous bone, can be explained by inclusion of soft tissue. The higher calcium content of mammalian cortical bone, as compared with that of the corresponding human bone, clearly cannot be explained thus. It must, however, be remembered that samples of cortical bone, cleaned by any of the methods so far devised, will include the organic contents of Haversian, Volkman and other canals together with those of the bone cells. Thus we cannot claim to have proved that, in the mammals we have examined, the organic matrix of the cortical bone is more highly calcified than that in human bone; it may only be that there is less 'canal space'. This suggestion is supported by the agreement between the values for cancellous bone of animals and that of man. In the examination of the trabeculae results are not confused by the inclusion of adventitious internal organic matter, since no canals are present therein. Such an argument suggests that the agreement between the calcium content of cortical and cancellous

bone in the human being is due rather to the relative size and contents of the spaces within the bones than to a true similarity in degree of calcification.

The variation in composition of the alveolar bone of the three species of mammal is particularly interesting in relation to function. This bone is probably subjected to a greater number of changes in stress and strain than any other bone in the body. Also, of the three species examined, the rabbit has continuously growing molars, the rat has continuously growing incisors only, and the cat has a permanent secondary dentition. The relation of composition of the bone to these functions cannot, of course, be interpreted from the present results.

SUMMARY

1. The mean calcium content of the cortical femoral bones taken from groups of adult cats,

rabbits and rats varies only from 27.3 to 27.9%. These values are higher than the value quoted for the comparable human bone.

2. The cancellous femoral bone from the three species of animal contains significantly less calcium than the cortical bone, ranging from 25.1 to 26.2%.

3. The nitrogen contents of both cortical and cancellous femoral bone from the cat are higher (4.13 and 5.00%, respectively) than those for the corresponding bones in the rabbit (3.36 and 4.43%) and rat (3.35 and 3.94%).

4. The mean calcium and nitrogen contents of the alveolar bone differ from species to species, ranging from 28.0 and 2.94%, respectively, for the rabbit to 25.3 and 4.83% for the cat.

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Studies on the Metabolism of Progesterone and Related Steroids *in vitro*

1. THE METABOLISM OF PREGNANE-3 α :20 α -DIOL BY LIVER PREPARATIONS

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It has been established that when progesterone is administered to human subjects by injection or orally a considerable proportion remains unaccounted for by the known progesterone metabolites, principally pregnane-3 α :20 α -diol, which are subsequently excreted in the urine (see Marrian (1949) and Sommerville & Marrian (1950*a*, *b*) for reviews of previous work). Recently, Sommerville & Marrian (1950*a*) found that when pregnane-3 α :20 α -diol was administered orally to human subjects the proportion of administered steroid subsequently excreted in the urine as 'pregnanediol' was no higher than when progesterone was administered to the same subjects by the same route. This finding led the

authors to suggest speculatively 'that administered progesterone may be largely reduced to pregnanediol in the body, and that the low recoveries of urinary pregnanediol following administration of the hormone, may be due to excretion of the reduction product by other routes or further metabolism into products so far unidentified or to a combination of both processes'.

In order to explore the possibility of the further metabolism in the body of pregnanediol, the present authors decided to investigate the effect of tissue preparations *in vitro* upon pregnane-3 α :20 α -diol. In the first instance experiments were carried out with liver preparations, since previous work by many