THE INFLUENCE OF HYDROCORTISONE ON THE ACTION OF EXCESS VITAMIN A ON LIMB BONE RUDIMENTS IN CULTURE

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It is well known that large doses of cortisone retard fibroplasia in animals (for a review of the literature see Pintar, 1960).

It affects the skeleton by inhibiting its growth in young animals (Follis, 1951) and greatly delays the healing of fractures (Blunt *et al.*, 1950). Cortisone also much reduces the growth rate in culture of the cartilaginous limb bone rudiments from 6- to 7-day-old chick embryos (Buno and Goyena, 1955; Sobel and Freund, 1958). Recently Whitehouse and Lash (1961) have studied the effect of cortisone, hydrocortisone, and related compounds on chondrogenesis induced in somites cultivated *in vitro*, and found that the hormones greatly reduced the amount of cartilage formed; this seemed to be due to inhibited sulfation of chondroitin.

Excess vitamin A causes rarifaction of bone *in vivo* (Wolbach, 1947) and in culture (Fell and Mellanby, 1952; Fell and Thomas, 1960). It also accelerates the resorption of cartilage *in vivo* and in culture (Wolbach 1947; Fell and Mellanby, 1952) and removes the metachromatic material from the matrix (Thomas *et al.*, 1960; Fell and Mellanby, 1952; Fell and Thomas, 1960).

Selye (1958) found that the simultaneous administration of cortisone and excess vitamin A to rats produced extensive resorption of bone by the 20th day of treatment, a stage at which no effect is seen in animals given either agent alone; from this result he concluded that there is a "sensitisation of the skeleton to vitamin A overdosage by cortisol." In animal experiments, however, it is seldom possible to determine whether a given effect is produced by the direct action of an agent on a particular tissue, or whether it is due to indirect systemic reactions.

To investigate this question, we have studied the influence of hydrocortisone on limb bone rudiments from chick and mouse embryos, grown in medium with and without added vitamin A. Our results show that in culture the hormone retards the effect of the vitamin on skeletal tissue. This suggests that the remarkable potentiation of the effect observed by Selye (1958) in animals treated with both agents together may have been due to systemic factors that are eliminated in organ culture.

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After this investigation had been begun, H. B. Fell found some notes by the late Sir Edward Mellanby, dated November 26, 1951, in which he described preliminary experiments on the effects of cortisone alone and in conjunction with vitamin A, on late fetal mouse bones in culture; his results, like ours with hydrocortisone, showed that the simultaneous addition of cortisone to the medium delayed but did not arrest the effect of the vitamin. Mellanby did not pursue this study further and his observation remained unpublished.

Material and Methods

Material.—Limb bone rudiments were removed from 7-day-old chick embryos and mouse fetuses near term.

Culture Methods.—The standard watch glass method was used, the procedure being the same as that described in the previous paper (Fell and Thomas, 1960). For the mouse bones, the embryo extract was made with Tyrode supplemented with 2 per cent (*w/v*) glucose instead of 4 per cent as in the earlier experiments.

Addition of Hydrocortisone and Vitamin A to the Medium.—Hydrocortisone sodium succinate (solu-cortef, Upjohn Co., Kalamazoo) was dissolved in sterile distilled water and added to the plasma to give a concentration in the final medium of either 7.5 μg or 75.0 μg/ml of clot.

Vitamin A alcohol (Roche Products, Ltd., London) was dissolved in ethanol and added to the plasma exactly as described by Fell and Thomas (1960). The final medium contained 10 I.U. vitamin A/ml of clot.

The different media whose effects were to be compared all contained the same quantity of water and/or ethanol, in order to make the experimental conditions as nearly uniform as possible.

Measurement.—In all experiments the explants were drawn at 2-day intervals with the aid of a camera lucida, and their lengths measured with string infiltrated with paraffin wax (see Fell and Mellanby, 1952).

Design of Experiments.—The designs of the various experiments and the number of explants used in each are shown in Tables I and II. To compare the effects of two experimental treatments the bone rudiments from one side of each embryo (set a) were exposed to one treatment and those from the opposite side of the same embryo (set b), to the other treatment.

Histology.—The explants were fixed in Zenker's fluid, washed, dehydrated, cleared, and embedded as described by Fell and Thomas (1960).

Serial sections were cut and stained with toluidine blue (0.5 per cent in 5 per cent ethanol), or with celestine blue, Mayer's acid hemalum and van Gieson's stain.

RESULTS

A. Experiments on Chick Limb Bone Rudiments (Table I)

1. Controls in Normal Medium.—The long bones of 7-day embryos vary somewhat in their size and stage of development according to the time of year and other factors. They consist of a cartilaginous rod with small celled epiphyses each separated by a broad proliferative zone of flattened cells from the middle region of hypertrophic chondrocytes. On the surface of the hypertrophic cartilage there is a layer of periosteal bone which varies in thickness and degree of calcification in different embryos. The bone is covered by a periosteum composed of an outer fibroblastic and an inner osteoblastic layer. The explants

used in these experiments included the cartilage, bone, periosteum, and a little attached muscle.

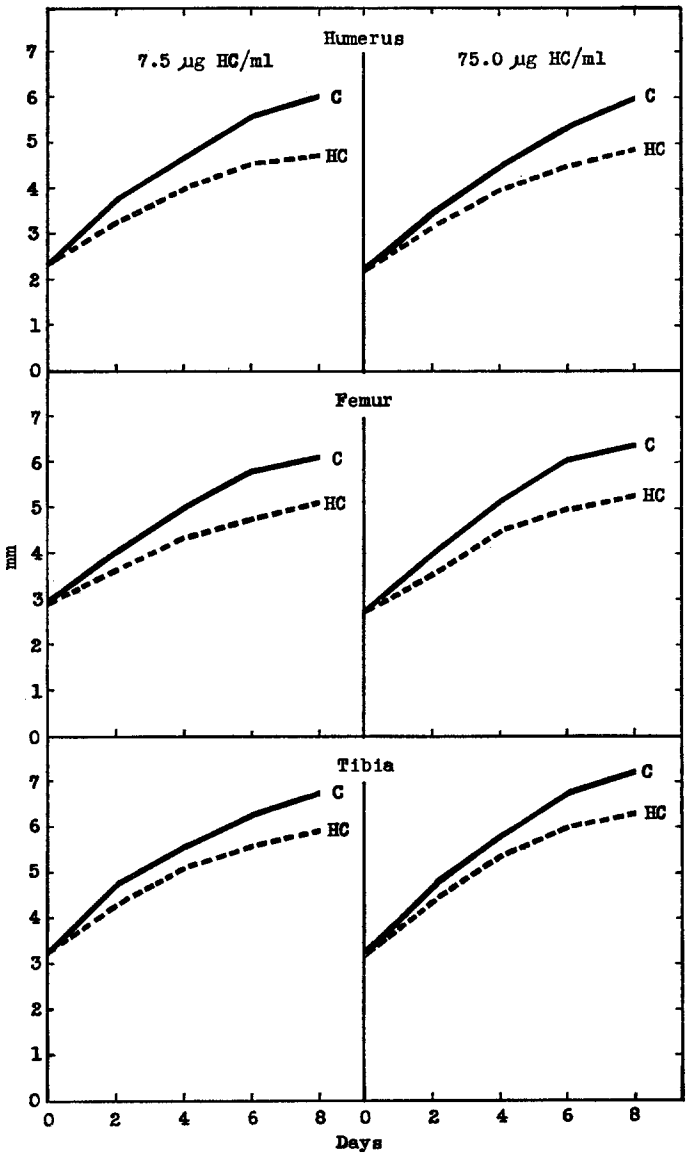
During 8 days' cultivation in normal medium (Experiments 311, 319) the controls (24 explants) enlarged 2 to 2½ times their original length; the layer

TABLE I

Experiments on 7-Day Chick Bone Rudiments Grown in Medium Containing Hydrocortisone (μg/ml of Medium), Vitamin A (10 I.U./ml) or Both Agents

Experiment	No. pairs	Culture medium		Culture period	Subsequent treatment			
		Set *a*	Set *b*		Immediate fixation	To normal medium	To HC medium	Culture period
				days				*days*
311	2 HFT	HC 7.5 μg	Control	8	*a*, *b*	—	—	—
	2 "	" 75.0 μg	"	8	", "	—	—	—
319	2 "	" 7.5 μg	"	8	", "	—	—	—
	2 "	" 75.0 μg	"	8	", "	—	—	—
281	1 "	A + HC 7.5 μg	A	8	", "	—	—	—
	1 "	"	"	8	—	*a*, *b*	—	4
	1 "	A + HC 75.0 μg	"	8	*a*, *b*	—	—	—
	1 "	"	"	8	—	*a*, *b*	—	4
285	1 "	A + HC 7.5 μg	"	8	*a*, *b*	—	—	—
	1 "	"	"	8	—	*a*, *b*	—	4
	1 "	A + HC 75.0 μg	"	8	*a*, *b*	—	—	—
	1 "	"	"	8	—	*a*, *b*	—	4
288	1 "	A + HC 7.5 μg	"	8	*a*, *b*	—	—	—
	1 "	"	"	8	—	*a*, *b*	—	4
	1 "	A + HC 75.0 μg	"	8	*a*, *b*	—	—	—
	1 "	"	"	8	—	*a*, *b*	—	—
300	2 "	A + HC 7.5 μg	HC 7.5 μg	8	*a*, *b*	—	—	—
	2 "	A + HC 75.0 μg	HC 75.0 μg	8	", "	—	—	—
308	2 "	A + HC 7.5 μg	A + HC 7.5 μg	8	—	*a*	*b* 7.5 μg	4
	2 "	A + HC 75.0 μg	A + HC 75.0 μg	8	—	*a*	*b* 75.0 μg	4
290	2 "	A	A	6	—	*a*	*b* 7.5 μg	6
	2 "	A	A	6	—	*a*	*b* 75.0 μg	6
321	4 "	A	A	4	—	*a*	*b* 7.5 μg	4

H, humerus; F, femur; T, tibia; HC, hydrocortisone; A, vitamin A. Set *a* and Set *b*, rudiments from opposite sides of the same embryo.



TEXT-FIG. 1. Curves showing the effect of hydrocortisone (HC) on the growth in length of the humerus, femur, and tibia in culture (Experiments 311, 319). Explants from 7-day chick embryos. Each curve represents the average growth of 8 explants.

Both doses of hydrocortisone (HC) reduce the growth rate to about the same extent as compared with that of the controls (C) in normal medium.

of periosteal bone became much thicker, the three cellular zones of the cartilage continued to differentiate fairly normally, and the amount of matrix greatly increased. The structure and behavior of such explants have been described in more detail elsewhere (Fell and Mellanby, 1952).

2. The Effect of Hydrocortisone Alone.—No significant difference could be detected between the effects of 7.5 μg/ml (12 explants) and 75.0 μg/ml (12 explants), respectively, so the two series will be described together.

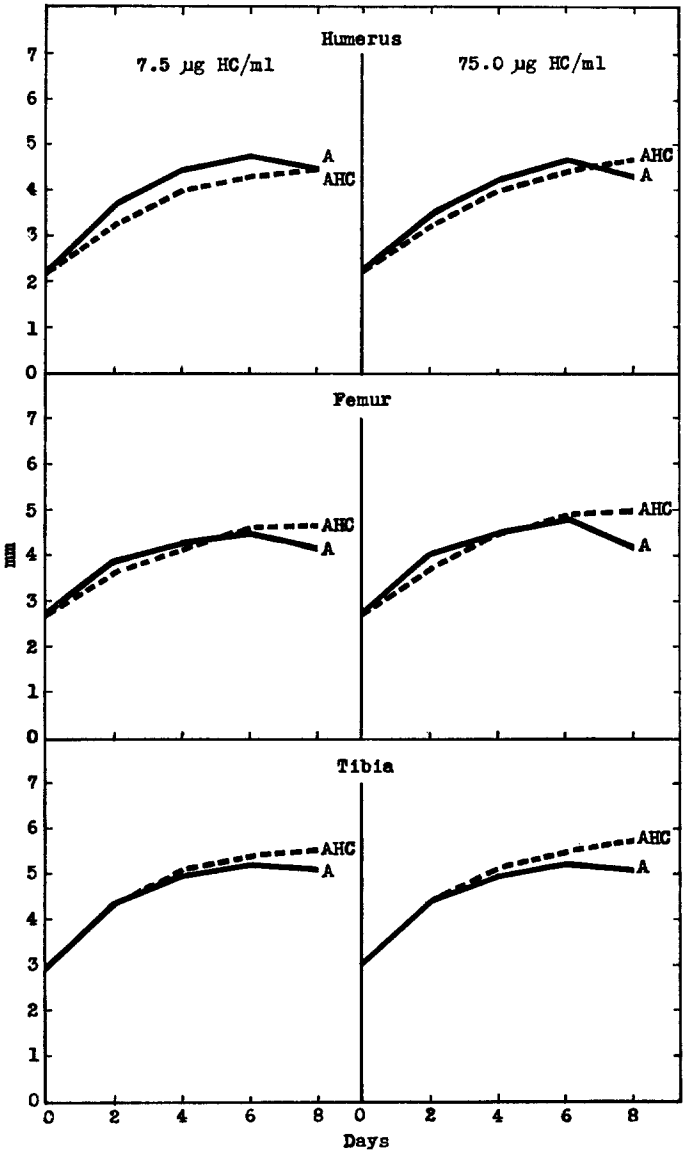
The hydrocortisone-treated rudiments (Experiments 311, 319) grew more slowly than their controls (Text-fig. 1) (*cf.* Buno and Goyena, 1955; Sobel and Freund, 1958), but appeared healthy and preserved their normal shape during 8 days' cultivation. On histological examination after 8 days' growth, the cells of both epiphyses and diaphysis were found to be smaller than in the controls (Fig. 1*a*, and 1*b*) (*cf.* Sobel and Freund, 1958) and in the epiphyses the intercellular partitions were narrower and gave a more intense metachromatic stain with toluidine blue.

3. The Effect of Vitamin A Alone.—The 36 explants treated with vitamin A alone and fixed after 8 days (Experiments 281, 285, 288) showed the characteristics previously recorded (Fell and Mellanby, 1952; Fell and Thomas, 1960), *viz.* shrinkage, distortion, and, in sections stained with toluidine blue, loss of metachromasia from the cartilage matrix.

In the present experiments the humeri which were the least affected of the rudiments, had doubled their average length by the 6th day after which they suddenly began to shrink (Text-figs. 2 and 4). The femora and tibiae, which grew less rapidly than the humeri, also reached their maximum average length by the 6th day and then began to diminish. As previously noted (Fell and Mellanby, 1952; Fell and Thomas, 1960), the regions most sensitive to the action of vitamin A were those where the cells of the proliferative zones were becoming transformed into the young hypertrophic chondrocytes; here there was an early reduction and rapid disappearance of metachromasia and extreme compression of the cells. The softened cartilage was often invaded by connective tissue cells which sometimes completely detached the terminal cartilage from the shaft. The camera lucida drawings of the living rudiments (Text-fig. 3) showed that by the 8th day, this sensitive region had collapsed at one or both ends in 32 of the 36 vitamin A–treated explants; this phenomenon was largely responsible for the sudden decrease in length between the 6th and 8th day. There was also loss of metachromatic material throughout the cartilage, especially from the shaft and from the periphery of the epiphyses.

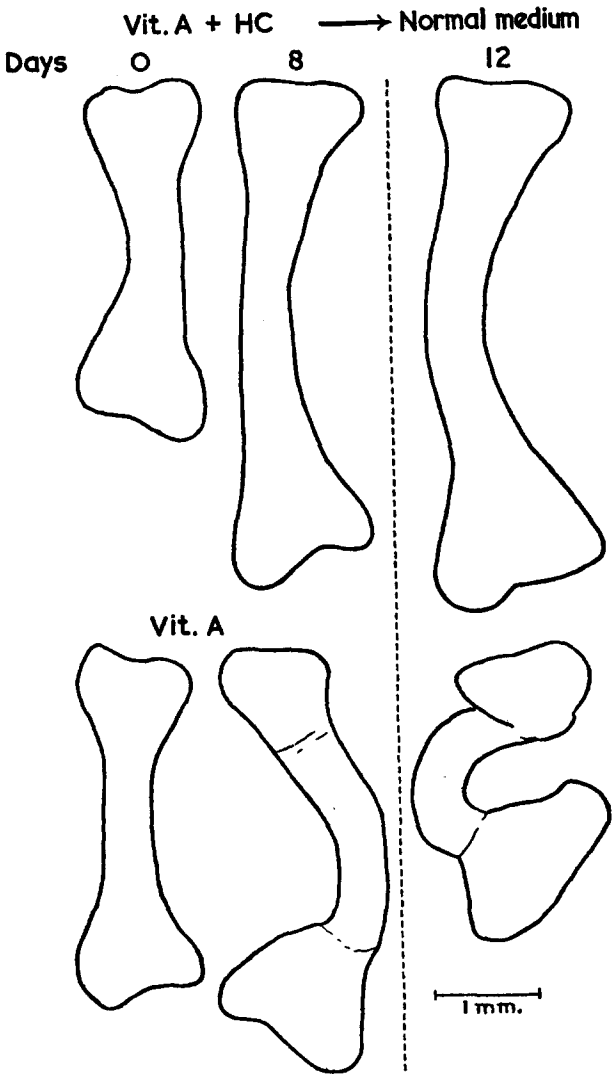
As stated above, embryos of the same age vary in their degree of development; vitamin A affected the rudiments from earlier embryos more drastically than those from more advanced chicks.

4. The Effect of Vitamin A + Hydrocortisone.—Observations on the living explants during 8 days' cultivation (Experiments 281, 285, 288, 300, 308)



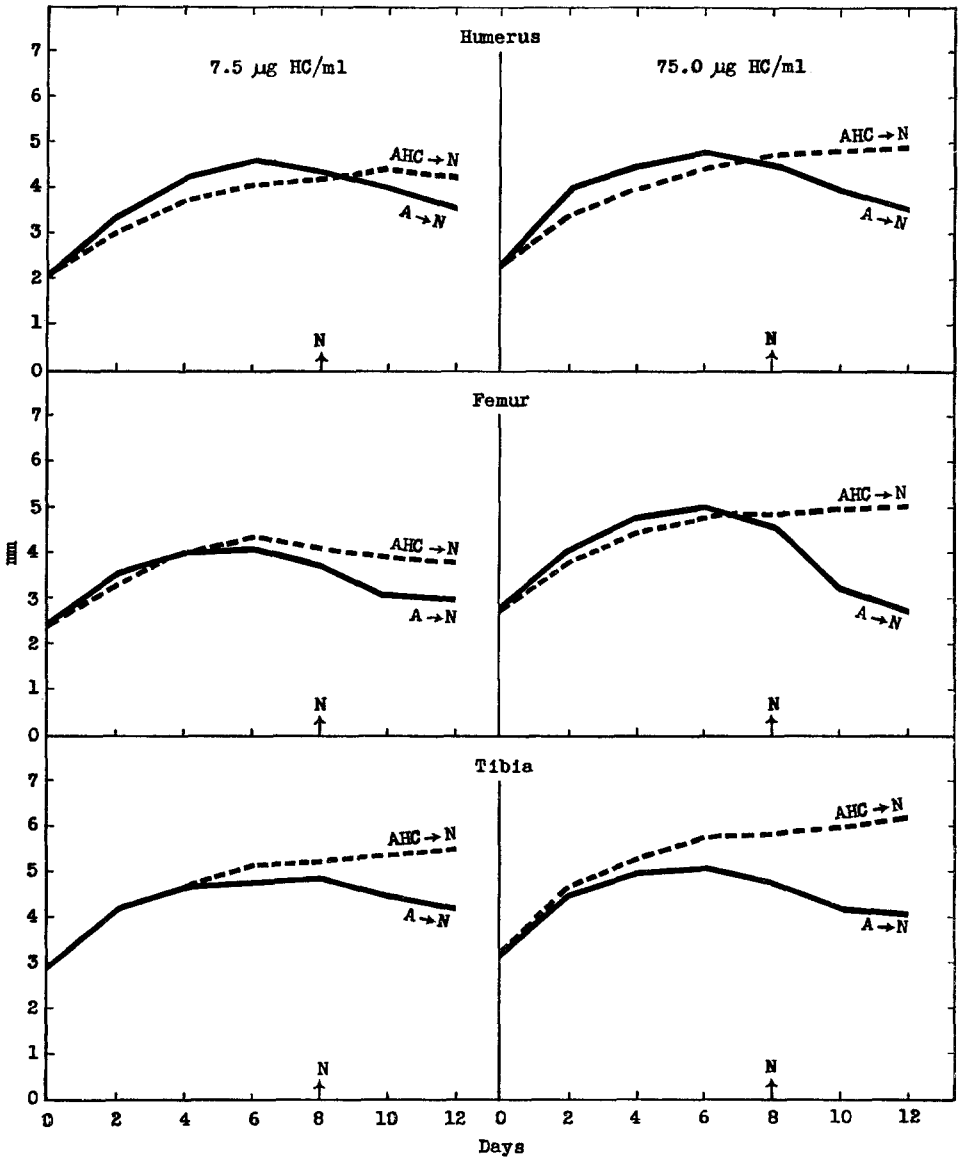
TEXT-FIG. 2. Curves showing the effect of vitamin A alone (A) as compared with that of vitamin A + hydrocortisone (AHC) on the growth in length of the humerus, femur, and tibia of 7-day chick embryos. (Experiments 281, 285, 288). Each curve represents the average growth of 6 explants.

Between the 6th and 8th day the growth curve of the vitamin A-treated explants is deflected downwards owing to shrinkage of the cartilage, whereas that of the rudiments exposed to vitamin A + hydrocortisone continues to rise slowly.



TEXT-FIG. 3. Camera lucida drawings of a pair of femora from the same 7-day chick embryo (Experiment 285). One explant was grown for 8 days in medium containing both vitamin A (10 I.U./ml) and hydrocortisone (7.5 μg/ml) and the other in medium to which only vitamin A had been added. After 8 days, both explants were transferred to normal medium for a further 4 days.

The explant pretreated with vitamin A only is much more severely affected than that pretreated with both agents.



TEXT-FIG. 4. Curves showing the growth in length of paired humeri, femora, and tibiae from 7-day chick embryos, grown first for 8 days either in medium to which only vitamin A had been added or in medium containing both the vitamin and hydrocortisone, and then transferred to normal medium for 4 days (Experiments 281, 285, 288). Each curve represents the average growth of 3 rudiments.

The explants pretreated with hydrocortisone as well as vitamin A, are less severely affected than those exposed to vitamin A alone.

showed that in 70 out of 72 rudiments grown in medium containing both vitamin A and hydrocortisone, vitamin A failed to produce the collapse of the sensitive region at the junction of the proliferative and hypertrophic zones, so that the explants treated with both agents remained intact with little or no distortion during this period. (Text-fig. 3). There was little difference in the growth rates (Text-fig. 4) of the vitamin A- and vitamin A + hydrocortisone–treated rudiments during the first 6 days, but when the sensitive region in the explants exposed to the vitamin alone began to collapse, the average growth curve was deflected downwards whereas that of the series treated with both the hormone and the vitamin continued slowly to rise.

Histological examination after 8 days' cultivation revealed a striking contrast between the vitamin A- and the vitamin A + hydrocortisone-treated rudiments. In paired explants from the same embryo (Experiments 281, 285, 288), the rudiment grown in medium containing both agents was without exception much less affected by the vitamin than the corresponding rudiment grown in the presence of the vitamin alone. In many of the pairs (Fig. 2b) the member exposed to vitamin A + hydrocortisone appeared almost normal in sections stained with toluidine blue; there was usually diminished metachromasia at the margin of the sensitive region mentioned above, and in a narrow peripheral zone of the epiphyses, but elsewhere the metachromatic staining was intense. In the vitamin A–treated member on the other hand (Fig. 2a) the metachromasia was greatly reduced throughout and had almost completely gone from the shaft, while the cartilage of the sensitive region was greatly compressed and sometimes disintegrating. Some of the explants given vitamin A + hydrocortisone showed a more advanced vitamin A effect than that described above, but their vitamin A–treated controls were altered to a correspondingly greater degree. The higher dose of the hormone (75 μg/ml) gave a slightly better protection against the action of the vitamin than the lower concentration, but the difference was not great.

In one experiment (300) the effect of the two agents together was directly compared with that of hydrocortisone alone. The two humeri treated with 7.5 hydrocortisone + vitamin A grew at about the same rate as the opposite rudiments treated with the hormone alone, but in the remaining 10 pairs of explants, the rudiments exposed to both compounds were much shorter than those treated with hydrocortisone only, especially in the 6 pairs of femora and tibiae. The explants in vitamin A + hydrocortisone medium displayed a moderate vitamin A change on histological examination; as before, the higher dose of hydrocortisone gave a slightly better protection than the lower. In sections stained with toluidine blue, the matrix was much more intensely metachromatic in the series treated with hydrocortisone than in that treated with both compounds.

These experiments showed that the effect of vitamin A was much retarded but not arrested by the simultaneous addition of hydrocortisone to the medium.

5. The Effect of Transferring Vitamin A- and Vitamin A + Hydrocortisone-Treated Explants to Normal Medium.—In 18 pairs of rudiments (Experiments 281, 285, 288), one member of each pair was grown for 8 days in medium containing the vitamin only and the other in medium to which both the vitamin and hydrocortisone had been added (7.5 or 75.0 μg hydrocortisone/ml); both were then transferred to normal medium for a further 4 days. After transfer, the vitamin A change progressed in both series, but far more rapidly in the vitamin A–treated than in the corresponding vitamin A + hydrocortisone–treated explants (Text-figs. 3 and 4), so that by the end of the culture period the former were much more shrunken and distorted than the latter; histological examination confirmed the difference seen in the living cultures. After transfer to normal medium there was no obvious difference in behaviour between explants previously grown in the presence of vitamin A + 7.5 μg hydrocortisone/ml and those in vitamin A + 75.0 μg hydrocortisone/ml.

6. The Effect of Transferring Explants from Medium Containing Vitamin A + Hydrocortisone to Medium with Hydrocortisone Only.—In one experiment (308) an attempt was made to see if the vitamin A changes could be completely arrested by transferring vitamin A + hydrocortisone–treated explants (12 pairs) to medium containing hydrocortisone alone. Both members of each pair were grown in medium with vitamin A + hydrocortisone (7.5 or 75.0 μg hydrocortisone/ml) for 8 days; one rudiment was then transferred to normal medium and the other to medium containing hydrocortisone only (7.5 or 75.0 μg/ml). Transfer to medium with hydrocortisone failed to arrest the vitamin A changes in any of the explants. The 6 rudiments transferred to medium containing 7.5 μg hydrocortisone/ml were less severely altered than the opposite rudiments transplanted to normal medium, but there was no difference between those in 75.0 μg hydrocortisone/ml and in normal medium. This suggests that under these conditions the lower dose of the hormone was more effective in retarding the vitamin A effect than the higher, but more experiments with a wider dose range and longer culture period would be required to settle this point.

7. The Effect of Transferring Vitamin A–Treated Explants to Medium Containing Hydrocortisone.—In two experiments (290, 321), paired rudiments were grown in vitamin A medium for 6 (Experiment 290) or 4 days (Experiment 321); one of each pair was then transferred to a normal clot and the other to one containing hydrocortisone for a further 6 (Experiment 290) or 4 days (Experiment 321). The object of these experiments was to see whether treatment with hydrocortisone would hasten recovery from the vitamin A effect which, as described above, progresses for some days after withdrawal of the added vitamin.

In the living cultures there was little difference in appearance or growth rate between the explants transferred to normal and to hydrocortisone medium respectively. In sections stained with toluidine blue, the cartilage matrix was rather more intensely metachromatic in the hydrocortisone-treated rudiments than in those transplanted to the normal clot, but otherwise the two series were similar.

These results provided no evidence of accelerated recovery in response to treatment with hydrocortisone following exposure to excess vitamin A.

TABLE II

Experiments on Late Fetal Mouse Bones Grown in Medium Containing Hydrocortisone (μg/ml of Medium), Vitamin A (10 I.U./ml) or Both Agents

Experiment	No. pairs	Culture medium		Culture period	Subsequent treatment			
		Set *a*	Set *b*		Immediate fixation	To normal medium	To HC medium	Culture Period
				days				*days*
316	4 R, U, T	HC 7.5 μg	Control	6	*a*, *b*	—	—	—
318	4 ", ", "	" " "	"	6	", "	—	—	—
298	2 ", ", "	A + HC 7.5 μg	A	6	", "	—	—	—
	2 ", ", "	A + HC 75.0 μg	"	6	", "	—	—	—
317	4 ", ", "	A + HC 7.5 μg	"	6	", "	—	—	—
320	4 ", ", "	A	"	4	—	*a*	*b* 7.5 μg	6

R, radius; U, ulna; other lettering as for Table I.

B. Experiments on Limb Bones from Late Fetal Mice (Table II)

1. Controls in Normal Medium.—The limb bones from late fetal mice are at a much more advanced stage of development than those of 7-day chick embryos. A mouse bone at this stage consists of a bony shaft covered by the usual two-layered periosteum and enclosing a marrow cavity. Each end of the shaft is occupied by a partially eroded mass of hypertrophic cartilage; this merges with the proliferative zone of flattened cells beyond which is the cartilaginous epiphysis where hypertrophy of the chondrocytes has not yet begun. Of the three bones studied, the radius is the smallest, the ulna the longest, and the tibia the stoutest.

Twenty-four explants (Experiments 316, 318) were cultivated for 6 days in normal medium. During this period they elongated very little, unlike the actively growing chick rudiments, but the terminal cartilage enlarged somewhat. Histological examination showed that the cells were healthy except in some areas of the old hypertrophic cartilage, and mitosis was abundant especially among the osteoblasts and marrow reticulum cells. There were large erosion bays in the hypertrophic cartilage (Figs. 3a and 5a) both at the surface next

to the marrow, and laterally as a result of invasion from the periosteum; most of the cells lining these bays were osteoblasts and marrow reticulum cells but a few osteoclasts were present. In sections stained with toluidine blue, areas of matrix adjacent to erosion cavities had often lost their metachromasia, which suggested that digestion of the matrix was in progress (*cf.* Fell and Thomas, 1960). In places new bone was being deposited by the periosteum.

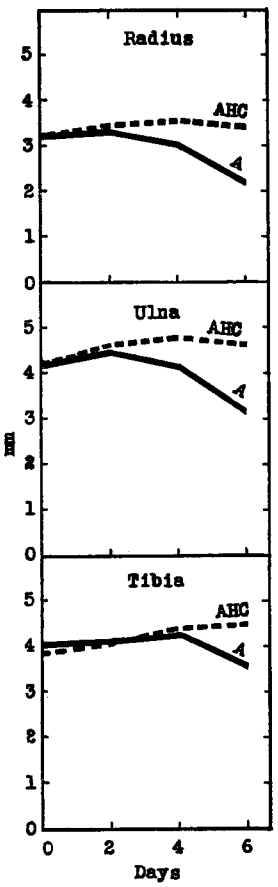
2. *The Effect of Hydrocortisone Alone.*—During 6 days' cultivation, the growth of the mouse bones (24 explants) exposed to hydrocortisone (Experiments 316, 318) was similar to that of the corresponding controls in normal medium; in this respect the slowly growing mouse bones differed in their response from the rapidly growing chick rudiments treated with the same dose (7.5 μg/ml) of hydrocortisone.

The chief histological difference between the hydrocortisone-treated and control explants, was the arrest of cartilage excavation in the former (Figs. 3, and 5). The resorption bays that were so conspicuous in the controls, were small or absent in the bones exposed to the hormone and the surface of the hypertrophic cartilage next to the marrow appeared almost straight in longitudinal section. After toluidine-blue staining, the zone of colourless matrix often seen in the controls in regions of active resorption, were rarely observed in the bones grown in the presence of hydrocortisone.

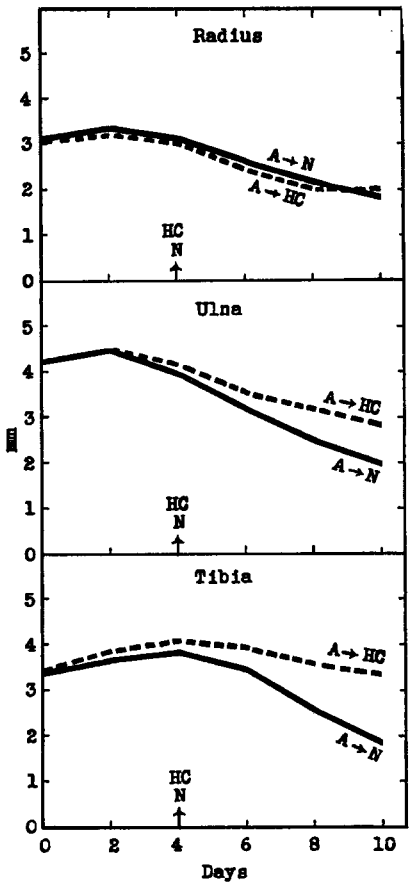
3. *The Effect of Vitamin A Alone.*—The effect of vitamin A on late fetal mouse bones has been described in detail elsewhere (Fell and Mellanby, 1952; Fell and Thomas, 1960). In the present experiments (298, 317), the 24 bones grown for 6 days in vitamin A medium showed the usual response *viz.* rapid shrinkage and resorption of the cartilage with loss of metachromasia, and speedy dissolution of the bone (Figs. 4 and 6), so that by the 6th day the explants had largely disintegrated (Text-fig. 5). Most of the cells, however, remained healthy and active except in some places in the interior of the explant where the cells had become densely packed owing to the collapse of the bone and cartilage; mitosis was abundant among the osteoblasts and marrow reticulum cells, and quite common among the chondrocytes of the dwindling cartilage.

The radius, being the smallest rudiment, was the most affected by the vitamin; the relatively stout tibia was the least changed and though greatly reduced in size by the 6th day, it had largely preserved its original shape.

4. *The Effect of Vitamin A + Hydrocortisone.*—All the 18 bones grown in the presence of both vitamin A and 7.5 μg hydrocortisone/ml, differed strikingly from the corresponding explants cultivated in medium to which only vitamin A had been added (Experiments 298, 317). The effect of the vitamin was much retarded by hydrocortisone (Text-fig. 5; Fig. 4), so that after 6 days in culture the explants treated with both agents had shrunk very little and in life appeared almost normal, whereas those in vitamin A medium were either fragmentary (radius and ulna) or greatly reduced (tibia). The contrast between



TEXT-FIG. 5.



TEXT-FIG. 6.

TEXT-FIG. 5. Curves showing the effect of vitamin A (A) on the length of the radius, ulna, and tibia from late fetal mice as compared with that of vitamin A + hydrocortisone (7.5 μg/ml) (AHC) (Experiments 317, 298). Each curve represents the average change in length of 6 bones.

Vitamin A alone produces a severe shrinkage which does not appear when hydrocortisone also is present in the medium.

TEXT-FIG. 6. Paired radii, ulnae, and tibiae of late fetal mice were grown for 4 days in medium containing 10 I.U. vitamin A/ml; one of each pair was then transferred to normal medium and the other to medium containing 7.5 μg hydrocortisone/ml (Experiment 320). Each curve shows the average change in length of 4 bones.

Hydrocortisone had no effect on the shrinkage of the radii, but retarded the vitamin A effect on the larger ulnae and tibiae.

the bones exposed to both vitamin A and hydrocortisone and those treated with the vitamin only, was greater in the radii and ulnae than in the tibiae; as stated above, of the three bones used the tibia is the most resistant to the action of the vitamin.

Although the effect of vitamin A was greatly retarded by hydrocortisone it was not arrested. In histological preparations (Fig. 6b), the terminal cartilage of the bones treated with both agents showed loss of metachromasia from the peripheral matrix, and resorption of both bone and cartilage was greater than in rudiments grown in normal medium.

Six bones were grown in medium containing 75 μg hydrocortisone/ml + vitamin A (Experiment 298); they were similar to the explants grown with the vitamin + 7.5 μg hydrocortisone/ml.

5. The Effect of Transferring Explants from Medium Containing Vitamin A to Medium with Hydrocortisone.—Twelve pairs of bones (Experiment 320) were grown for 4 days in vitamin A medium. One of each pair was then transferred to medium with hydrocortisone (7.5 μg/ml) and the other to a normal clot; after a further 6 days' cultivation, they were fixed for histological study.

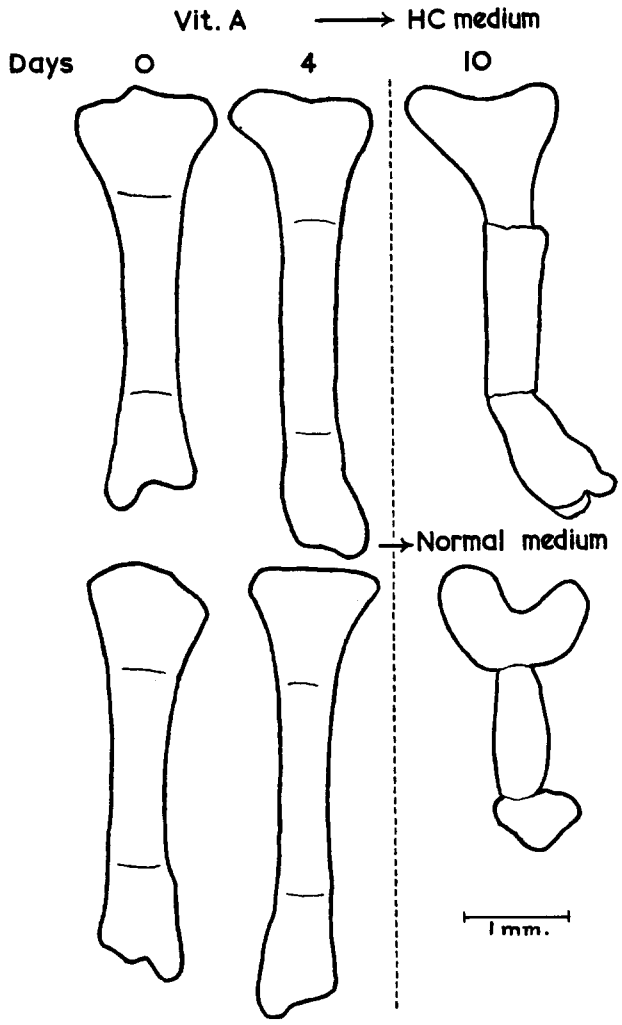
In the living cultures, the usual vitamin A changes were observed during the first 4 days (Text-figs. 6 and 7). After transfer to normal medium, the radii and ulnae continued to disintegrate and were fragmentary by the 8th day. The tibia fared rather better. Both the bony and the cartilaginous parts of the shaft rapidly shortened, until finally the broad end of the terminal cartilage was sitting squarely on a short tube of periosteal bone (Text-fig. 7); in three of the tibiae, this residual cartilage continued to enlarge at the proximal end of the bone and in all 4 explants it acquired a curious bilobed shape.

The radii and ulnae transferred to normal medium, showed all the histological changes characteristic of exposure to excess vitamin A. The cartilage had almost gone, and the interior of the dilapidated bony shaft was filled with cells which seemed to be mainly osteoblasts and narrow reticulum cells; mitosis was plentiful and even occurred in the shrunken remnants of the cartilage. In the tibiae, the surviving terminal cartilage was quite strongly metachromatic; the chondrocytes were nearly all in the early hypertrophic stage and represented the epiphysial ossification centre, but the small celled articular cartilage that normally covers the centre, had almost completely disappeared. In all four explants, bone was being deposited on the articular surfaces of the hypertrophic cartilage.

The radii transferred to medium with hydrocortisone did not differ significantly from those placed in normal medium (Text-fig. 6), and retardation of the vitamin A effect was seen in only one explant, the largest of the 4. In all the ulnae, however, hydrocortisone considerably retarded the vitamin A changes. The greatest inhibitory effect of the hormone was in the tibiae all of which were much larger at the end of the culture period than the corresponding rudiments transferred to a normal clot.

Histological examination confirmed the observations on the living cultures treated with hydrocortisone. The sections also showed evidence of diffuse ossification in the very cellular marrow cavity, and bone was being deposited on the articular surfaces of the tibiae.

From these results it was clear that after 4 days' exposure to vitamin A, the



TEXT-FIG. 7. Camera lucida drawings of a pair of tibiae from a late fetal mouse (Experiment 320). Both bones were grown for 4 days in medium containing 10 I.U. vitamin A/ml; one was then transferred for a further 6 days to medium to which hydrocortisone (7.5 μg/ml) had been added and the other to normal medium.

The hydrocortisone has retarded the progress of the vitamin A effect.

relatively stout tibia which is the most vitamin A–resistant of the three bones, gave the best response to hydrocortisone, that the small highly susceptible radius was unaffected by the hormone, and that the medium-sized ulna gave an intermediate reaction. Although the vitamin A changes were much retarded in the ulna and tibia, however, they were not arrested by hydrocortisone.

DISCUSSION

In our experiments, hydrocortisone alone severely inhibited the growth of the cartilaginous limb bone rudiments from 7-day chick embryos; these results confirmed previous work on the action of cortisone and hydrocortisone on embryonic chick cartilage in culture (Buno and Goyena, 1955; Sobel and Freund, 1958; Whitehouse and Lash, 1961). Neither Buno and Goyena nor Sobel and Freund record any difference in the matrix between hydrocortisone-treated and control explants. On the other hand Whitehouse and Lash found that the synthesis of matrix was much inhibited by cortisone and hydrocortisone during chondrogenesis induced in somite cultures; our results agree with these findings, since the intercellular partitions of the epiphysial matrix were considerably narrower in the hydrocortisone-treated rudiments than in the controls. Rather to our surprise, however, in sections stained with toluidine blue, the metachromasia was more intense in the hydrocortisone-treated cartilage; the significance of this is not known. In our explants, as in those of Sobel and Freund, the diaphysial cartilage cells were smaller in the rudiments grown in medium with hydrocortisone.

The question arises as to what factors are responsible for the reduced growth of chick cartilage cultivated in the presence of hydrocortisone or cortisone. Buno and Goyena attribute the reduced growth rate to diminished cell division. Whitehouse and Lash, however, found that the increase in deoxyribonucleic acid (DNA) was the same in both treated and control explants, and state that "the effects of hydrocortisone cannot therefore be simply explained as an inhibition of cell multiplication and loss of viability"; their results suggest that the lowered growth rate is due to depressed synthesis of matrix. In our material also, diminished synthesis of matrix must account at least partly for the growth inhibition, but it is probable that the failure of the diaphysial chondroblasts to hypertrophy normally is a contributory factor; we have no information about the DNA content of the hydrocortisone-treated and control explants. Retardation of skeletal growth has also been described by Moscona and Karnofsky (1960) in chick embryos treated *in vivo*.

The explants of the well developed but slowly growing mouse bones, displayed another effect of hydrocortisone *viz.* an inhibition of cartilage resorption of the cells of the marrow cavity; this effect also is mentioned by Mellanby in his unpublished notes. A similar inhibition of the excavation of cartilage has been observed *in vivo*. Follis (1951), who investigated the effect of cortisone on the growing bones of young rats, found that "a dense zone composed of spicules

of calcified cartilaginous matrix encased in bone appears at the growing ends of the bone," and he concludes that "there is an inhibition or retardation in normal osteolytic sequences." Moscona and Karnofsky (1960) record a similar phenomenon in the limb bones of chick embryos treated with cortisone *in vivo*; at a stage when the cartilage had been excavated from the shafts of the long bones in the controls, in the hydrocortisone-treated chicks "the shaft consisted of a continuous core of cartilage surrounded by perichondral bone." The arrested resorption of the terminal cartilage in the fetal mouse bones exposed to hydrocortisone in culture, shows that this "retardation in normal osteolytic sequences" is due to a direct action of the hormone on the tissue.

Thus hydrocortisone or cortisone has essentially the same effect on skeletal tissues in culture as it has on the growing skeleton of the intact animal, and inhibits normal growth and resorption both *in vitro* and *in vivo*. The combined action of hydrocortisone or cortisone and vitamin A, however, is strikingly different in culture from that in the body: in the former the hormone retards and in the latter it accelerates the action of the vitamin on skeletal tissue. The accelerating influence of the hormone on the effect of the vitamin was first shown by Selye (1958) in rats, and in the Strangeways Laboratory similar results (unpublished) have recently been obtained by G. Weissmann on *Xenopus* larvae.

In view of the fact that skeletal resorption is inhibited by hydrocortisone alone, both *in vivo* and in organ culture, it might have been expected that the processes of skeletal dissolution set in motion by excess vitamin A, would be retarded *in vivo* as they are in organ culture, and the apparent additive effect of the two agents in the body is puzzling. *In vivo*, however, systemic factors operate that are excluded from cultures *in vitro*. Thus Clark and Colburn (1955) showed that in rats put on a vitamin A–deficient diet, the administration of cortisone reduced the amount of vitamin A stored in the liver to 20 per cent of its original value in 13 days, whereas there was no measurable depletion in the controls during this brief period. G. Weissmann (personal communication) observed a striking additive effect of the two agents on the histological structure of the liver in *Xenopus* larvae; either compound alone produced little change, but both together caused partial atrophy. Moreover Jackson and his colleagues (Wang, Glass, Goldenburg, Stearns, Kelly, and Jackson, 1954) found that the administration of pituitary corticotropin to patients with rheumatic fever raised the level of vitamin A in the blood stream. These observations suggest that *in vivo* the hormone may hasten the action of the vitamin by preventing it from being stored in the liver, so that a high concentration is rapidly produced in the blood. It should be emphasized that under the conditions of our experiments, hydrocortisone merely delays but never arrests the action of the vitamin, and there is little difference in protective action between a dose of 7.5 and one of 75.0 μg hydrocortisone/ml.

As yet nothing is known of the mechanism whereby hydrocortisone retards

the effect of excess vitamin A on skeletal rudiments in culture. Recent work (Fell and Thomas, 1960; Dingle, Lucy, and Fell, 1961; Lucy, Dingle, and Fell, 1961; Dingle, 1961) indicates that vitamin A increases the proteolytic activity of the chondrocytes, and that the degradation of the cartilage matrix may be due to the liberation of a protease (with other enzymes) from the lysosomes (Dingle, 1961). If this view is correct, it is possible that hydrocortisone may partially inhibit the activity, the synthesis or the release of the protease. Further experiments on animals and organ cultures should resolve the discrepancy between the results obtained *in vivo* and *in vitro*.

SUMMARY

The effect of hydrocortisone has been studied in organ cultures of the cartilaginous long bone rudiments from 7-day chick embryos and of the well ossified limb bones from late fetal mice. In the chick rudiments, which grow rapidly in culture, the growth rate was much reduced by hydrocortisone, less intercellular material was formed, and the hypertrophic cells of the shaft were much smaller than in the controls in normal medium. In the late fetal mouse bones, which grow very little in culture, hydrocortisone had no obvious effect on growth but arrested resorption of the cartilage. These effects resemble those described by others in the skeleton of animals treated with cortisone or hydrocortisone.

The influence of hydrocortisone on the response of the chick and mouse explants to excess vitamin A was investigated. In the presence of excess vitamin A, cartilage (chick, mouse) and bone (mouse) rapidly disintegrated, but when hydrocortisone also was added to the medium, this dissolution of the intercellular material was much retarded, though not suppressed.

The retardative action of hydrocortisone on the changes produced by excess vitamin A in skeletal tissue in culture, contrasts sharply with the strongly additive effect of the two agents on the skeleton in the intact animal (Selye, 1958). It is suggested that this discrepancy between the results obtained *in vitro* and *in vivo* is probably due to systemic factors that operate in the body but are eliminated in organ cultures.

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EXPLANATION OF PLATES

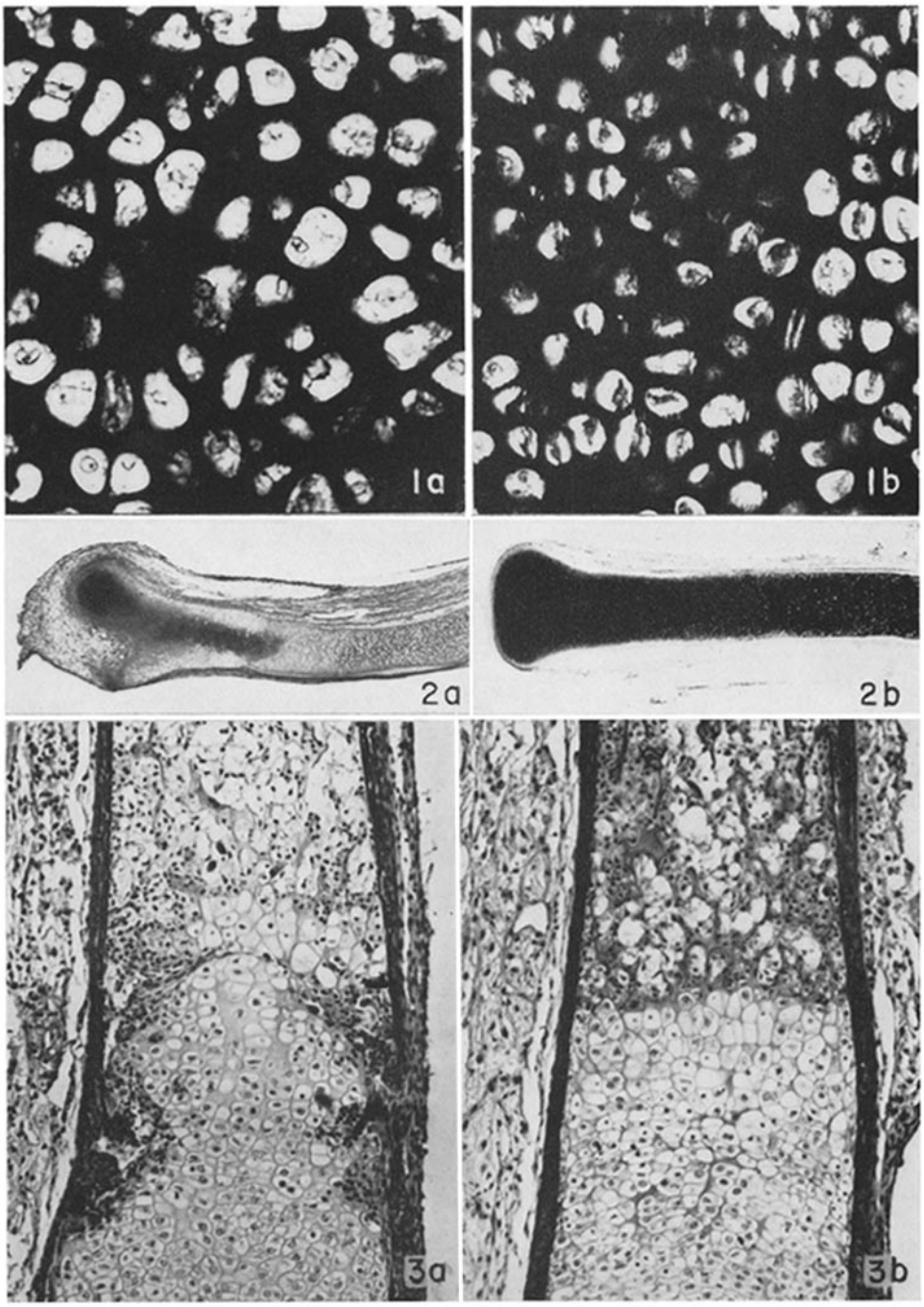
The photographs in Fig. 4 are by Mr. V. C. Norfield and the remaining photographs by Mr. M. F. Applin.

PLATE 34

FIG. 1. Hypertrophic cartilage in humeri from a 7-day chick embryo, after 8 days' cultivation in control medium (Fig. 1 *a*) and medium containing 7.5 μg of added hydrocortisone Fig. 1 *b* (Experiment 311). The cells are much smaller in Fig. 1 *b* than in Fig. 1 *a*. Toluidine blue. × 1300.

FIG. 2. The proximal ends of femora from a 7-day chick embryo, grown for 8 days in medium containing 10 I.U./ml of added vitamin A alcohol (Fig. 2 *a*) and in medium to which had been added both the vitamin and 7.5 μg/ml of hydrocortisone (Fig. 2 *b*) (Experiment 285). The severe loss of metachromasia from the cartilage matrix of Fig. 2 *a* has been inhibited in Fig. 2 *b* by the hormone. Toluidine blue. × 20.

FIG. 3. Fig. 3 *a*, distal ends of the ulnae of a mouse fetus near term after 6 days' cultivation in control medium (Fig. 3 *a*) and medium containing 7.5 μg of added hydrocortisone Fig. 3 *b* (Experiment 316). Note the active invasion and resorption of the cartilage in 3 *a*; these processes are arrested in 3 *b*. Mayer's acid hemalum, celestine blue, and van Gieson's stain. × 335.



(Fell and Thomas: Hydrocortisone and limb bone rudiments)

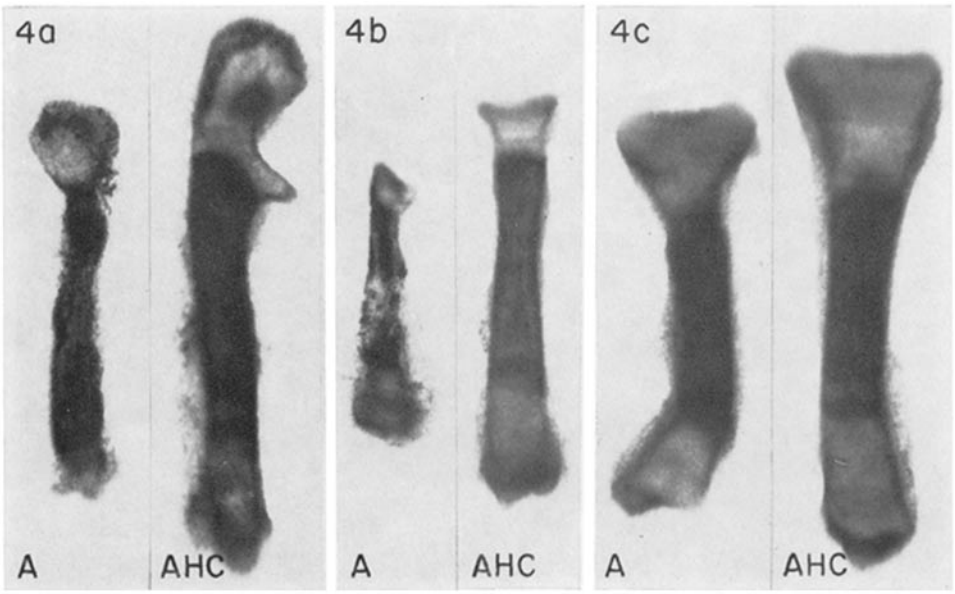
PLATE 35

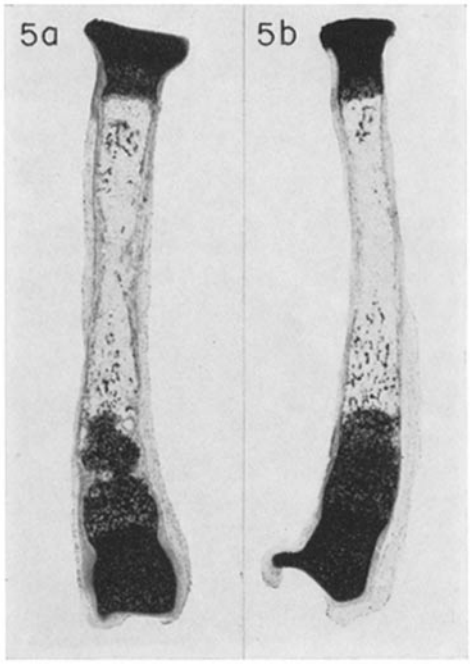
All the explants shown in this plate are from fetal mice near term.

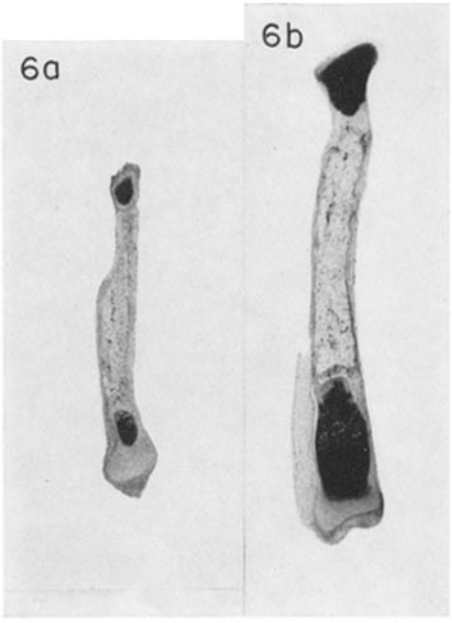
FIG. 4. Three pairs of living bone rudiments from the same fetus photographed after 6 days' cultivation (Experiment 317). One of each pair (*A*) was grown in medium to which 10 I.U./ml of vitamin A had been added, and the other (*AHC*) in medium containing both the vitamin and 7.5 μg/ml of hydrocortisone. In all three pairs the hydrocortisone has greatly retarded the action of the vitamin. Fig. 4 *a* ulnae; Fig. 4 *b* radii; Fig. 4 *c* tibiae. × 15.

FIG. 5. Radii from the same fetus after 6 days' growth in control medium (Fig. 5 *a*) and medium containing 7.5 μg/ml of hydrocortisone (Fig. 5 *b*) (Experiment 316). Note the extensive invasion and resorption of the distal cartilage in Fig. 5 *a*; these processes are arrested in Fig. 5 *b*. Toluidine blue. × 20.

FIG. 6. Radii from the same foetus after 6 days' growth in medium to which had been added 10 I.U./ml of vitamin A of (Fig. 6 *a*) and both the vitamin and 7.5 μg/ml of hydrocortisone (Fig. 6 *b*). The dissolution of the cartilage and bone which is advanced in Fig. 6 *a* is greatly retarded though not arrested in Fig. 6 *b*; note some loss of metachromasia from the distal cartilage in Fig. 6 *b* as compared with the control shown in Fig. 5 *a*. Toluidine blue. × 20.







(Fell and Thomas: Hydrocortisone and limb bone rudiments)