and the succinoxidase activity of homogenates and slices of rat tissues have been investigated.

2. Their inhibitory action on urease increases to a maximum on allowing their solutions to oxidize by exposure to air, thus indicating an intermediate oxidation product as the actual inhibitor. Aniline and benzidine, which are not easily oxidized by exposure to air, are non-inhibitory. All the *p*-diamines tested are strongly inhibitory. *m*-Phenylenediamine shows only slight inhibitory activity.

3. In general those amines which inhibit urease also inhibit succinoxidase, but with this enzyme system the oxidation of the amines by the cytochrome oxidase component is so rapid that maximum inhibitory activity is obtained with freshly prepared solutions, and on exposure of the solutions to air a decrease in activity occurs.

4. In the succinoxidase system with addition of cytochrome *c* the higher concentrations of the inhibitory amines (10^{-3}–10^{-4} M) generally give initially an increase in O_2 uptake followed by complete inhibition of the enzyme activity. With lower concentrations (10^{-5}–10^{-6} M) only a gradually increasing inhibitory effect is observed. Without addition of cytochrome *c* results are less uniform in that the higher concentrations cause inhibition only in some of the livers; the lower concentrations are often inactive.

5. The succinoxidase of the liver of animals which had been fed *p*-dimethylaminoazobenzene, even in tumour-free parts of the liver of tumour-bearing animals, showed the same general behaviour towards the amines as that of control animals.

6. The initial increase in O_2 uptake is due to the catalytic oxidation of the amines by the tissues, their oxidation in absence of tissue being negligible.

7. When the amines are added to the enzyme 20–30 min. before the succinate their complete inhibitory activity is observed immediately on addition of the succinate.

8. On tissues of low oxidative capacity no inhibitory effect on the O_2 uptake in presence of succinate is produced by amines which inhibit tissues of high succinoxidase content.

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The Calcium and Nitrogen Content of Human Bone Tissue Cleaned by Micro-dissection

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The object of the work here reported was to obtain as accurate a figure as possible for the proportion of calcium salt to organic (collagenous) matrix in human bone substance. We have been unable to find any satisfactory series of figures in the literature, one of the chief sources of error being the inclusion of variable amounts of the soft tissue within the bone.

The material we have studied is mainly normal human bone with a view to establishing criteria for use in a further study of pathological bone; one group of pathological bone (sequestra) is also included.

Fully formed bone consists of a collagenous (protein) matrix impregnated with calcium salts in a finely dispersed state. As is well known (Tomes & de Morgan, 1853; Müller, 1858; Pommer, 1885) the protein matrix is deposited first and is then rendered hard by the deposition of calcium salts.

The growth of bone takes place by the deposition of layers of non-calcified bone matrix ('osteoid tissue') by the osteoblastic cells on the bone surfaces. In normal bone calcification takes place rapidly, so that even during rapid growth there is never more than a very thin layer (5–12 μ) of non-calcified osteoid tissue on the bone surfaces. Under pathological conditions (rickets or osteomalacia), produced either by vitamin D deficiency or by lack of phosphates, there is interference with the calcifying process in the bones so that the osteoid tissue fails to calcify or calcifies imperfectly; bone which is formed under these conditions may therefore contain little or no calcium and appears histologically as osteoid tissue which remains soft and flexible. Large numbers of chemical analyses have been made on bones in rickets and osteomalacia, and it is well established that they may show a gross deficiency in calcium content as indicated by the ratio of inorganic to organic substance. Apart from vitamin D deficiency or disturbances of phosphate metabolism, no disease is known in which there is histological evidence of calcium deficiency in the bone substance. A bone consists partly of bone tissue and partly of spaces filled with soft tissues (e.g. blood, blood vessels, connective tissue, marrow cells, etc.); the amount of soft tissue in proportion to the bony tissue varies within wide limits in different bones and also in the same bone in different individuals according to age and build. Many pathological conditions produce changes in the relative amounts of bony and soft tissue; only the two mentioned above are known to affect the calcium content of the bone tissue. Under standardized conditions the radiographic density of a bone depends on the amount of calcium salt per unit area intervening between the tube and the film. A reduction in image density can be produced by either (1) reduced calcium content of the bone tissue or (2) reduced amount of bone tissue relative to the soft tissues within the bone. The radiologist often uses the term 'decalcification' for conditions in which the radiographic density of a bone is reduced, but reduction in amount of bone tissue is much the commonest condition so that the term 'de-ossification' is usually the correct description. Histologically there is no evidence that *de*calcification, in a strict sense, exists; in vitamin D deficiency and similar conditions the process is a deficiency in calcification or what might be called 'hypo-calcification'. Histologically the process by which the quantity of bony tissue is reduced is de-ossification, the bone being removed by phagocytes (osteoclasts), so that both protein matrix and calcium are removed together. There is no satisfactory histological evidence of any biological process which removes the calcium and leaves behind the bone matrix; it used to be supposed that this occurred in rickets and osteomalacia, the term 'halisteresis' being used for such hypothetical 'salt removal'. Virchow (1853) and Müller (1858) showed that the essential lesion in rickets was not removal of calcium but failure to calcify, and Pommer (1885) extended this conclusion to osteomalacia. Recent discoveries in this field have not in any way invalidated these conclusions. As regards the term 'fibrosis of bone' the process by which a bone becomes fibrous, e.g. in osteitis fibrosa, consists in de-ossification with the development of fibrous tissue in place of bone tissue; the bone tissue itself does not become fibrous. The histological aspects of these questions are discussed in more detail by one of us elsewhere (Baker, 1939).

The difference between non-calcified or slightly calcified osteoid tissue and calcified bone is easily seen histologically, but there is no reason to suppose that one could detect even fairly marked changes in the calcium content of the calcified portions. The question therefore arises as to whether in normal bone the calcification process always proceeds to a constant end-point and whether under pathological conditions grades of calcium deficiency or excess exist which cannot be discovered histologically.

In most of the published results of the chemical analysis of bone no particulars are given of the exact source of the bone (in some cases it is not even stated whether it is of human or animal origin) nor of the method used for cleaning and preparing the specimen for analysis. It is difficult to separate all the soft tissues from the bone and to clean them out from all the spaces within the bone; unless this is done, however, the results of the analysis will include a variable amount of soft tissue and lead to a variable and unknown error in the results. This error should certainly be small with the compact cortex of a normal long bone, but it might be large with cancellous bone or porosed cortical bone. In the work here reported the first aim was to obtain bony tissue as free as possible from the soft tissues within the bone.

METHODS

Selection of material and choice of methods. The material for analysis of femur and ribs was obtained at autopsy. No conditions likely to affect the bones were present, and the specimens would be passed as 'normal bone' from the pathologist's point of view. A segment of about 1½ in. of the upper one-third of the shaft of the femur was removed from each of six adults and used for analysis of cortex and cancellous bone of femur (cortex nos. 1–6, cancellous 7–12,

Table 1). The anterior two-thirds of the 5th right rib of nine other adults and eleven infants was used. The sequestra consisted of pieces of dead bone removed surgically from abscess cavities in bone; five were from the jaw and one from the tibia. In the case of the femur, portions of (A) compact cortex and (B) cancellous bone were analyzed; in the case of ribs, many of which had little compact bone, cancellous bone (C and D) was analyzed. The sequestra (E) were composed of compact bone. Pieces of each type of bone were cleaned as described below, and divided into four samples, two of which were used for calcium estimations and two for nitrogen.

Our problem was to determine the amount of calcium salt per unit (weight) of dried bone matrix. Although some variation is reported in the proportion of phosphate to carbonate in the bone salt (Logan & Taylor, 1937), there is so little difference between the equivalent weights of the phosphate and carbonate radicle that the percentage of calcium in various recent formulae varies only between 38·88 and 38·9%; the calcium is therefore a measure of the total bone salt. An estimation of the total nitrogen will give a measure of the bone matrix, since this is almost entirely protein (collagen). An analysis of bone matrix decalcified in acid gave N = 16·18% of dry organic substance. This figure is taken as a basis for the estimation of protein in Table 2.

Micro-methods on small samples (15 mg. approx.) were used for two reasons: (1) the labour of cleaning the bone (see below), and (2) a method adaptable for small biopsy specimens or the examination of small localized lesions was desirable (some of the sequestra weighed less than 200 mg.).

Cleaning. The bone, taken at necropsy, was preserved in 80% ethanol until required. It was then cleaned of muscle and connective tissue by ordinary dissection, cut into small blocks with a fret-saw and the cancellous bone removed from the compact with an old scalpel. The compact bone was then cut into small pieces (about 10–15 mg.) with bone forceps and cleaned in 80% ethanol under a dissecting microscope using a scalpel and hypodermic needle as a scraper. The cancellous bone was cut into laminae with a scalpel and cleaned in 80% ethanol by means of a hypodermic needle and camel's-hair paint-brush cut down by about two-thirds to give short stiff hairs. The laminae (about 5 mg.) had to be thin so that one could see into crannies with the microscope. The compact bone was much easier to clean than the cancellous. On an average it took about 6 hr. to clean about 100 mg. of bone. The cleaned bone fragments were defatted in a mixture of equal parts of ether and absolute ethanol for two days.

Weighing. The cleaned, defatted bone fragments were washed with fresh solvent and then dried in an air oven at 105° overnight. They were cooled in a desiccator and weighed on a micro-balance in small platinum boats, contained in stoppered weighing bottles.

Calcium estimation. The sample (usually about 15 mg.) was ashed by placing the platinum boat in a porcelain crucible and heating over a strong bunsen flame or in a muffle furnace at dull red heat. The ash was then dissolved in 10 ml. N-HCl and 2 ml. portions used for the determination of calcium. The method adopted was that used by Kramer & Tisdall (1921) for the micro-titration of calcium in urine and a blank estimation on the reagents alone was done for each series of analyses. The washing technique of Clark & Collip (1925) was found to be satisfactory provided a weak solution (0·02%) of phenol red was used as indicator (if stronger solutions of phenol red are used the indicator is incompletely removed from the calcium oxalate precipitate and the washing technique of Kramer & Tisdall (1921) must then be followed). The washed precipitate of calcium oxalate was dissolved in 2 ml. N-H_2SO_4 and titrated in the usual way with 0·01 N-$KMnO_4$ from a micro-burette. Duplicate analyses were made on each sample of bone and as a rule two samples of the same bone were analyzed. The percentage of calcium is given by: Unknown – blank (ml. 0·01 N-$KMnO_4$) × 0·2 × 5 × 100/wt. of bone (mg.).

Nitrogen estimations. The weighed sample of dry, defatted bone (about 15 mg.) was transferred to a pyrex tube (6 × 1 in.) and digested with 1 ml. conc. H_2SO_4 (A.R.) over a micro-burner for 1 hr. and the solution cleared by means of 30% (100 vol.) hydrogen peroxide (A.R.), the heating being continued for 20–30 min. after the addition of peroxide. A blank determination was made with each series of analyses. The clear digest was transferred quantitatively into a micro-Kjeldahl apparatus by washing out the tube three times with 5 ml. portions of distilled water. 10 ml. of NaOH solution (40% w/v) were added and the liberated ammonia distilled in steam into 10 ml. 0·01 N-H_2SO_4. In order to obtain a sharp end-point in the titration, 2 ml. KI solution (5% w/v), and 5 drops of a saturated solution of KIO_3 were added to the distillate and the liberated iodine titrated with 0·01 N-$Na_2S_2O_3$ solution. The percentage of nitrogen is given by: Blank – unknown (ml. 0·01 N-$Na_2S_2O_3$) × 14/wt. of bone (mg.).*

RESULTS

Expression of results. The results are set out in Table 1, the bone samples being grouped (A, B, C, D and E) according to site, type of bone (compact or cancellous) and age (adult or baby). The sequestra were all adult compact bone and are grouped together. Each number (1–38) refers to one or more samples taken from a particular bone specimen as described above. The bones were from different individuals except in groups A and B (Table 1), where the compact and cancellous samples were from the same person, i.e. nos. 1 and 7, 2 and 8, etc., were from the same individuals. The results are expressed as percentages of calcium and nitrogen in dried defatted bone.

Calcium results. In most cases two samples were taken and two estimations were made on each sample. There was no significantly greater difference between samples from the same specimen than between the duplicate analyses of the same sample. The mean of the set of analyses (usually four) two from each of two samples) was therefore taken as the calcium figure (Table 1, col. 4). The figures for nos. 16, 19, 23 and 30 are based on two analyses

* Since this work was started it has been shown by Chibnall, Rees & Williams (1943) and Hoch & Marrack (1945) that digestion with H_2O_2 is inadequate for conversion of all nitrogen to ammonia. This indicates that our absolute values for nitrogen are probably a little too low; it does not affect our figures for protein of bone matrix.

Table 1. *Calcium and nitrogen content of bone*

	No.	Sex	Age (yr.)	Calcium (%)	Nitrogen (%)
A. Femur cortex	1	F.	69	26·14	4·43
	2	F.	61	26·20	4·34
	3	F.	43	24·56	4·63
	4	M.	37	26·20	4·17
	5	F.	53	25·82	4·38
	6	F.	32	24·59	4·60
B. Femur cancellous	7	F.	69	24·77	4·75
	8	F.	61	24·14	4·84
	9	F.	43	25·47	4·50
	10	M.	37	25·27	4·42
	11	F.	53	25·19	4·45
	12	F.	32	26·08	4·42
C. Rib cancellous, adult	13	F.	52	24·01	4·89
	14	M.	53	25·30	4·84
	15	M.	40	26·07	5·09
	16	M.	15	26·83	5·27
	17	F.	69	24·91	5·14
	18	M.	75	26·50	5·05
	19	M.	64	25·88	5·05
	20	F.	25	25·73	5·18
	21	F.	36	25·10	5·18
			(mo.)		
D. Rib cancellous, baby	22	F.	3	25·92	5·47
	23	M.	22	24·71	5·19
	24	F.	4	22·29	5·66
	25	M.	11	24·31	5·74
	26	M.	11	23·05	5·01
	27	M.	5½	23·97	5·28
	28	M.	8	26·19	5·04
	29	M.	3½	23·70	5·73
	30	F.	21	24·24	5·32
	31	M.	13	24·93	5·33
	32	M.	9½	24·94	5·09
E. Sequestra	33			26·56	4·39
	34			26·33	4·40
	35			26·38	4·45
	36			26·03	4·25
	37			25·89	4·21
	38			25·99	4·27

The figures give the calcium and nitrogen content of dried defatted bone. Each calcium figure is the mean of two bone samples, the figure for each sample being (usually) the mean of two analyses. The nitrogen figure is (in all but two cases) the mean of two samples on each of which one analysis was done.

The standard deviation of the differences between each pair of samples was for Ca $\pm$0·2%, for N $\pm$0·09%.

only, those for nos. 12, 13, 14, 18, 24, 27, 28 and 31 on three analyses and the remainder on four analyses. The mean calcium for each group is given in Table 2.

Nitrogen results. These are analyses of samples from the same bone regions as used for calcium estimations. A single result was obtained from each of two samples, except nos. 22 and 24, where only one sample of each was examined. The mean nitrogen and the calcium/nitrogen ratio for each group is given in Table 2. Comparisons were made by using the method of analysis of variance (Fisher, 1936), and applying t as a test of significance. The

Table 2. *Average calcium and nitrogen content of groups of bone samples from different sources*

(The total composition of the dried defatted bone is shown in the second half of the table)

Group	Type of bone	Calcium (%)	Nitrogen (%)	Calcium/ nitrogen
A	Femur cortex	25·59	4·43	5·8
B	Femur cancellous	25·15	4·56	5·5
C	Rib cancellous, adult	25·59	5·08	5·1
D	Rib cancellous, baby	24·39	5·35	4·6
E	Sequestra	26·20	4·33	6·0

		Calcium salt* (%)	Protein† (%)	Residue	Calcium salt/ Protein
A	Femur cortex	65·8	27·4	6·8	2·4
B	Femur cancellous	64·7	28·2	7·1	2·3
C	Rib cancellous, adult	65·8	31·4	2·8	2·1
D	Rib cancellous, baby	62·7	33·0	4·3	1·9
E	Sequestra	67·4	26·8	5·8	2·5

* Calculated as $(Ca_3(PO_4)_2)_3.CaCO_3$.
† Calculated as $6{\cdot}18 \times N$.

results are summarized in Table 3. The probability, p, that the groups compared (e.g. A and B) come from the same 'population' was obtained from the table of t given by Kendall (1943). Thus, comparing A and B (compact and cancellous bone of femur), the probability that the two groups could be drawn from the same population is 1 in 3 (0·34) for calcium, and 1 in 5 (0·20) for nitrogen. If a probability of 1 in 20 (0·05) or less is taken to indicate a significant difference between the groups, then it is

Table 3. *Summary of statistical comparisons between mean results of analyses of the different types of bone given in Table 2*

Comparison	Degrees of freedom	Calcium t	Calcium p	Nitrogen t	Nitrogen p
A with B. Femur cortex: femur cancellous	10	1·027	0·34	1·348	0·20
C with D. Rib cancellous adult: rib cancellous baby	18	2·577	0·018	2·770	0·012
B with C. Femur cancellous: rib cancellous adult	13	1·055	0·31	6·144	$<$0·001
A with E. Femur cortex: sequestra	10	1·782	0·104	1·200	0·26

evident that groups A and B, and A and E (living and dead compact bone), respectively, are not significantly different, but C and D (adult and infant ribs) are significantly different. In the case of groups B and C (cancellous femur and cancellous rib bone) the calcium figures are not significantly different, but those for nitrogen are.

DISCUSSION

Our figures indicate that normal adult human bone, cleaned as thoroughly as possible, defatted and dried, has a calcium content lying between 24 and 27 % (mean 25·5) and a nitrogen content between 4·2 and 5·3 % (mean 4·7). There is no statistically significant difference between the mean figures for compact and cancellous bone nor between those for dead (sequestra) and living bone. Analyses of babies' ribs show a calcium figure between 22·3 and 26·2 % (mean 24·4) and a nitrogen content between 5·0 and 5·7 % (mean 5·35). Comparing mean values of infantile and adult ribs there was a significant difference both in calcium and nitrogen, infants' ribs showing a rather lower calcium and a rather higher nitrogen resulting in a lower calcium/nitrogen value (Table 2). In adults bone growth is slow compared with that in children, whose bones show histological evidence of greater growth activity by the presence of more numerous and wider osteoid seams. Our findings of a lower calcium/nitrogen ratio in young children might, therefore, be expected on histological grounds. The comparison between cancellous femur and cancellous rib was made on account of the structural and histological similarity of the bone from these sites. The significant difference between the nitrogen figures is, therefore, unexpected. We cannot offer any explanation of this finding, which we hope to investigate further.

Individual differences in calcium and nitrogen content of normal femur. Since a statistical analysis of our figures shows that there is no significant chemical difference between compact cortex and cancellous bone of adult femur, it seems legitimate to group together the analyses of cortex and cancellous bone of each individual. This gives us mean figures for femur based on eight analyses for calcium and four for nitrogen for each of these six individuals (1 + 7, 2 + 8, etc., Table 1). If this is done, figures for femur for different individuals range from 25·01 to 25·73 % calcium and from 4·15 to 4·59 % nitrogen. This suggests a fairly constant composition.

Results of other workers. Among the numerous accounts of analyses of bone for calcium, only one (Shear & Kramer, 1928) has been found where the technical methods were similar to ours. These workers analyzed small samples (10 mg.) of cleaned, defatted, dried and powdered bone. They give figures for two normal human femurs, (1) calcium = 24·35 % (mean of ten estimations, min. = 23·9, max. = 25·2) and (2) calcium = 24·03 % (mean of six estimations, min. = 24·0, max. = 24·7). Their material was apparently not cleaned as thoroughly as ours, which may account in part for the slightly lower calcium figure.

In general the calcium figures given in the literature have been too low, no doubt owing to the analysis of relatively gross fragments of bone which always include a proportion of soft tissue. The results of Booher & Hansmann (1931) illustrate this point. Their technique was designed to estimate the total amount of calcium in the tibias of infants, for which it was quite suitable. The bones were cleaned externally, crushed, dried and defatted, but no internal cleaning was done; they gave a very constant calcium content between 16 and 17·1 % (mean 16·48). Comparing this with our calcium figures for infants' rib bone it appears that these analyses of tibias must have included a considerable amount of soft non-fatty tissue within the bone, and therefore give little indication of the calcium content of the bone tissue itself.

Calcium/nitrogen ratio. As noted above the process of calcification of bone consists in the deposition of calcium salts in a protein matrix and the measure of the degree of calcification of the matrix will be given by the calcium/nitrogen ratio. The upper limit for the calcium/nitrogen ratio for a normal bone in our series was 6; this was given by one individual as the mean figure for the femur (mean of cortical and cancellous bone, nos. 4 and 10, Table 1). If this is taken as fully calcified (i.e. 100 %) the figures for groups A, B and E are 97, 92 and 100 % respectively, but these differences are not statistically significant. Group C (85 %) is significantly less calcified than A, B and E, and group D (77 %) significantly less than C.

The bone matrix will also contain a small percentage of inorganic material other than calcium salts which will be included in the residue (Table 2).

Calcium content of sequestra (dead bone). Dead bone can be recognized histologically only by the loss of nuclei from the bone cells; the bone matrix shows no visible alteration and may remain in the body for long periods without visible structural change. It has often been stated that dead bone in the form of a sequestrum acquires a higher calcium content than normal bone, the reason for this statement being that in a radiograph it usually appears denser than the surrounding bone. While the possibility that additional calcium may be deposited in dead bone cannot be ruled out, the more probable explanation of the relative radiographic density of a sequestrum is that the surrounding bone has undergone an osteoclastic destruction of inflammatory origin while the

sequestrum, being non-vascular and inaccessible to the osteoclasts has remained unchanged. In clinical radiography judgements are based on relative rather than on absolute image densities.

Our figures show a slight and statistically non-significant increase in the calcium content of sequestra (group E) as compared with normal compact bone (group A). It may be argued that this is suggestive ($p = 0{\cdot}1$) and that a small increase in calcium content would account for an increased radiographic density. We have investigated this point by taking X-rays of a series of slices of femur cortex of different thicknesses, and have found that under the conditions of clinical radiography it would require at least a 50% increase in calcium content to give any clearly visible difference in radiographic density. Nothing of this sort occurs in sequestra.

SUMMARY

1. An estimation has been made of the calcium and nitrogen content of a series of samples of human bone tissue cleaned as thoroughly as possible under a dissecting microscope, defatted and dried.

2. Tables are given showing calcium and nitrogen content of groups of samples of normal bone from (A) compact cortical bone of adult femur, (B) cancellous bone of adult femur, (C) adult rib, (D) infant rib, and of abnormal bone (E) in the form of sequestra composed of dead compact bone.

3. No statistically significant differences were found between groups A and B or between groups A and E. There was a significant difference between groups C and D, infantile rib (D) showing a lower calcium and a higher nitrogen content than adult rib (C). This could be explained by the greater amount of osteoid tissue seen on histological examination in the rapidly growing bones of infants. There was a significantly greater amount of nitrogen in adult rib (C) as compared with adult femur (A and B); no explanation of this can be offered at present.

4. Taking the femur (average of compact and cancellous bone) of six individuals, the figures range from 25·01 to 25·73% calcium and from 4·15 to 4·59% nitrogen, suggesting a fairly constant composition.

5. The degree of calcification of the collagenous (protein) bone matrix may be expressed by the calcium/nitrogen or by the calcium salt/protein ratio. The calcium salt/protein ratio averaged 2·4 for compact bone of femur cortex, 2·3 for cancellous bone of femur, 2·1 for cancellous bone of adult rib, 1·9 for cancellous bone of infant rib and 2·5 for compact bone of sequestra.

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The Cerimetric Determination of Glucuronic Acid, using the Conway Burette

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As a preliminary to a study of β-glucuronidase, it was necessary to be able to estimate small amounts of glucuronic acid in the presence of excess unhydrolyzed glucuronides. This can be done by determining reducing sugar by one of the methods for blood glucose. Thus Masamune (1934) used the Hagedorn-Jensen technique, while Fishman (1939) employed a modification of the cerimetric method of Miller & Van Slyke (1936). None of the methods used by previous workers, however, was sufficiently