Skeletal Depletion in Hens Laying on a Low-calcium Diet

By T. G. TAYLOR and J. H. MOORE
Department of Agricultural Chemistry, University of Reading

(*Received* 5 *October* 1953)

It is generally accepted that the skeletons of birds and mammals act as a reservoir of minerals, in particular calcium and phosphorus, which can be drawn upon when dietary sources are inadequate for requirements. In birds these bone reserves are especially important during the egg-laying period. In the laying hen the rate of calcium deposition in the shell is often greater than the rate of absorption from the intestine, even when the calcium level of the diet conforms to accepted standards for egg production (Tyler, 1940). The immediate source of the additional mineral is thought to be the medullary bone contained in the marrow cavities of the long bones of the legs (Kyes & Potter, 1934; Bloom, Bloom & McLean, 1941; Bloom & Domm, 1941) and in other parts of the skeleton (Taylor & Moore, 1953). This highly specialized bone undergoes rapid changes during the egg-laying cycle, periods of intense bone formation alternating with periods of equally intense bone destruction (Bloom *et al.* 1941) and in birds that are in calcium balance the mineral reserves lost during the period of shell calcification are restored when active shell formation is not taking place. On a low-calcium diet the minerals for the calcification of the newly formed medullary bone are derived from structural bone (Benoit & Clavert, 1945), which becomes progressively depleted, and presumably a similar state of affairs exists in birds that are in negative calcium balance, even though their ration supplies theoretically adequate amounts of calcium (Morgan, Mitchell, Ringrose & Lease, 1942).

There is a limit to the amount of mineral matter that can be supplied by the skeleton, and as this limit is approached the egg-shells beome progressively thinner, until eventually soft-shelled eggs are laid (Common, 1938) or laying ceases altogether (Deobald, Lease, Hart & Halpin, 1936). Common (1938) has shown that 24% of the total body calcium of hens laying on a low-calcium diet can be withdrawn from the skeleton for shell formation, but the extent to which individual bones are depleted under these conditions does not appear to have been studied, and this is one of the objects of the present experiment. A second object of study is the effect of calcium depletion on the minerals of both cortical and medullary bone.

EXPERIMENTAL

The birds and their treatment

Eight Light Sussex pullets of the same strain, judged as being due to lay in about a month, were placed in separate metabolism cages on 15 October 1952. Their daily ration consisted of 100 g basal meal to which were added 5·5 g calcium carbonate and 2 ml. cod-liver oil. This mixture was made into a crumbly mash with 80 ml. of a solution containing 0·5% (w/v) NaCl and 0·0125% (w/v) $MnSO_4 \cdot 4H_2O$. After 1 week the daily meal allowance was increased to 110 g/bird, and this level of feeding was maintained throughout the rest of the experiment. (The birds were also given radioactive calcium and phosphorus and the experimental results concerning these radioactive elements will be reported elsewhere.) The basal meal was designed to be as low in Ca as possible, and its composition in parts by weight was as follows: maize 60, middlings 15, bran 10, groundnut meal 10 and dried yeast 10. The meal as fed contained 15·7% crude protein, 0·054% Ca and 0·546% P. After a 48 h preliminary period weekly collections of droppings were made and these continued until laying began, after which droppings were collected daily. Drinking water (distilled) was supplied *ad lib.* but no grit was given.

After they had laid three eggs on the normal-calcium ration, six of the birds were given a low-Ca diet by withholding the supplement of calcium carbonate from the standard ration. The other two birds, which were to act as controls, were killed immediately after laying their third egg on the normal-Ca ration. Of the remaining birds, two were allowed to lay two eggs, two four eggs, and two six eggs on the low-Ca

Table 1. *Days on the low-calcium diet, initial and final live weights and final weights of the dry fat-free skeletons of the experimental birds*

Bird no.	1	2	3	4	5	6	7	8
No. of eggs laid on the low-Ca diet	6	6	0	4	2	4	2	0
Days on the low-Ca diet	10	12	—	4	3	4	2	—
Initial live weight (g)	2359	2205	2138	2398	2240	2487	2290	2030
Final live weight (g)	2415	2242	2450	3188	2562	2622	2721	2290
Final weight of dry fat-free skeleton (g)	69·52	63·31	95·50	85·41	87·07	85·10	74·42	79·60

ration, and all were killed immediately after the required number of eggs had been laid. All eggs were recovered intact with the exception of the final ones laid by the six-egg birds. The shells of these eggs were thin and the birds began to eat them, but they were recovered from the crop and gizzard for analysis within a few minutes. The experimental lay-out, together with initial and final live weights and dry fat-free skeleton weights, are summarized in Table 1.

Preparation of bone for analysis

Each bird was skinned and eviscerated after it was killed, and as much muscular tissue as could be dissected from the carcass was removed. The skeleton was completely freed from soft tissue by heating it gently in liquid paraffin for 5–6 h during which time the temperature did not rise above 100°. The individual bones were then placed on filter-paper in an oven at 100–105° and subsequently extracted with a boiling alcohol-benzene mixture (2:1, v/v) for 24 h in a Soxhlet apparatus. They were then dried in the oven, cooled in a desiccator and weighed. The manner in which the skeleton was divided for weighing and for subsequent analysis is shown in Table 4.

The medullary bone was then separated from the structural bone. This was readily achieved with the tibia, femur, radius, ulna, coracoid and scapula by splitting the bone and scraping out the fine powdery medullary bone from the marrow cavity.

Other bones were first ground in a Wiley mill using a 20-mesh sieve, and the medullary was separated from the cortical bone by means of a 300-mesh sieve. With some samples it was necessary to grind gently in a small mortar after milling, as not all the medullary bone was fine enough to pass the 300-mesh sieve without this additional treatment. Using these techniques it was possible to achieve a fairly complete separation of the two types of bone as judged by appearance. Before analysis the cortical and scraped medullary-bone samples were also ground to pass a 300-mesh sieve.

Preliminary treatment of materials

Droppings, egg contents and egg-shells were subjected to a nitric acid-perchloric acid digestion by the method of Gerritz (1935) before the determination of calcium and phosphorus. Droppings were first dried in an oven at 100–105° for 24 h and then ground in the laboratory mill. Weighed samples of egg contents were taken for digestion after mixing them in a Waring Blendor. The complete shells were digested separately.

Weighed samples of dry 300-mesh bone were ashed at 600° in an electric muffle furnace for 24 h and, after weighing, the ash was dissolved in N-nitric acid.

Analytical methods

Calcium. Determinations on droppings and egg-shell digests were made by precipitation as oxalate and titration with permanganate, and on egg contents by the semi-micro method of Wheatley (1944). The Ca of bone-ash solutions was precipitated as oxalate in 15 ml. centrifuge tubes and, after washing, the precipitate was dissolved in 2 ml. 0·1 N-ceric sulphate, the excess being titrated with 0·025 N-ferrous sulphate using *o*-phenanthrolene ferrous complex as indicator.

Phosphorus. Phosphorus was determined by the colorimetric method of Koenig & Johnson (1942) as modified by Kitson & Mellon (1944) using a Spekker absorptiometer.

RESULTS

Pre-laying storage of minerals. The retention of Ca and P during the 10 days or so before laying was calculated from the balance results. The figures are given in Table 2. Birds 1 and 2 came into lay sooner than expected, and it is probable that pre-laying storage of minerals began before the experimental period, so that the weights recorded represent minimum values. The Ca:P ratio of the mineral matter retained during this period varied from 2·93 to 6·79 in individual birds with a mean of 4·25, figures of the same order as those reported by Common (1936) for pullets fed a ration adequately supplied with calcium.

Egg-shell Ca. The weight of shell Ca in the eggs laid on the low-Ca ration, together with the same values expressed as a percentage of egg weight, are given in Table 3. The eggs laid by each bird were lettered in sequence, eggs *A*, *B* and *C* being laid on the normal-Ca diet. In general, there was a reduction in the percentage of shell Ca immediately the birds were placed on the low-Ca diet, a reduction which became progressively greater with each egg laid. These results are in agreement with those reported by Common (1938).

Skeletal depletion during the low-Ca régime. Since the intake of Ca and P and the losses in eggs and droppings were known, the amounts of these elements lost from the

Table 2. *Calcium and phosphorus stored before laying and lost during the low-calcium period, and calculated percentage losses from the skeleton during the low-calcium period*

	No. of eggs laid on low-Ca diet: 0		2		4		6	
	Bird no. 3	Bird no. 8	Bird no. 5	Bird no. 7	Bird no. 4	Bird no. 6	Bird no. 1	Bird no. 2
Information about storage before laying								
Storage period (days)	11	12	14	15	10	13	10	8
Ca retained (g)	6·52	4·90	4·43	6·75	7·53	5·52	4·65	4·31
P retained (g)	1·36	1·27	1·51	1·67	1·56	0·81	1·14	1·16
Ca : P ratio of retained mineral	4·80	3·85	2·93	4·03	4·84	6·79	4·07	3·72
Ca balance								
Total loss of body Ca (g)	—	—	3·61	3·53	6·19	6·83	9·02	8·37
Ca in skeleton at beginning of low-Ca period (g)	—	—	23·77	20·33	25·64	26·20	23·54	21·80
Ca in skeleton at slaughter (g)	22·54	18·97	20·16	16·80	19·45	19·37	14·52	13·43
P balance								
Total loss of body P (g)	—	—	1·19	1·63	2·35	2·47	2·67	2·53
P in skeleton at beginning of low-Ca period (g)*	—	—	10·89	9·31	11·74	12·00	10·78	9·98
P in skeleton at slaughter (g)	10·31	8·70	9·25	7·86	8·99	9·10	6·99	6·42
P lost from skeleton (g)	—	—	1·64	1·45	2·75	2·90	3·79	3·56
P stored in soft tissues (g)	—	—	0·45	−0·18	0·40	0·43	1·12	1·03
Loss of Ca from skeleton (%)	—	—	15·2	17·4	24·2	26·1	38·3	38·4
Loss of P from skeleton (%)	—	—	15·1	15·6	23·4	24·1	35·1	35·7
Ca : P ratio of total loss	—	—	2·20	2·44	2·25	2·36	2·38	2·35

* Calculated on the assumption that the Ca : P ratios of the skeletons were 2·184, the mean for the control birds.

Table 3. *Shell calcium in eggs laid by birds placed on the low-calcium ration, expressed as weight and as a percentage of egg weight*

		Two-egg birds				Four-egg birds				Six-egg birds			
		Bird no. 5		Bird no. 7		Bird no. 4		Bird no. 6		Bird no. 1		Bird no. 2	
Diet	Egg	Wt. (g)	Percentage of egg wt.	Wt. (g)	Percentage of egg wt.	Wt. (g)	Percentage of egg wt.	Wt. (g)	Percentage of egg wt.	Wt. (g)	Percentage of egg wt.	Wt. (g)	Percentage of egg wt.
Normal-Ca	*A*	1·37	3·44	1·56	3·63	1·61	3·53	1·68	3·33	1·60	3·48	1·54	3·54
	B	1·66	4·04	1·71	3·97	1·83	3·51	1·88	3·83	1·78	3·48	1·65	3·78
	C	1·62	3·73	1·80	3·91	1·85	3·71	1·79	3·41	1·78	3·61	1·84	3·72
Low-Ca	*D*	1·68	3·73	1·75	3·49	1·56	3·29	1·75	3·28	1·53	3·14	1·63	3·61
	E	1·79	3·84	1·52	3·42	1·73	3·45	1·66	3·16	1·71	3·25	1·90	2·72
	F	—	—	—	—	1·43	2·84	1·61	3·10	1·57	3·22	1·47	2·97
	G	—	—	—	—	1·24	2·50	1·57	2·96	1·55	3·20	1·20	2·59
	H	—	—	—	—	—	—	—	—	1·35	2·52	0·99	1·99
	I	—	—	—	—	—	—	—	—	1·02	2·04*	0·77	1·54*
Total weight on low-Ca diet		3·47	—	3·27	—	5·96	—	6·59	—	8·72	—	7·97	—

* Estimated (egg broken).

body during the low-Ca period could be calculated. As approximately 98% of the body Ca of pullets is present in the bones (Halnan, 1936; Common, 1938) and since the amount of Ca finally present in the skeleton was determined, the percentage loss of skeletal Ca in this period could also be calculated. The percentage loss of P from the skeleton could not be calculated in this way as 20% or so of the body P is present in the soft tissues, nor could it be assumed that the ovaries contained the same amount of vitellin and phospholipid P at the end of the low-Ca period as at the beginning. In addition, the birds were gaining weight during the experiment. The amount of P originally present in the skeletons can be calculated indirectly, however, on the assumption that their Ca:P ratios at the beginning of the low-Ca régime were the same as in the two control birds, which were respectively 2·19 and 2·18. Using the mean of these figures the percentage loss of P during the low-Ca period can be estimated. Data on the skeletal loss of Ca and P are presented in Table 2.

The percentage loss of P from the skeleton was in all birds slightly less than the percentage loss of Ca. With the exception of bird 7, the calculated loss of skeletal P was greater than the negative balance, which means that not all of the P lost from the bones was excreted and it must presumably have been stored in the soft tissues. With birds 1 and 2 which laid six eggs on the deficient diet the difference between the amount of P calculated to have been lost from the skeleton and that excreted in eggs and droppings amounted to over 1 g. Even if the Ca:P ratio of the skeletons of these birds at the beginning of the low-Ca period had been 2·25 instead of 2·18, the calculated loss of skeletal P would have been 0·80 g and 0·75 g greater than the negative P balance for birds 1 and 2 respectively.

Ash weights of individual bones. Mineral depletion can most usefully be studied by comparing the total amount of ash present in individual bones. As the experimental birds differed somewhat in both live weight and skeletal weight a common basis of comparison was necessary and the standard chosen was a dry, fat-free skeleton weighing 100 g at the time the third egg was laid on the normal-Ca diet when the control birds were killed and the low-Ca régime began. The controls had, respectively, 23·6 and 23·8% Ca in their skeletons and the mean of these figures was taken as the Ca percentage of the skeletons of the other birds at the end of the normal-Ca period. Using this figure their standard skeleton weights at the end of the normal-Ca period were calculated, as the weight of Ca present at this time could be deduced from the balance and slaughter data.

The mean ash weights for the bones of the two birds on each treatment, corrected to the standard skeletal weight, are given in Table 4, together with the ash weights of bones from depleted birds expressed as a percentage of the ash weights of the controls.

It will be observed that the loss of minerals from the skeleton was far from uniform. Initially the ilium, ischium and pubis, ribs, femur and fibula suffered the greatest depletion with the thoracic and coccygeal vertebrae and the sternum losing only slightly less. During the calcification of the second two eggs on the low-Ca diet the tibia, coracoid, coccygeal vertebrae and wing bones showed the greatest percentage loss of ash, but the bones that were most severely depleted earlier (with the exception of the coccygeal vertebrae) did not suffer much further loss at this stage. A much more generalized loss of minerals took place during the calcification of the fifth and sixth eggs, almost all bones having made some contribution; the cervical and lumbo-sacral vertebrae, scapula, coracoid, clavicle and sternum lost particularly heavily. The bones that suffered the greatest overall percentage loss were the ribs, sternum, ilium, ischium and pubis, coccygeal vertebrae and fibula, all of which lost at least 50% of the minerals originally present, with the ribs and sternum having lost approximately 60%.

Table 4. *Mean ash weights (g) of the bones of the birds in each treatment corrected to the standard skeletal weight* (A) and mean ash weights of the bones of depleted birds expressed as a percentage of the ash weights of the control birds (B)*

	A				B			
	No. of eggs laid on low-Ca diet							
Bone	0	2	4	6	0	2	4	6
Femur	7·25	5·06	4·48	4·00	100	69·8	61·7	55·1
Tibia	8·59	7·66	6·65	5·93	100	89·1	77·4	69·0
Fibula	0·60	0·42	0·32	0·31	100	69·9	52·5	50·5
Metatarsus	4·00	3·95	3·52	3·19	100	98·7	87·9	79·7
Toes	2·26	2·41	2·04	2·15	100	106·6	90·3	95·0
Humerus	4·93	4·95	4·14	3·34	100	100·5	84·1	67·8
Radius	1·14	1·08	0·97	0·83	100	95·0	85·1	73·1
Ulna	2·70	2·48	2·06	1·71	100	91·8	76·2	63·4
Metacarpals and wing digits	1·82	1·79	1·57	1·18	100	98·3	86·2	64·5
Skull	2·49	2·33	2·28	2·23	100	93·7	91·5	89·4
Cervical vertebrae	4·32	3·83	3·96	3·21	100	88·8	91·8	74·3
Thoracic vertebrae	2·10	1·51	1·28	1·12	100	72·0	61·1	53·5
Lumbo-sacral vertebrae	1·85	1·51	1·60	1·03	100	81·7	86·4	55·7
Coccygeal vertebrae	0·51	0·40	0·25	0·25	100	78·2	49·7	49·7
Sternum	4·01	3·06	2·72	1·67	100	76·4	67·8	41·5
Clavicle	0·60	0·50	0·45	0·35	100	82·4	74·2	57·1
Ilium, ischium and pubis	5·59	2·95	3·16	2·53	100	52·7	56·5	45·2
Ribs	2·42	1·45	1·33	0·96	100	59·8	55·1	39·8
Coracoid	2·15	1·97	1·64	1·25	100	92·0	76·4	58·3
Scapula	1·34	1·37	1·18	0·90	100	102·1	88·3	67·3
Total	60·65	50·67	45·61	38·12	100	83·5	75·2	62·8

* See p. 116.

The bones that most successfully maintained their integrity were the skull and the toes, in which mineral losses were negligible. The cervical and lumbo-sacral vertebrae were not appreciably affected until more than four eggs had been laid.

It is interesting to observe which bones were responsible for providing the greatest absolute amounts of minerals during the different stages of skeletal depletion. The percentage contribution of the individual bones is given in Table 5. The femur, ilium, ischium and pubis together supplied almost 50% of the minerals for the calcification of the first two eggs, with the tibia, ribs and sternum each having contributed over 9%. During the period when the third and fourth eggs were calcified the tibia, humerus and femur gave up the greatest amounts, and in the final stage the sternum made the greatest contribution, while a large number of different bones supplied from 5 to 10% of the total. Over 50% of the total mineral matter released from the skeleton for the calcification of the six eggs was supplied by the tibia, femur, sternum, ilium, ischium and pubis together.

Medullary bone. The amount of medullary bone present in the skeletons of the experimental birds was surprisingly large. Previous workers (Bloom *et al.* 1941; Kyes & Potter, 1934) have reported its presence exclusively in the long bones of the legs, except for Zondek (1936) who found it also in the vertebral column of young cockerels after treatment with oestrogen. In the pullets used in this experiment it was found in all bones, although the skull, humerus, metacarpals, wing digits, metatarsus and toes contained only small amounts (Taylor & Moore, 1953). Particularly large amounts were present in the sternum, ribs, ilium, ischium and pubis. The percentage of medullary bone in the individual bones of four birds (one from each treatment) are

Table 5. *Mean ash losses for individual bones expressed as a percentage of total skeletal loss at each stage of skeletal depletion*

Bone	Stage of depletion induced by the laying on the low-Ca diet of the: First two eggs	Second two eggs	Third two eggs	All six eggs
Femur	21·5	10·6	6·3	14·4
Tibia	9·2	18·2	9·5	11·8
Fibula	1·8	1·9	0·2	1·3
Metatarsus	0·5	7·9	4·3	3·6
Toes	—	6·7	—	0·5
Humerus	—	14·6	10·6	7·0
Radius	0·6	2·1	1·8	1·4
Ulna	2·2	7·7	4·5	4·4
Metacarpals and wing digits	0·3	4·0	5·2	2·9
Skull	1·6	1·0	0·7	1·2
Cervical vertebrae	4·7	—	10·0	4·9
Thoracic vertebrae	5·8	4·1	2·1	4·3
Lumbo-sacral vertebrae	3·3	—	7·5	3·6
Coccygeal vertebrae	1·0	2·6	—	1·1
Sternum	9·3	6·3	13·9	10·4
Clavicle	1·1	1·0	1·4	1·2
Ilium, ischium and pubis	26·0	—	8·3	13·6
Ribs	9·6	2·1	4·9	6·5
Coracoid	1·7	6·1	5·1	4·0
Scapula	—	3·4	3·7	1·9
Total	100·2	100·2	100·0	100·0

given in Table 6. The effect of laying on a low-Ca diet was to increase the proportion of medullary bone, the birds that laid six eggs on this diet having almost twice as much medullary bone, when expressed as a percentage of total bone weight, as the controls. There was, however, a tendency for the medullary bone to be less highly calcified as skeletal depletion advanced, but in spite of the fact that all birds were killed at the same stage of the laying cycle there was a considerable variation in its ash percentage quite unrelated to treatment, as can be seen from Table 7, which shows the ash percentage of medullary bone from the tibiae, femora, and entire skeleton of the eight pullets. The figures for total medullary ash weight, corrected to standard skeletal weight (Table 8), convey most clearly the effect of the low-Ca diet, and although the amounts are somewhat variable, there appears to be a tendency for the level of medullary ash to be maintained in spite of a drastic fall in the amount of cortical ash. This observation was not unexpected in view of the accepted function of medullary bone to act as a readily available source of Ca for the egg shell, particularly when dietary sources are inadequate.

Table 6. *Percentage of medullary bone in individual bones of one bird from each treatment*

(Dry, fat-free basis)

Bone	No. of eggs laid on low-Ca diet: 0, Bird no. 3	2, Bird no. 5	4, Bird no. 4	6, Bird no. 1
Femur	21·8	32·8	33·8	39·1
Tibia	12·4	13·1	19·7	34·5
Fibula	7·9	19·0	11·9	24·5
Metatarsus	5·1	3·6	2·7	4·5
Toes	1·3	4·0	1·6	2·8
Humerus	4·6	6·8	8·6	14·7
Radius	3·6	3·6	8·4	7·9
Ulna	4·0	6·7	10·6	23·9
Metacarpals and wing digits	4·3	6·5	4·5	17·9
Skull	7·2	8·3	2·0	7·5
Cervical vertebrae	7·0	10·0	9·0	13·9
Thoracic vertebrae	10·3	15·9	22·6	31·7
Lumbo-sacral vertebrae	6·7	21·8	30·1	16·3
Coccygeal vertebrae	20·5	16·0	34·0	56·8
Sternum	15·5	18·3	20·8	28·7
Clavicle	12·2	14·7	14·5	16·0
Ilium, ischium and pubis	20·8	32·0	27·4	36·5
Ribs	29·2	39·9	39·8	51·8
Coracoid	3·7	10·3	13·8	21·3
Scapula	16·1	9·8	14·1	14·7
Total skeleton	11·7	15·0	16·6	23·6

Table 7. *Percentage ash in medullary bone from tibiae and femora and from the entire skeletons of all the birds*

	No. of eggs laid on low-Ca diet											
	0			2			4			6		
Bone	Bird no. 3	Bird no. 8	Mean	Bird no. 5	Bird no. 7*	Mean	Bird no. 4	Bird no. 6	Mean	Bird no. 1	Bird no. 2*	Mean
Tibiae	38·3	48·8	43·6	40·6	17·4	29·0	43·6	49·1	46·3	28·0	17·8	22·9
Femora	41·1	48·8	45·0	46·9	17·4	32·2	36·4	41·8	39·1	30·7	17·8	24·3
Entire skeleton, weighted mean	46·7	51·3	49·0	47·3	35·6	41·5	41·4	47·5	44·5	36·4	30·9	33·6

* Bone from tibiae and femora bulked for analysis.

Ash percentage in cortical bone. Ash-percentage figures for some of the bones that suffered the most severe mineral depletion (as shown by total ash weights) are compared in Table 9 with figures for those that suffered least. With the exception of the femur there was a tendency for the bones subject to severe depletion to have a lower ash percentage as the degree of depletion became more extreme, but the reduction was not great and might well have been due to contamination of the cortical bone with non-osseous organic matter (particularly with haematopoietic tissue) or with medullary bone. Reduction in ash percentage due to contamination of this nature would become increasingly great with increasing loss of total ash, and it is perhaps significant that the femur, in which contamination was unlikely to be great, did not show a consistent fall in ash percentage with increasing severity of depletion.

Table 8. *Effect of laying on a low-calcium diet on the amount of medullary bone and medullary bone ash of all the birds*

	No. of eggs laid on low-Ca diet											
	0			2			4			6		
Variable studied	Bird no. 3	Bird no. 8	Mean	Bird no. 5	Bird no. 7	Mean	Bird no. 4	Bird no. 6	Mean	Bird no. 1	Bird no. 2	Mean
Medullary bone expressed as percentage of total skeletal weight	11·7	14·0	12·9	15·0	13·9	14·5	16·6	23·1	19·8	23·6	20·1	21·8
Medullary ash expressed as percentage of total ash	9·1	10·0	9·6	12·1	8·5	10·3	11·6	18·7	15·2	15·6	11·3	13·5
Total medullary ash corrected to standard skeletal weight (g)	5·47	6·09	5·78	6·16	4·31	5·24	5·37	8·45	6·91	6·00	4·28	5·14
Total cortical ash corrected to standard skeletal weight (g)	54·8	54·9	54·9	44·6	46·3	45·5	40·6	36·8	38·7	32·3	33·6	33·0

Table 9. *Ash percentage of bones that suffered the most severe mineral depletion and of those that suffered least*

	No. of eggs laid on low-Ca diet											
	0			2			4			6		
Bone	Bird no. 3	Bird no. 8	Mean	Bird no. 5	Bird no. 7	Mean	Bird no. 4	Bird no. 6	Mean	Bird no. 1	Bird no. 2	Mean
	Most depletion											
Ribs*	60·4	61·7	61·1	60·0	61·4	60·7	60·3	60·3	60·3	57·0	57·1	57·1
Sternum*	60·4	61·7	61·1	59·3	61·4	60·4	59·7	60·6	60·2	57·7	57·1	57·4
Ilium, ischium and pubis	59·1	61·7	60·4	60·8	61·4	61·1	58·5	58·8	58·7	56·9	57·1	57·0
Thoracic vertebrae	59·7	58·6	59·2	58·1	60·0	59·1	59·2	59·7	59·5	55·9	55·9	55·9
Femur	68·9	67·1	68·0	65·9	68·0	67·0	66·9	65·6	66·3	67·9	68·8	68·4
	Least depletion											
Skull	60·5	58·6	59·6	56·3	60·0	58·1	59·5	59·7	59·6	61·6	61·6	61·6
Metatarsus	59·9	57·3	58·6	58·2	57·8	58·0	61·6	61·6	61·6	59·4	58·8	59·1
Radius	62·3	63·8	62·1	62·4	63·0	62·7	63·8	65·7	64·8	61·6	63·4	62·5
Entire skeleton, weighted mean	62·1	62·4	62·3	60·4	61·9	61·2	61·5	62·0	61·8	60·4	61·1	60·8

* In birds nos. 2, 7 and 8 these bones were bulked before ashing.

Composition of bone ash. Weighted mean figures for the percentages of Ca and P in the ash of medullary and cortical bone, together with the corresponding Ca:P ratios are given in Table 10. In general, medullary ash had slightly less Ca and more P than cortical ash and, as might be expected, depletion of the body of Ca resulted in the ash of both medullary and cortical bone becoming richer in P and poorer in Ca. In

Table 11 the Ca:P ratios of cortical bone from severely depleted bones are compared with those of bones that suffered only slightly, and it is clear from these figures that a Ca-rich fraction of bone mineral was removed during the course of the skeletal depletion, leaving behind a mineral with a Ca:P ratio that became progressively lower with increasing severity of depletion. It is interesting to observe that although the skull, metatarsus and toes lost quite small amounts of ash, their Ca:P ratios followed the same trend as those for the bones that suffered severely.

Table 10. *Composition of cortical and medullary bone ash (weighted means) of all the birds*

		Ash of cortical bone				Ash of medullary bone			
No. of eggs laid on low-Ca diet		0	2	4	6	0	2	4	6
Percentage of Ca	Individual birds	39·2	39·8	39·2	38·5	38·6	38·4	38·1	36·4
		39·1	38·9	38·9	38·8	38·2	37·1	38·3	36·5
	Mean	39·2	39·4	39·1	38·7	38·4	37·8	38·2	36·5
Percentage of P	Individual birds	17·9	18·2	18·0	18·3	17·9	18·1	18·4	18·6
		17·9	18·1	18·2	18·4	17·6	18·4	18·3	18·9
	Mean	17·9	18·2	18·1	18·4	17·8	18·3	18·4	18·8
Ratio, Ca : P	Individual birds	2·19	2·19	2·18	2·10	2·15	2·13	2·07	1·96
		2·18	2·15	2·13	2·11	2·16	2·02	2·10	2·04
	Mean	2·19	2·17	2·16	2·11	2·16	2·08	2·09	2·00

Table 11. Ca:P *ratios of cortical bone ash from bones that suffered the most severe mineral depletion and from those that suffered least, for all the birds*

	No. of eggs laid on low-Ca diet											
	0			2			4			6		
Bone	Bird no. 3	Bird no. 8	Mean	Bird no. 5	Bird no. 7	Mean	Bird no. 4	Bird no. 6	Mean	Bird no. 1	Bird no. 2	Mean
	Most depletion											
Ribs	2·18	2·18	2·18	2·12	2·11	2·12	2·10	2·11	2·11	1·98	2·04	2·01
Sternum	2·17	2·18	2·18	2·17	2·11	2·14	2·16	2·10	2·13	2·02	2·04	2·03
Ilium, ischium, pubis	2·17	2·18	2·18	2·16	2·11	2·14	2·10	2·08	2·09	1·98	2·04	2·01
	Least depletion											
Skull	2·18	2·17	2·18	2·17	2·14	2·16	2·20	2·13	2·17	2·11	2·08	2·10
Metatarsus	2·22	2·23	2·23	2·22	2·19	2·21	2·23	2·20	2·22	2·17	2·16	2·17
Toes	2·24	2·23	2·23	2·25	2·23	2·24	2·22	2·20	2·21	2·17	2·20	2·19

Table 12 gives the Ca:P ratios of medullary bone from the tibiae and femora, obtained quite uncontaminated with cortical bone. Apart from bird no. 7 which was exceptional, having both low total ash and low Ca:P ratio, the fall in Ca:P ratio was not marked until six eggs had been laid on the Ca-deficient diet.

DISCUSSION

During the pre-laying period all eight pullets retained minerals with a Ca:P ratio much higher than that of normal bone, and Common (1933, 1936, 1938) observed the same phenomenon in some of his birds when a high-Ca ration (approx. 2% Ca) was fed, but not on a low-Ca ration (0·26%Ca). A satisfactory explanation for these findings has yet to be put forward. It has been suggested that the new bone laid down during this period might have a higher Ca:P ratio than normal bone, but in view of the finding that the mineral composition of both medullary and cortical bone from the control birds in the present experiment was normal, this possibility appears to be most unlikely.

A second possible explanation is that during the pre-laying period the Ca:P ratio of the whole skeleton increases considerably. Using the mean weights of Ca and P found to have been stored during the pre-laying period in the present experiment and assuming that an average skeleton contains 24·3 g Ca and 10·8 g P at the end of this period, it can be calculated that the Ca:P ratio would have to change from 1·97 to 2·25. A change of this magnitude in less than a fortnight is difficult to conceive, but in view of the observed Ca:P ratios of 2·08 and 2·09 found for the complete skeletons of birds 1 and 2 after they had laid six eggs on the low-Ca diet, compared with control values of 2·18 and 2·19, this possibility cannot be dismissed. Unfortunately there is no reliable method of calculating the Ca:P ratios of the skeletons of our experimental

Table 12. Ca:P *ratios of medullary bone from tibiae and femora for all the birds*

	No. of eggs laid on low-Ca diet											
	0			2			4			6		
Bone	Bird no. 3	Bird no. 8	Mean	Bird no. 5	Bird no. 7	Mean	Bird no. 4	Bird no. 6	Mean	Bird no. 1	Bird no. 2	Mean
Tibiae	2·11	—	—	2·06	—	—	2·10	2·10	2·10	1·91	—	—
Femora	2·13	—	—	2·11	—	—	2·04	2·09	2·07	1·93	—	—
Mean for both bones	2·12	2·16*	2·14	2·09	1·79*	1·94	2·07	2·10	2·09	1·92	1·76*	1·84
Ca in last egg on low-Ca diet %	—	—	—	3·84	3·42	—	2·50	2·96	—	2·04	1·54	—

* Bulked for analysis.

birds before and after pre-laying storage as both skeletal and soft-tissue metabolism contribute to the observed P balances.

A third possibility is that P from extra-skeletal sources is available for bone mineralization at this time, a suggestion tentatively put forward by Common (1938) and which is perhaps strengthened by the observation that P appears to have been retained in the soft tissues of the pullets that were laying on a Ca-deficient diet in the present experiment. Further work is in progress to investigate the whole question of pre-laying storage and of the possible storage of P in the soft tissues.

As a result of histological studies (McLean & Bloom, 1940; Bloom *et al.* 1941) it has been suggested that the removal of minerals from the skeleton can occur in vivo only as a result of bone resorption, in which both organic matrix and mineral matter are removed together. If this view is correct loss of minerals should not bring about a reduction in ash percentage, and the results of the present investigation tend to support it. The mean ash content of the femora of the six-egg birds that had lost 45 % of their mineral matter was actually higher than that of the controls. If, however, the progressive reduction in ash percentage observed in the ribs, sternum, ilium, ischium and pubis with increasing severity of depletion was in fact due to demineralization and not to the presence of non-osseous organic matter, it could have taken place only to a limited extent, as a loss in total ash of over 50% was in these bones associated with a reduction in ash percentage of little more than 5%.

Sobel, Rockenmacher & Kramer (1945), in an experiment in which groups of rats were fed on rations deficient in Ca, P, or both, have shown that the composition of bone mineral is related to the composition of the blood serum from which it is precipitated, and it seems likely that the blood picture of the hens on the Ca-deficient diet was not unlike that of the rats of the low-Ca, high-P treatment. If this was so, the low Ca:P ratios of the medullary bone from birds 1, 2 and 7 were reflexions of low diffusible Ca, high inorganic phosphate and low bicarbonate levels in the blood induced by egg laying on the Ca-deficient ration. Similarly, the fact that the Ca:P ratios of the medullary bone of the birds that laid two and four eggs on the low-Ca diet were not in general greatly depressed, suggests that these birds were fairly successful in maintaining the diffusible Ca level in their blood, a suggestion which is strengthened by the fact that these birds were still capable of laying eggs with reasonably good shells. The ash content of the medullary bone from birds 2 and 7 was very low (mean 17·63%) and the extremely low Ca:P ratio of this bone might be due in part to the presence of considerable amounts of organically combined P.

The two birds that laid six eggs on the deficient ration each lost over 38% of the total skeletal Ca initially present, one in 10 and the other in 12 days, a severity of depletion quite remarkable in such a short time. Common's (1938) low-Ca birds lost 24% of their skeletal Ca, one after laying eleven eggs in 40 days and the other after laying thirteen eggs in 69 days, but his ration contained 0·260% Ca compared with 0·054% in the present experiment. Whereas Common's birds were laying soft-shelled eggs at the time they were killed, the final eggs laid by birds 1 and 2 of the present experiment were by no means soft-shelled, so that it seems probable that a still higher degree of depletion could have been achieved. The fact that the total medullary bone of birds 1 and 2 contained respectively 2·17 and 1·43 g of Ca at slaughter is further evidence that calcification of the egg-shell was still possible.

After birds 1 and 2 had laid six eggs on a Ca-deficient diet their complete skeletons had Ca:P ratios of 2·08 and 2·09 respectively, compared with values of 2·18 and 2·19 for the controls. From the balance data and bone analysis (Table 2) it has been calculated that the Ca:P ratios of the mineral matter lost during the low-Ca régime were 2·38 and 2·35 respectively. It appears therefore that the bones of laying pullets are capable of losing Ca and P at a higher Ca:P ratio than normal bone, as Tyler (1940) had already inferred from balance experiments. A detailed chemical study of the changes taking place during skeletal depletion is in progress.

SUMMARY

1. Using balance and slaughter techniques the effects of progressive mineral depletion on the skeletons of pullets have been studied by allowing them to lay no, two, four or six eggs on a calcium-deficient diet. During the pre-laying period and up to the time the third egg was laid the birds were maintained on a high (1·9%) Ca ration.

2. The mean weights of Ca and phosphorus stored by the eight pullets during the pre-laying period were 5·58 and 1·31 g respectively, giving a mean Ca:P ratio of 4·25.

3. There was a reduction in the percentage of shell Ca when the birds were placed on the low-Ca diet. The reduction became progressively greater with each egg laid.

4. After the laying of two, four or six eggs on the deficient diet the mean skeletal losses of Ca were 16·3, 25·2 and 38·4 % respectively. The percentage loss of P was in all birds slightly less than the percentage loss of Ca.

5. During the low-Ca period five out of six birds excreted less P than was calculated to have been lost from the skeleton. This P, which amounted to over 1 g in the birds that had laid six eggs on the deficient diet, is thought to have been stored in the soft tissues.

6. The mean ash weights of individual bones from the two birds on each of the low-Ca treatments were compared with controls. The bones that suffered the greatest proportional losses were the ribs, sternum, ilium, ischium and pubis, coccygeal vertebrae, and fibula, all of which, in the birds that had laid six eggs on the deficient ration, lost over 50 % of the mineral matter originally present. The bones that suffered least were the skull, metatarsus and toes.

7. The level of medullary bone ash tended to be maintained during the low-Ca régime in spite of a severe fall in the amount of cortical ash.

8. The percentage of ash in the depleted bones showed a slight reduction in most birds.

9. Severely depleted bones showed a much greater reduction in the Ca:P ratio than those bones that had suffered only slightly.

10. The significance of the high Ca:P ratio of the mineral stored in the pre-laying period and of the changes in bone composition consequent on mineral depletion is discussed.

REFERENCES

Benoit, J. & Clavert, J. (1945). *C.R. Soc. Biol., Paris*, **139**, 737.

Bloom, W., Bloom, M. A. & McLean, F. C. (1941). *Anat. Rec.* **81**, 443.

Bloom, W. & Domm, L. V. (1941). *Anat. Rec.* **81**, 91 (Suppl.)

Common, R. H. (1933). *J. agric. Sci.* **23**, 555.

Common, R. H. (1936). *J. agric. Sci.* **26**, 85.

Common, R. H. (1938). *J. agric. Sci.* **28**, 347.

Deobald, H. J., Lease, E. J., Hart, E. B. & Halpin, J. G. (1936). *Poult. Sci.* **15**, 179.

Gerritz, H. W. (1935). *Industr. Engng Chem. (Anal.)*, **7**, 167.

Halnan, E. T. (1936). *World's Poult. Congr.* VI. *Berlin*, **1**, 53.

Kitson, R. E. & Mellon, M. G. (1944). *Industr. Engng Chem. (Anal.)*, **16**, 379.

Koenig, R. A. & Johnson, C. R. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 155.

Kyes, P. & Potter, T. S. (1934). *Anat. Rec.* **60**, 377.

McLean, F. C. & Bloom, W. (1940). *Anat. Rec.* **78**, 333.

Morgan, C. L., Mitchell, J. H., Ringrose, R. C. & Lease, E. J. (1942). *Poult. Sci.* **21**, 212.

Sobel, A. E., Rockenmacher, M. & Kramer, B. (1945). *J. biol. Chem.* **159**, 159.

Taylor, T. G. & Moore, J. H. (1953). *Nature, Lond.*, **172**, 504.

Tyler, C. (1940). *Biochem. J.* **34**, 202.

Wheatley, V. R. (1944). *Analyst*, **69**, 207.

Zondek, B. (1936). *Lancet*, **231**, 842.