Br. J. Nutr. (1969), **23**, 523

A study on the effect of vitamin D in rats maintained on diets with different calcium and phosphorus content but with the same high ratio of calcium to phosphorus

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(*Received* 29 *October* 1968—*Accepted* 28 *January* 1969)

1. Eight groups of ten weanling rats were maintained for 60 days on diets containing calcium and phosphorus in the ratio 10:1 at four different levels of mineral, namely 0·8, 1·19, 2·29 and 3·33% Ca.

2. At the two lower levels of mineral intake the provision of vitamin D reduced final body-weight. Increasing the mineral intake increased final body-weight except at the highest level.

3. Increasing the dietary mineral content had no effect on serum Ca, but serum P was higher in the groups receiving the two higher levels of dietary mineral. Vitamin D raised the serum Ca level in the rats receiving the two lower levels of mineral, and serum P was raised by the vitamin at all levels of mineral intake.

4. Provision of vitamin D at the two lower levels of mineral intake decreased the fresh weight, dry weight and volume of the humerus but had no significant effect on the absolute amount of mineral ash in the bone. Consequentially there was an increase in percentage of ash, overall density and the ratio ofweight of ash to organic matter in dry fat-free bone (A:R value). With increasing mineral intake there was an increase in all the bone measurements except volume.

5. The presence of vitamin D had no effect on tooth mass or ash content. Increasing the mineral intake caused an increase in tooth mass and ash except at the highest level of intake.

6. The ratio of tooth ash to bone ash was very much greater at the lower levels of mineral intake than at the higher levels.

7. In the animals on the two lower levels of mineral intake the bones were frankly rachitic, and the presence of vitamin D ameliorated the condition. With increase in mineral intake histological signs of rickets were reduced, but even at the highest level of dietary mineral the epiphyseal discs appeared slightly wider than normal.

8. The incisor teeth of animals on the two lower levels of dietary mineral had wide predentine, and the presence of vitamin D reduced the amount of predentine. At the two higher levels of mineral intake the dentine appeared normal when vitamin D was present in the diet.

9. Quantitatively, the bones were much more affected than the teeth by a low intake of mineral at a Ca to P ratio of 10:1.

The mineralized tissues of the rat are affected by lack of dietary calcium and phosphorus in two ways: first, by a deficiency in absolute terms, and secondly, by changes in the ratio in which the two minerals are present. The effect of the presence or absence of vitamin D will, in the rat, vary with the state of mineral nutrition of the animal. It is also of interest to note that tooth and bone react differently to the same disturbance of mineral deficiency (Ferguson & Hartles, 1963, 1964, 1966). We have reported the effects on the bone and tooth of young rats that received diets containing equal but suboptimal amounts of Ca and P, with and without vitamin D (Harrand, Green & Hartles, 1966). We have also studied the effects of the vitamin in rats

maintained on diets containing different amounts of Ca and P in the ratio 1:10 (Harrand & Hartles, 1968).

In the experiments now reported weanling rats were maintained on diets with or without vitamin D and at four different levels of dietary minerals but with a constant Ca to P ratio of 10:1. The four levels of dietary Ca were 0·8, 1·2, 2·3 and 3·3%. The lowest level of Ca was therefore higher than that considered necessary for the rat, whilst even the highest level of P (0·33%) was below the generally accepted optimum. The effects of these treatments and that of adding vitamin D were determined by measuring final body-weight, serum Ca and P, weight, volume, length and ash content of the humeri and weight and ash content of the mandibular incisor teeth. The femurs and maxillary incisors were examined histologically.

EXPERIMENTAL

Animals. Eighty black and white weanling rats (24–28 days old) from our own colony were distributed in ten blocks of eight animals according to weight and sex. There were therefore five blocks of males and five of females, the weights of the animals within each block being as similar as possible.

Treatments. One animal from each block was distributed at random to each of eight treatment groups designated 1, 1D, 2, 2D, 3, 3D, 4 and 4D. Each group consisted of five male and five female rats.

Diets. The basal diet contained sucrose (1340 g), egg albumin (400 g) and groundnut oil (100 g). This mixture contained 0·026% Ca and 0·06% P. The Ca and P contents of the diets were adjusted by the addition of appropriate mixtures of AR grade salts. In all diets, to each 1840 g of basal diet were added 80 g of salt mixture containing neither Ca nor P (Harrand *et al.* 1966), 40 ml of water-soluble vitamin solution (Hartles & Leaver, 1961) and 40 mg biotin.

The following salts were added to the diets for the several groups:

Groups	$CaCO_3$ (g)	KH_2PO_4 (g)
1 and 1D	38·75	1·76
2 and 2D	58·75	5·27
3 and 3D	118·75	15·80
4 and 4D	178·75	26·30

In groups 1–4, the groundnut oil contained vitamins A, E and K; in groups 1D, 2D, 3D and 4D the oil also contained ergocalciferol (Hartles & Leaver, 1961).

The main features of the eight dietary treatments were:

Group	Ca (%)	P (%)	Ergocalciferol (μg/kg diet)
1	0·80	0·08	None
1D	0·80	0·08	200
2	1·19	0·12	None
2D	1·19	0·12	198
3	2·29	0·23	None
3D	2·29	0·23	191
4	3·33	0·33	None
4D	3·33	0·33	185

Husbandry. The experimental regime was exactly as described by Harrand & Hartles (1968).

Serum, bone and tooth examinations. The techniques used for chemical analysis and histological examination were those described by Harrand & Hartles (1968).

RESULTS

The terminal body-weights and findings relating to serum and bone are given in Table 1, and those for the incisor teeth are shown in Table 2.

The results were subjected to an analysis of variance in which the total treatment variation was divided into that due to treatments, that due to blocks and the residual or error variation. The treatment variation was then subdivided into that due to vitamin D, that due to alteration in dietary Ca and P, and that due to the interaction of these two factors.

The following comparisons were made:

C_1 No added vitamin D (groups 1–4)	*v.*	Added vitamin D (groups 1 D, 2 D, 3 D, 4 D)
C_2 Lower mineral intake (groups 1, 1 D, 2, 2 D)	*v.*	Higher mineral intake (groups 3, 3 D, 4, 4 D)
C_3 0·8% Ca, 0·08% P (groups 1, 1 D)	*v.*	1·2% Ca, 0·12% P (groups 2, 2 D)
C_4 2·3% Ca, 0·23% P (groups 3, 3 D)	*v.*	3·3% Ca, 0·33% P (groups 4, 4 D)

The following interactions were also calculated: C_1 *v.* C_2; C_1 *v.* C_3; C_1 *v.* C_4. The significance levels for all comparisons are shown in Table 3.

Histological examination

Femurs. The animals in group 1 presented frankly rachitic signs. The epiphyseal cartilage was wide and irregular, and there were large amounts of osteoid in both shaft and trabeculae. The walls of the shaft and trabeculae were thin.

In group 1 D, the addition of vitamin D to the diet caused a reduction in the width of the epiphyseal cartilage, although the zone was more irregular than in the normal animal. Osteoid was still present but in less quantity than in group 1. The shafts and trabeculae were thinner than normal.

The increased mineral intake in the animals of group 2 improved the appearance of the epiphyseal disc but it was still wider than normal. Some osteoid seams were visible.

The addition of vitamin D (group 2 D) reduced the width of epiphyseal cartilage to almost normal proportions but the shaft and trabeculae were thinner than normal.

In the animals of groups 3, 3 D, 4 and 4 D the appearance of the femur was very close to the normal, but there were still signs of abnormality in the epiphyseal disc.

Incisor teeth. The dentine in the animals of group 1 was highly abnormal, and the predentine was wide. The disturbance was greatest in the apical region.

The addition of vitamin D (group 1 D) caused an improvement in the incisal region

Table 1. *Effect of vitamin D and increasing mineral intake at a calcium to phosphorus ratio* 10:1 *on the composition of serum and bone*

(Mean values with standard errors for groups of five ♂ and five ♀ rats)

Ca content of diet (%)...	0·8		1·2		2·3		3·3		
Group*...	1	1D	2	2D	3	3D	4	4D	SE
Serum Ca (μg/ml)	121·0	132·2	112·7	150·1	128·8	125·1	127·3	122·5	5·90
Serum P (μg/ml)	36·3	45·0	31·7	47·2	57·7	61·9	59·2	64·6	5·38
Fresh wt of humeri (mg)	403·5	332·0	424·8	358·4	483·0	495·3	483·2	505·9	19·20
Volume of humeri (μl)	297·8	224·6	294·8	233·8	276·6	280·0	272·8	282·7	14·35
Length of humerus (mm)	22·2	22·6	23·1	23·0	24·2	24·3	23·9	24·2	0·30
Fresh wt/volume (g/ml)	1·36	1·48	1·46	1·54	1·75	1·78	1·78	1·80	0·02
Dry wt of humeri (mg)	182·6	164·4	216·7	194·6	311·1	319·8	321·8	335·8	11·45
Wt of ash in humeri (mg)	75·2	83·6	111·6	108·2	196·6	205·2	207·1	218·1	7·40
Ash as % of fresh humeri	18·2	25·2	26·8	30·4	40·6	41·3	42·9	43·1	0·79
Ash as % of dry humeri	41·1	50·7	51·7	55·6	63·1	64·1	64·4	64·9	0·78
Ratio D : R†	0·84	1·00	1·05	1·21	1·81	1·82	2·00	1·98	0·04
Ratio A : R‡	0·71	1·04	1·08	1·26	1·71	1·79	1·77	1·85	0·04
Final body-wt (g)	143	128	155	137	173	180	169	177	9·56
Fresh wt of humeri/body-wt (mg/g)	2·83	2·61	2·77	2·63	2·83	2·81	2·87	2·89	0·06
Dry wt of humeri/body-wt (mg/g)	1·29	1·29	1·43	1·43	1·82	1·81	1·91	1·92	0·04
Wt of ash in humeri/body-wt (mg/g)	0·54	0·66	0·74	0·80	1·15	1·17	1·23	1·25	0·03

* D denotes addition of vitamin D to the diet.
† Ratio of dry weight to weight of fat and water in fresh bone.
‡ Ratio of weight of ash to weight of organic matter in dry, fat-free bone.

Table 2. *Effect of vitamin D and increasing mineral intake at a calcium to phosphorus ratio* 10:1 *on the composition of the mandibular incisor tooth*

(Mean values with standard errors for groups of five ♂ and five ♀ rats)

Ca content of diet (%)...	0·8		1·2		2·3		3·3		
Group*...	1	1D	2	2D	3	3D	4	4D	SE
Dry wt of incisor tooth (mg)	58·5	58·9	61·9	62·7	68·3	65·5	65·9	67·4	1·23
Wt of ash in incisor tooth (mg)	44·4	44·8	46·5	48·1	52·7	50·3	51·3	51·5	0·98
Ash as % of dry tooth	75·9	76·1	75·3	76·8	77·2	76·7	77·8	77·5	0·46
Ratio A : R†	3·16	3·19	3·05	3·33	3·42	3·32	3·51	3·29	0·09
Dry wt of tooth/body-wt (mg/g)	0·42	0·47	0·42	0·46	0·41	0·38	0·40	0·39	0·02
Wt of ash in tooth/body-wt (mg/g)	0·32	0·35	0·32	0·35	0·31	0·30	0·31	0·30	0·02
Dry wt of tooth/dry wt of humerus (mg/mg)	0·64	0·72	0·58	0·65	0·45	0·42	0·41	0·41	0·02
Wt of ash in tooth/wt of ash in humerus (mg/mg)	1·19	1·08	0·84	0·89	0·55	0·50	0·50	0·48	0·02

* D denotes addition of vitamin D to the diet. † See footnote to Table 1.

Table 3. *Significance levels* (P) *for the effects on bones and teeth of rats of vitamin D, increased dietary calcium and phosphorus content at a constant ratio of* 10:1 *and their interaction* (*I*)

	Vitamin D	Groups 1 and 2 *v.* 3 and 4	I	Group 1 *v.* group 3	I	Group 3 *v.* group 4	I
Serum Ca	0·025	NS	0·001	NS	0·025	NS	NS
Serum P	0·001	0·001	NS	NS	NS	NS	NS
Fresh wt of humeri	0·01	0·001	0·001	0·05	NS	NS	NS
Volume of humeri	0·001	0·01	0·001	NS	NS	NS	NS
Length of humerus	NS	0·001	NS	0·001	NS	NS	NS
Fresh wt/volume	0·001	0·001	0·001	0·001	NS	NS	NS
Dry wt of humeri	NS	0·001	0·01	0·01	NS	NS	NS
Wt of ash in humeri	NS	0·001	NS	0·001	NS	NS	NS
Ash as % of fresh humeri	0·001	0·001	0·001	0·001	0·01	0·01	NS
Ash as % of dry humeri	0·001	0·001	0·001	0·001	0·001	NS	NS
Ratio D : R*	0·01	0·001	0·001	0·001	NS	0·001	NS
Ratio A : R*	0·001	0·001	0·001	0·001	0·01	0·025	NS
Final body-wt	NS	0·001	0·01	NS	NS	NS	NS
Fresh wt of humeri/body-wt	0·01	0·001	0·01	NS	NS	NS	NS
Dry wt of humeri/body-wt	NS	0·001	NS	0·001	NS	0·001	NS
Wt of ash in humeri/body-wt	0·001	0·001	0·01	0·001	NS	0·001	NS
Dry wt of incisor tooth	NS	0·001	NS	0·01	NS	NS	NS
Wt of ash in incisor tooth	NS	0·001	NS	0·01	NS	NS	NS
Ash as % of dry tooth	NS	0·001	NS	NS	NS	NS	NS
Ratio A : R	NS	0·01	0·025	NS	NS	NS	NS
Dry wt of tooth/body-wt	0·05	0·001	0·001	NS	NS	NS	NS
Wt of ash in tooth/body-wt	0·05	0·001	0·001	NS	NS	NS	NS
Dry wt of tooth/dry wt of humerus	0·001	0·001	0·001	0·001	NS	0·05	NS
Wt of ash in tooth/wt of ash in humerus	0·05	0·001	NS	0·001	0·001	NS	NS

NS, not significant.
* See footnote to Table 1.

where the ratio of dentine to predentine was normal. The apical region was similar to that in group 1.

In group 2, the predentine was very wide both apically and incisally. There was again an improvement on addition of vitamin D to the diet (group 2 D) and in general the appearance of the teeth was similar to that in animals of group 1 D.

The incisors of animals in groups 3 and 4 were almost normal in appearance, although there were some qualitative disturbances. In groups 3 D and 4 D the teeth appeared normal.

DISCUSSION

Growth of animals

When the final body-weights of all animals that had received vitamin D were compared with those of all animals denied the vitamin there was no significant difference between the two. However, inspection of the results (Table 1) shows that, at the two lower levels of mineral intake, the addition of vitamin D to the diet lowered the final body-weight, whereas at the two higher levels of intake provision of the vitamin raised the final body-weight slightly. Hence, overall, there was no significant change. The comparison of the animals receiving the two lower levels of dietary mineral with those on the two higher levels reveals a highly significant difference in body-weight. Since no significant difference in body-weight was found between rats on either of the two lower levels of mineral intake or between those on the two higher levels, the major effect on increase in body-weight occurred when the dietary P content was raised from 0·12 to 0·23%. In addition there is a significant interaction between the effect of the vitamin and the effect of increased mineral intake. This lends support to the validity of the deduction that the addition of vitamin D to the diet containing the two lower levels of mineral at Ca : P ratio of 10 : 1 caused a reduction in the final body-weight. When the dietary content was raised to 0·23% or 0·33% at the same Ca : P ratio this effect was no longer observed.

This result is quite different from that observed when the Ca : P ratio is 1 : 10 (Harrand & Hartles, 1968), when the addition of vitamin D increased final body-weight at all levels of mineral intake studied. The statement that vitamin D promotes a general stimulation of growth (Bicknell & Prescott, 1946) thus requires qualification, for it does not apply to all dietary circumstances. Schneider & Steenbock (1939) reported that vitamin D retarded growth in rats when the dietary Ca : P ratio was high and suggested that the effect was caused by the vitamin mobilizing P from the soft tissues to the bone. Our results go some way to confirm this, for when the dietary P was increased the effect was no longer apparent even though the Ca : P ratio remained high.

Campbell & Douglas (1965) fed to puppies diets containing 0·08–0·10% Ca, 0·13–0·15% P and very little vitamin D. Addition of vitamin D to these diets caused a reduction in weight gain and the authors suggested that this might be attributed to some degree of vitamin D intoxication. However, in approximately similar conditions, Harrand *et al.* (1966) found that when the Ca : P ratio was 1 : 1 and the diet contained either 0·12 or 0·24% Ca the addition of vitamin D caused an increase in body-weight.

Thus we conclude that the addition of vitamin D to a diet results in a lowering of body-weight only when the dietary Ca:P ratio is high and there is a gross deficiency of P with an adequate intake of Ca.

Serum Ca and P

Comparison of all groups receiving vitamin D with those denied the vitamin shows an effect on serum Ca which was significant at the 0·025 level. Inspection of Table 1 reveals that this effect was in fact confined to the two lower levels of dietary mineral. The effect of vitamin D at the two lower levels of dietary mineral was to increase serum Ca, the greatest increase being observed when the diet contained 0·12% P. At the two higher levels of mineral intake the vitamin had no effect on serum Ca. Varying the mineral intake *per se* had no significant effect on the values for serum Ca. This is not surprising since even the lowest level of dietary Ca was above the commonly accepted optimum.

Vitamin D had a consistent and highly significant effect in raising serum phosphate at all levels of dietary mineral but the effect was greatest at the lower levels of mineral intake.

Bones and incisor teeth

A comparison of results from all the groups receiving vitamin D with those from groups denied the vitamin reveals several interesting differences. The over-all effect of vitamin D was to cause a significant fall in fresh weight of the humeri, but inspection of Table 1 shows that in fact this fall was again confined to the groups receiving the two lower levels of dietary mineral. Similar results were obtained for the effect on bone volume. The total effect of the vitamin on dry weight in the humeri was not significant although again inspection of Table 1 shows a pattern similar to that obtained for fresh weight, namely a slight drop in dry weight in the groups receiving the two lower mineral intakes. The vitamin had no significant effect on weight of ash. Thus when the diet is low in P (0·08% or 0·12%) at a Ca:P ratio of 10:1 the major effect of vitamin D is to reduce the total mass by approximately 15% and the volume by about 20%. The dry weight is reduced by only 10% and the weight of ash is not significantly altered. The cumulative effect of these changes is to increase the percentage of ash in the bone and to raise the A:R ratio. It is important to realize, however, that the change in these latter factors is not due to any great increase in the deposition of mineral but to a reduction in the amount of non-mineral in the bone. When the intake of P was increased to 0·23 and 0·33% this 'anti-growth' effect was no longer apparent.

The effect of the vitamin in reducing bone mass and volume was therefore confined to groups 1D and 2D in which the intake of P was lowest; in the absence of the vitamin (groups 1 and 2) histological examination revealed a frankly rachitic situation, which was ameliorated by the presence of vitamin D in the diet.

McGowan (1933) and Underwood, Fisch & Hodge (1951) concluded that the effect of vitamin D in rachitic animals is to mobilize P from the soft tissues and so aid in the mineralization of bone. Our results suggest that when P is in short supply the effect of the vitamin is not primarily on its increased mobilization but on the organization

of its proper utilization. In an earlier study (Harrand & Hartles, 1968) we found that when the diets had a Ca : P ratio of 1 : 10 the vitamin had a significant effect in increasing the amount of mineral present in bone, except at the lowest level of dietary Ca (0·08%). Fig. 1 shows diagrammatically the differing effects of vitamin D on bones of rats receiving diets with a low or a high Ca : P ratio. As with body-weight, vitamin D exerts its 'anti-growth' effect only when the diet is grossly deficient in P, and there is a reversal of this effect when the P intake is raised, even though the Ca : P ratio remains high. Examination of the ratio of fresh weight of humeri to body-weight (Tables 1

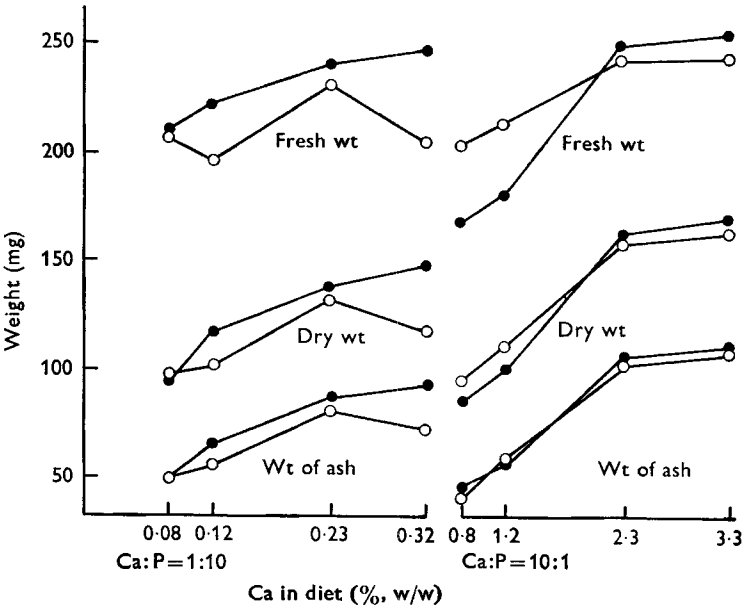

Fig. 1. Effect of vitamin D on the fresh weight, dry weight and weight of ash of the humeri of rats at different levels of mineral intake and with a Ca:P ratio of 1 : 10 (left) and 10 : 1 (right). O–O, without vitamin D; ●–●, with vitamin D. Values for the Ca:P ratio of 1 : 10 are derived from Harrand & Hartles (1968).

and 3) shows that vitamin D exerts a greater 'anti-growth' effect on bone than on general body-weight. It has no effect on the ratio of dry weight of humeri to body-weight and the effect is reversed in relation to the ratio of weight of ash in humeri to body-weight. These simple calculations confirm that when the P intake is low the main effect of vitamin D is not solely on the accumulation of mineral in the bone, but in the reduction in the non-mineral portion, particularly the fat and water content (see D : R ratio, Table 1). The conclusion can be drawn therefore that the vitamin is more able to improve the mobilization of mineral to the bones when the Ca to P ratio is low than when it is high.

A comparison of the groups receiving the diets with the two lower amounts of mineral with those receiving the two higher levels of mineral (groups 1, 1D, 2, 2D *v.* groups 3, 3D, 4, 4D) showed a significant increase in every factor studied with the exception of serum Ca concentration. Increasing the dietary P from 0·08 to 0·12%

resulted in significant changes in all bone measurements studied except volume. Increasing the dietary P from 0·23 to 0·33% had less effect on bone. There were, however, significant increases in the ash expressed as a percentage of fresh weight, and in D : R and A : R ratios. In these experiments the greatest effect on the accumulation of mineral in bone, i.e. actual weight of ash, occurred in the transition from diets containing 0·12% P to those containing 0·23% P (Ca:P = 10:1). This is relatively a much greater change than that governed by transition from a diet containing 0·12% Ca to one containing 0·23% Ca at a Ca to P ratio of 1 : 10 (Harrand & Hartles, 1968). Fig. 1 shows these effects graphically.

Examination of the values in Tables 2 and 3 shows that vitamin D had no significant effect on the dry weight, weight of ash, percentage of ash or A : R ratio of the incisor teeth. Nevertheless despite this lack of quantitative effect, microscopic examination revealed that the vitamin had a qualitative effect on the organization of the dentine.

Comparison of the values for the two lower with those for the two higher dietary mineral intakes shows a small but significant rise in the above measurements. These results emphasize the differences in reaction of the bones and growing teeth to the same disturbance of mineral intake. Even at the lowest intake of mineral the tooth contained over 85% of the mineral found in the tooth formed on the highest mineral intake. On the other hand, the bone formed on the lowest mineral intake contained less than 35% of the mineral found in that formed on the highest mineral intake. These differences are further illustrated by examination of the ratio of weight of ash in tooth to weight of ash in humerus (Table 2), at the lowest level of dietary mineral the ratio was twice that at the highest intake. This is an even greater difference than that found by Harrand & Hartles (1968) in rats receiving diets with a Ca to P ratio of 1 : 10.

From all these results certain clear-cut conclusions can be drawn. The forming teeth are less severely affected than bone by mineral deficiency at Ca to P ratios of both 10 : 1 and 1 : 10. When the Ca to P ratio is 10 : 1 vitamin D has no significant effect on the absolute amount of mineral deposited in the bone and tooth. When the Ca to P ratio is 1 : 10, vitamin D increases the mineral deposited in bone and tooth. Thus, vitamin D promotes the deposition of bone mineral if the lack is of Ca, but not if the deficiency is one of P.

The authors wish to thank Mr R. P. Williams, Mr R. Connel and Miss Greta Rixom for their skilled technical assistance.

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Printed in Great Britain