The Effect of Normal Development and of Severe Undernutrition on some Minor Components of Cortical Bone

By R. A. McCANCE, D. A. T. SOUTHGATE, P. J. SPENCER AND P. D. WESTON*
Medical Research Council Department of Experimental Medicine, University of Cambridge

(Received 29 October 1965)

1. The amounts of calcium, magnesium, sodium and citric acid in the bones of undernourished pigs 1 year old were compared with the amounts in the bones of smaller newborn animals, normal animals of the same weight aged 4 weeks and of the same age weighing 170 kg. 2. The differences that were found between 4 weeks and 1 year of age in the normal animals were expected as effects of aging. However, between birth and 4 weeks of age the changes in composition were in the opposite direction to those between 4 weeks and 1 year. 3. Undernutrition produced a bone that resembled chemically that of an animal 1 year old.

Undernutrition has been shown to produce characteristic changes both in the morphology and the chemistry of the bones of the pig (Dickerson & McCance, 1961). Sodium, magnesium and citric acid are among the minor constituents of bone and they have been relatively little investigated in undernutrition, and so it was decided to study them because of their importance in the chemical exchanges of bone.

The skeleton contains 30–45% of the total body sodium in man (Forbes, Tobin & Lewis, 1959) and can part with a considerable amount of it in the event of an acidosis or a sodium deficiency (Bergstrom & Wallace, 1954; Bergstrom, 1955; Woodbury, 1956; Rolf, Armstrong, Steiger, Audia & White, 1959; Bergstrom & Ruva, 1960). The sodium in whole bone is generally reckoned to be present in two forms; first and predominantly adsorbed to the surface of the bone crystals, and secondly dissolved (with chloride and bicarbonate) in the extracellular water. It is difficult to put all the information in the literature together satisfactorily because of the composite nature of bone and because authors have not all analysed the same parts or expressed their results in the same way (Munro, Satoskar & Wilson, 1957; Widdowson & Dickerson, 1964). However, the concentration of sodium in bone has been shown to rise with that of calcium as the percentage of water falls, and the cortical bone of an old animal contains more sodium and less water than that of a young animal, although the calcium/sodium ratio may not alter (Forbes, Mizner & Lewis, 1957; Forbes, 1960).

The magnesium, like the sodium, in bone is mainly associated with the bone crystals, and Neuman &

Neuman (1953) considered that it was adsorbed on to their surfaces. This should mean that the percentage of magnesium in bone goes up with age like that of calcium. Since the bone crystals also increase in size with development the calcium/magnesium ratio should increase (Widdowson & Dickerson, 1964). Hammett (1925*a,b*) and Orent, Kruse & McCollum (1934) found that the percentage of magnesium in rat bone did indeed rise during growth but they did not find any consistent trend in the calcium/magnesium ratio. Pickering, Foran, Scott & Crane (1956) found an increase in the calcium/magnesium ratio in the long bones of rats from the time they were weaned at 24 days to the age of 75 days but no significant change in the percentage of magnesium. Bowes & Murray (1935) and Murray (1936) established that the calcium/magnesium ratio in teeth varied much more from one species to another and from one tooth to another than did the percentage of calcium. The ratio varied, for example, from 8.5 in an elephant's tusk to about 30 in dentine from the sound premolar teeth of children, and 83 in the enamel of these teeth.

Over half the citrate in the body is to be found in the skeleton (Freeman, 1958), and it may amount to 1% of the weight of the bone (Fourman, 1960) and certainly plays an active part in its metabolism (Eeg-Larsen, 1951). The percentage is low in a vitamin D deficiency (Harrison, 1956; Dixon & Perkins, 1956) and rises with treatment, but it cannot be raised by the ingestion of citrate (Waasjö & Eeg-Larsen, 1951). The rise in the citrate concentration in bone after the administration of vitamin D is accompanied by a rise in that in the serum and both can be inhibited by cortisol (Carlsson & Hollunger, 1954; Harrison, Harrison & Park, 1958). The association, however, has recently been shown

* Present address: Department of Chemistry, University of Birmingham.

to be more complicated than was at first supposed (Hartles, 1965). Parathyroid hormone raises the serum citrate concentration and parathyroidectomy decreases it, and it was at one time held that parathyroid hormone also raised the citrate concentration in bone (Freeman, 1958; Fourman, 1960). Recently, however, this has been disputed (Forscher & Cohn, 1963; Jenkins, 1966), and, in the discussion of a paper by Budy (1962), W. D. Armstrong stated that the injection of oestrogens raised the citrate concentration in the femora of mice without altering the plasma concentration, so that the two may move independently given the appropriate stimulus. The citrate concentration in rat bones has been shown to fall from soon after weaning to maturity (Zipkin, Schraer, Schraer & Lee, 1963).

MATERIALS AND METHODS

The experimental animals were pigs and the undernourished animals were reared as described by McCance (1960) and by Cabak, Gresham & McCance (1962). Their diet was in itself a perfect one had enough of it been given. The weights of the undernourished animals when they were 1 year old were about 5.5 kg. This is about 3% of the weight to be expected in a pig of that age (McCance & Widdowson, 1962; McCance, 1964).

The animals were kept at 0° from the time of death and the bones were dissected out at once from the smaller animals and within a few hours from the larger ones. The humeri were dissected free of muscle and tendon and the periosteum was removed by scraping with a scalpel or a sharp knife. The central portion of the shaft was then cut out with a hacksaw, the two cuts being made so that the central portion taken for analysis was practically cylindrical and excluded any of the 'buttressing' at the two ends. The cylinder of bone was split longitudinally and the marrow and any spongy bone lining the marrow cavity were removed. In newborn pigs the outer layer of the bone was vascular and this vascular layer was also removed so that only compact cortical bone was employed in the subsequent analyses. The cortical bone from both humeri was used when the pigs were small or undernourished. The bone was dried to constant weight in an air-oven at 98–100°, defatted with light petroleum (b.p. 40–60°) and, after being redried, ground to a fine powder with a pestle in a mortar and stored in a stoppered tube. Immediately before sampling for analysis the tube and its contents were heated for a few hours in an oven and cooled in a desiccator.

For determination of calcium and magnesium, portions of bone powder were ashed at 475° in an electric muffle furnace and an ash extract was prepared in HCl as described by McCance, Widdowson & Shackleton (1936). Calcium was determined in this by the classical micro-method of Kramer & Tisdall as modified by Clark & Collip and described by Hawk, Oser & Summerson (1954). To minimize the coprecipitation of magnesium oxalate (Vogel, 1951), the precipitate of calcium oxalate was allowed to form for only 2 hr. before being centrifuged. Magnesium was measured in the calcium-free supernatant by precipitation as the ammonium phosphate according to the procedure described by Economou-Mavrou & McCance (1958). All analyses were

made in duplicate and the procedure described gave theoretical recoveries of both calcium and magnesium when standard solutions, made up to have the calcium/magnesium ratio in bone, were analysed alone or after their addition to an ash extract of bone powder. Portions of this standard were analysed with each series of bone-ash extracts and the mean recovery of magnesium was $99.8\% \pm 1.29$ (S.E.M.).

For the determination of sodium, portions of the bone powder were ashed in platinum crucibles or in silica crucibles with a perfect glaze. The ash was extracted with 0.1 N-HNO₃ and the sodium measured with a flame photometer (Evans Electro-Selenium Ltd., Halstead, Essex) after removal of calcium according to the procedure of Bergstrom & Wallace (1954).

For the determination of citric acid, bone powder was extracted at room temperature with 10% (w/v) trichloroacetic acid (100 mg. of bone plus 25 ml. of acid). The use of EDTA instead of trichloroacetic acid (Borden, 1960) has not been found to give higher results. After the extraction, 3 ml. of 50% (v/v) H₂SO₄ was added and the mixture was boiled until its volume was about 15 ml. It was then centrifuged, the supernatant was siphoned off and the bone gel washed once with 10% (w/v) trichloroacetic acid. The combined supernatant and washings were made up to a known volume and the solution used for the determination of citric acid by the procedure advocated by Buffa & Peters (1950), with the modification that the pentabromoacetone was allowed to react with thiourea in borate buffer for colour development as described by Natelson, Pincus & Lugovoy (1948). Occasionally the method gave erratic results and the preparation of the reagents was found to be very important. The bromine water must be freshly made up and the solution of thiourea should never be warmed above 50°. The petroleum spirit must be pure (Natelson *et al.* 1948) and the standard solution of citrate must be kept sterile. Care must also be taken to prevent 'bumping' during the process of evaporation. Standards were carried through with every determination. The citrate in serum was measured by the method of Natelson *et al.* (1948).

RESULTS

Table 1 shows the results obtained for normal newborn pigs weighing about 1 kg., normal pigs 4 weeks old weighing about 5.5 kg., undernourished animals with similar weights but 1 year old and normal animals 1 year old weighing about 170 kg.

In normal animals the mean percentage of bone calcium fell between the time of birth and 4 weeks, but the difference was not statistically significant. There was, however, a highly significant increase between 4 weeks and 1 year. This increase was the expected effect of age. The suggestion of a fall between birth and 4 weeks of age is perhaps an indication in the pig of the small fall that has been found in other animals during suckling (Widdowson & Dickerson, 1964). The mean percentage for the undernourished animals at 1 year was not significantly different from that for normal animals of the same age.

There was a significant rise in the percentage of bone magnesium between birth and 4 weeks, but

Table 1. *The composition of dry fat-free cortical bone from pigs of various ages*

State and age	Calcium (g./100 g.)	Magnesium (g./100 g.)	Sodium (g./100 g.)	Citric acid (g./100 g.)	Ca/Mg ratio	Ca/Na ratio	Ca/citric acid ratio
A: Normal; at birth	25.9 ± 0.25 (11)	0.38 ± 0.014 (11)	0.602 ± 0.065 (11)	0.61 ± 0.072 (11)	69.3 ± 6.67 (11)	43.4 ± 4.23 (11)	43.0 ± 5.70 (11)
B: Normal; 1 month old	25.2 ± 1.14 (10)	0.44 ± 0.050 (10)	0.585 ± 0.064 (9)	1.70 ± 0.249 (6)	57.3 ± 6.67 (10)	43.2 ± 5.32 (9)	15.0 ± 2.84 (6)
C: Undernourished; 1 year old	28.8 ± 0.57 (9)	0.42 ± 0.040 (9)	0.719 ± 0.033 (9)	0.58 ± 0.099 (8)	70.4 ± 6.74 (9)	40.3 ± 1.88 (9)	51.9 ± 9.39 (8)
D: Normal; 1 year old	28.1 ± 0.54 (7)	0.41 ± 0.027 (7)	0.716 ± 0.028 (7)	0.67 ± 0.062 (7)	68.5 ± 4.25 (7)	39.7 ± 2.84 (7)	44.4 ± 1.06 (7)

Significance of difference between means (*t* test):

A versus B	N.S.	<i>P</i> < 0.005	N.S.	<i>P</i> < 0.001	<i>P</i> < 0.001	N.S.	<i>P</i> < 0.001
A versus D	<i>P</i> < 0.001	N.S.	<i>P</i> < 0.001	N.S.	N.S.	N.S.	N.S.
B versus D	<i>P</i> < 0.001	N.S.	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.005	N.S.	<i>P</i> < 0.001
B versus C	<i>P</i> < 0.001	N.S.	<i>P</i> < 0.001	<i>P</i> < 0.001	<i>P</i> < 0.001	N.S.	<i>P</i> < 0.001
D versus C	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

thereafter the mean percentage declined again and there was no significant difference between the values at birth and at 1 year in normal animals or in undernourished animals.

There was a small fall in the mean values for percentage sodium in bone between birth and 4 weeks of age, but the difference was not significant. There was, however, a highly significant difference between the values at birth and at 4 weeks and the values for normal animals 1 year old or for undernourished animals of that age. There was no significant difference between the last two values.

There was a highly significant rise in the mean percentage of bone citric acid between birth and 4 weeks and thereafter a highly significant fall to 1 year of age, whether the animals were normal at that time or undernourished. The rise between birth and 4 weeks was not the effect of aging to be expected from the literature (Zipkin *et al.* 1963), but the subsequent fall was in keeping with it.

There was a highly significant fall in the calcium/magnesium ratio between birth and 4 weeks and a highly significant rise again to 1 year. The mean for the undernourished pigs was higher than that for the normal animals of the same age but the difference was not significant. There were no significant differences between the calcium/sodium ratios at different times but the fall in the calcium/citric acid ratio between birth and 4 weeks and the subsequent rise in this ratio to 1 year were highly significant. The calcium/citric acid ratio for the undernourished animals was higher than that for the normal animals 1 year old, but this difference was not significant.

Table 2 shows the mean concentration of citric acid in the sera of normal pigs at birth, at 4 weeks of age and 1 year old or over. There was a highly significant fall between 4 weeks and 1 year of age as

Table 2. *Concentration of citric acid in the sera of normal pigs*

Values are expressed as means ± S.D. The differences between the newborn and 4-week-old animals, and between the adults and the 4-week-old animals, were highly significant (*P* < 0.001) by the *t* test. The differences between the newborn and the adult animals were not significant.

Age	No. of animals	Citric acid (mg./100 ml. of serum)
Newborn	7	2.5 ± 0.56
4 weeks	6	7.3 ± 1.58
1 year or over	7	1.9 ± 0.85

in bone and, as in bone also, a highly significant rise between birth and 4 weeks.

DISCUSSION

From 4 weeks to 1 year of age the changes in the calcium, magnesium, sodium and citric acid concentrations and in their ratios were all in keeping with the effects of aging where these have been recorded in the literature, and therefore they were not unexpected. The changes in concentration between birth and 4 weeks, however, were all in the opposite direction to those between 4 weeks and 1 year, and indicate that considerable changes in the metabolic pattern of the bone, and possibly in the size of the bone crystals, were taking place at this time.

There is a critical time during the suckling period of many species when the deposition of calcium does not keep pace with the growth of the bone and the calcium/collagen ratio falls (Weidmann & Rogers, 1958; Dickerson, 1962). The raised concentration of citrate and the low calcium/citrate ratio in the bones at 4 weeks may therefore reflect a higher

metabolic activity associated with this rapid growth. There is no suggestion that the fluctuations in citric acid content could have been caused by deficiencies of vitamin D or in parathyroid activity. The changes in the concentrations of magnesium and sodium at this age are unexplained but may be reflections of metabolic activity at the surface of the bone crystals.

The high concentration of citrate in the bones at 4 weeks of age seems to be a new observation and to be connected with the rise in the serum concentration (Hartles, 1965). The use of the pig as the experimental animal may have contributed materially to the discovery of these effects, for they clearly belong only to a limited period of time. It is possible that the concentration of citrate in pig bone may be even higher either before or after the age of 4 weeks, and the age at which the highest value is reached may be difficult to pinpoint. This problem deserves further investigation by those interested in the physiology of bone.

Undernutrition produced a bone that was only slightly larger than that of an animal of the same weight (McCance & Widdowson, 1962; McCance, 1964), but that was very different chemically. The chemical differences between the bones of the undernourished animals 1 year old and of bones from normal animals of that age, on the other hand, were never significant. In this respect therefore the bone of the undernourished animal resembled that of an animal of the same age. This was the conclusion also reached by Dickerson & McCance (1961) in their study of calcium/collagen ratios in bone and by McCance & Misra (1966) in their investigation of phosphate exchange with bone powder *in vitro*.

The authors are grateful to Mr Terry Cowen for his care of the animals and to Miss G. A. Norden and Mr Peter John for their technical assistance. Dr John Dickerson has given us valuable advice and help.

REFERENCES

- Bergstrom, W. H. (1955). *J. clin. Invest.* **34**, 997.
 Bergstrom, W. H. & Ruva, F. D. (1960). *Amer. J. Physiol.* **198**, 1127.
 Bergstrom, W. H. & Wallace, W. M. (1954). *J. clin. Invest.* **33**, 867.
 Borden, J. (1960). *Arch. oral Biol.* **2**, 319.
 Bowes, J. H. & Murray, M. M. (1935). *Biochem. J.* **29**, 2721.
 Budy, A. M. (1962). In *Radioisotopes and Bone*, p. 261. Ed. by McLean, F. C., Lacroix, P. & Budy, A. M. Oxford: Blackwells Scientific Publications.
 Buffa, P. & Peters, R. A. (1950). *J. Physiol.* **110**, 488.
 Cabak, V., Gresham, G. A. & McCance, R. A. (1962). *Brit. J. Nutr.* **16**, 635.
 Carlsson, A. & Hollunger, G. (1954). *Acta physiol. scand.* **31**, 317.
 Dickerson, J. W. T. (1962). *Biochem. J.* **82**, 47.
 Dickerson, J. W. T. & McCance, R. A. (1961). *Brit. J. Nutr.* **15**, 567.
 Dixon, T. F. & Perkins, H. R. (1956). In *The Biochemistry and Physiology of Bone*, p. 309. Ed. by Bourne, G. H. New York: Academic Press Inc.
 Economou-Mavrou, C. & McCance, R. A. (1958). *Biochem. J.* **68**, 573.
 Eeg-Larsen, N. (1951). *Acta physiol. scand.* **25**, Suppl. 89, 17.
 Forbes, G. B. (1960). *J. Pediat.* **56**, 180.
 Forbes, G. B., Mizner, G. L. & Lewis, A. (1957). *Amer. J. Physiol.* **190**, 152.
 Forbes, G. B., Tobin, R. B. & Lewis, A. (1959). *Amer. J. Physiol.* **196**, 69.
 Forscher, B. K. & Cohn, D. V. (1963). *Amer. Ass. Advanc. Sci. Publ. no. 75: Mechanisms of Hard Tissue Destruction*, p. 577.
 Fourman, P. (1960). *Calcium Metabolism and the Bone*, pp. 26, 53. Oxford: Blackwells Scientific Publications.
 Freeman, S. (1958). In *Bone as a Tissue*, p. 315. Ed. by Rodahl, K., Nicholson, J. T. & Brown, E. M. New York and London: McGraw-Hill Book Co.
 Hammett, F. S. (1925a). *J. biol. Chem.* **64**, 409.
 Hammett, F. S. (1925b). *J. biol. Chem.* **64**, 685.
 Harrison, H. C., Harrison, H. E. & Park, E. A. (1958). *Amer. J. Physiol.* **192**, 432.
 Harrison, H. E. (1956). *Amer. J. Med.* **20**, 1.
 Hartles, R. L. (1965). In *Calcified Tissues*, p. 93. Ed. by Richelle, L. J. & Dallemagne, M. J. University of Liège.
 Hawk, P. B., Oser, B. L. & Summerson, W. H. (1954). *Practical Physiological Chemistry*, 13th ed., pp. 644-646. London: J. and A. Churchill Ltd.
 Jenkins, G. N. (1966). *The Physiology of the Mouth*, 3rd ed., p. 40. Oxford: Blackwells Scientific Publications.
 McCance, R. A. (1960). *Brit. J. Nutr.* **14**, 59.
 McCance, R. A. (1964). *J. Pediat.* **65**, 1008.
 McCance, R. A. & Misra, R. N. (1966). *Biochem. J.* **100**, 489.
 McCance, R. A. & Widdowson, E. M. (1962). *Proc. Roy. Soc. B*, **156**, 326.
 McCance, R. A., Widdowson, E. M. & Shackleton, L. (1936). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 213.
 Munro, D. S., Satoskar, R. S. & Wilson, G. M. (1957). *J. Physiol.* **139**, 474.
 Murray, M. M. (1936). *Biochem. J.* **30**, 1567.
 Natelson, S., Pincus, J. B. & Lugovoy, J. K. (1948). *J. biol. Chem.* **175**, 745.
 Neuman, W. F. & Neuman, M. W. (1953). *Chem. Rev.* **53**, 1.
 Orent, E. R., Kruse, H. D. & McCollum, E. V. (1934). *J. biol. Chem.* **106**, 573.
 Pickering, D. E., Foran, R. F., Scott, K. G. & Crane, J. T. (1956). *Amer. J. Dis. Child.* **92**, 276.
 Rolf, D., Armstrong, C. M., Steiger, B. W., Audia, M. & White, H. L. (1959). *Amer. J. Physiol.* **196**, 1109.
 Vogel, A. I. (1951). *Quantitative Inorganic Analysis*, 2nd ed., p. 110. London: Longmans, Green and Co.
 Waasjö, E. & Eeg-Larsen, N. (1951). *Acta physiol. scand.* **25**, Suppl. 89, 84.
 Widdowson, E. M. & Dickerson, J. W. T. (1964). In *Mineral Metabolism*, vol. 2a, pp. 89-110. Ed. by Comar, C. L. & Bronner, F. New York and London: Academic Press Inc.
 Weidmann, S. M. & Rogers, H. J. (1958). *Biochem. J.* **69**, 338.
 Woodbury, D. M. (1956). *Amer. J. Physiol.* **185**, 281.
 Zipkin, I., Schraer, R., Schraer, H. & Lee, W. A. (1963). *Arch. oral Biol.* **8**, 119.