COMPARISON OF THE EFFECTS OF PAPAIN AND VITAMIN A ON CARTILAGE

II. The Effects on Organ Cultures of Embryonic Skeletal Tissue

By HONOR B. FELL,* and LEWIS THOMAS,‡ M.D.

(From the Strangeways Research Laboratory, Cambridge, England, and the Department of Medicine, New York University-Bellevue Medical Center, New York)

Plates 58 to 62

(Received for publication, January 12, 1960)

When embryonic cartilage is grown in medium containing excess vitamin A, the most conspicuous effect of the vitamin is the disappearance of metachromatic material from the matrix (Fell and Mellanby, 1952). Autoradiographic studies, in which the explants were treated with labeled inorganic sulfate, $S^{35}O_4$, showed that this loss of metachromasia is preceded by an inability of the cartilage to fix sulfate, and is accompanied by a loss of the sulfate already present (Fell, Mellanby, and Pelc, 1956). It was not known whether this effect on chondroitin sulfate was the fundamental action of the vitamin on embryonic skeletal tissue in culture, or whether it would account for all the changes observed, including the quick resorption of fetal mouse bone (Fell and Mellanby, 1952).

Thomas (1956) found that crude papain injected into young rabbits caused a rapid loss of chondroitin sulfate from all the cartilage of the body. This was dramatically seen in the collapse of the ears which, 18 hours after injection, drooped like those of a spaniel. Histological examination of the affected cartilage showed that the intercellular partitions were much reduced and had lost their normal basophilia. The general picture was strikingly reminiscent of that seen in fetal mouse bones grown in medium containing excess vitamin A. Later it was found that this effect of the crude papain could be reproduced by the protease component (McCluskey and Thomas, 1958).

Since both papain and vitamin A removed the chondroitin sulfate from living cartilage, it seemed desirable to compare the effects of these two agents on the same material under the same experimental conditions; if the structural changes produced by the vitamin were due primarily to the removal of chondroitin sulfate from the tissue, they should be almost identical with those

* Royal Society Foulerton Research Fellow.

‡ Work done during tenure of a Fellowship awarded by the Commonwealth Fund.

produced by papain protease. In the preceding paper (Thomas, McCluskey, Potter, and Weissmann, 1960) experiments were described in which the effects of papain and vitamin A on the cartilage of young rabbits were compared; it was shown that the administration of large doses of vitamin A caused partial collapse of the ears and release of chondroitin sulfate from the cartilage matrix. The present paper deals with experiments in which limb bone rudiments from embryonic chicks and late fetal mice were grown as organ cultures and exposed to papain, or vitamin A, or to both substances simultaneously.

Material and Methods

Material.—Bone rudiments were dissected from the limbs of 7- and 13-day chick embryos, and of mouse fetuses near term.

Culture Methods.—The standard watch-glass method was used (Fell and Robison 1929; Fell and Mellanby, 1952). The culture medium consisted of 15 drops of cock plasma mixed with 5 drops of embryo extract, and the bones were transplanted to fresh medium every 2 days. For each subculture, 2 extracts were prepared from 13-day chick embryos, as described by Fell and Mellanby (1955). The extract for the culture medium was made of equal parts of tissue mince and Tyrode supplemented with 1 per cent (w/v) glucose for chick explants and 4 per cent glucose for mouse bones; a more dilute extract, in which the rudiments were finally washed before being explanted, was composed of 1 part of mince and 2 parts of Tyrode without extra glucose.

Addition of Papain Protease and Vitamin A to the Medium.—Crystalline papain protease was obtained from Nutritional Biochemicals Corporation, Cleveland. It was received as a non-sterile solution containing 0.03 M cysteine; in some experiments the cysteine was removed by dialysis. To obtain a sufficiently high concentration of the enzyme without diluting the medium too much, the following procedure was adopted. A measured amount of the papain solution was placed in a 6.5 × 2 cm. centrifuge tube and dried in a vacuum desiccator; absolute ethanol was added to sterilize the dried material, which was ground with a sterile glass rod until it formed a fine suspension in the ethanol. The alcohol was then evaporated in the desiccator. A measured volume of plasma was introduced into the tube, well mixed with the dry powder and transferred to a sterile wax tube to prevent clotting. The plasma containing the papain was allowed to stand at 4°C. for 2 days before use, during which time most of the powder dissolved. At the beginning of the investigation, irregular results were obtained because the plasma was used immediately or very soon after addition of the papain. Solid as yet undissolved material rapidly sank to the bottom of the pipettes and watch-glasses, so that the explants received variable doses. These experiments were rejected. However, the procedure indicated above proved satisfactory and gave reproducible results. Except in Experiments 252, 270 (Table I), 251 (Table II), and 253 (Table IV) the solution of papain protease also contained cysteine.

Synthetic vitamin A alcohol (Roche Products, Ltd., London) was used and was stored under nitrogen (at −20°C.). To introduce it into the plasma, a weighed quantity was dissolved in absolute ethanol; the strength of the alcoholic solution was adjusted so that the addition of 0.2 per cent to the plasma produced the required concentration of vitamin A. Since ethanol is not a biologically inert substance, 0.2 per cent was added to the plasma of all the culture media whose effects were to be compared with those of the media containing vitamin A.

Measurement.—In most experiments the explants were drawn with the aid of a camera lucida at the beginning of the experiment and thereafter at 2-day intervals. Each drawing was measured along its midline by means of a piece of string infiltrated with paraffin wax, which served as a flexible ruler (Fell and Mellanby, 1952).

Design of Experiments.—The designs of the individual experiments and the number of explants in each are recorded in Tables I to IV. To compare the effects of 2 media, the bone rudiments from one side of each embryo (set *a*) were explanted in one medium and those from opposite side (see *b*) in the other. Thus in Table II, the first line indicates that in Exp. 251, 2 pairs of humeri, femora, and tibiae (*i.e.* 12 rudiments) were explanted; the rudiments from one side of each chick were grown in medium containing 75 micrograms/ml. of papain without cysteine, those from the opposite side in normal (control) medium, and the explants were cultivated *in vitro* for 2 days.

Histology.—For histological study the explants were fixed for 30 minutes in 3 per cent acetic Zenker's fluid which was then replaced by Zenker's fluid without acetic acid. After a further hour's fixation, the rudiments were washed in several changes of tap water for 2 to 3 hours; they were left overnight in 70 per cent ethanol, then stained in bulk with eosin in 95 per cent ethanol to facilitate subsequent orientation, dehydrated, and cleared in 3 changes of cedar wood oil. The explants were infiltrated overnight with paraffin wax of m.p. 38°C. in an oven kept at 45°C., and transferred for 5 to 6 hours to paraffin wax of m.p. 56°C. containing a trace of beeswax (one change). For the study of glycogen (Exps. 265, 266, 267, 269) the explants were fixed in Rossmann's fluid; this, like other alcoholic fixatives, has the disadvantage of causing serious folding in the cartilage when the sections are flattened.

Serial sections (7 μ) were cut and stained with toluidine blue, with celestine blue, Mayer's acid hemalum, and Van Gieson's stain, or with Delafield's hematoxylin and chromotrop. For the demonstration of glycogen, sections were stained by the periodic acid–Schiff method (PAS); control sections from the same block were digested with diastase before staining.

RESULTS

A. The Effect of Single Exposure of Limb-Bone Rudiments from 7-Day Chick Embryos to Papain or Vitamin A

The experimental procedure is summarised in Table I. The femora, tibiae, humeri, and in one experiment (270) the radii and ulnae were used. At the time of explantation the rudiments consisted of a cartilaginous rod differentiated into the usual 3 zones: a middle segment of hypertrophic cartilage merging on either side with a relatively broad proliferative zone of flattened cells which in turn was continuous with the small-celled epiphyseal cartilage. A thin layer of periosteal bone covered the hypertrophic region.

Nine rudiments were incubated for 30 minutes (Exps. 237, 254) and 7 for 1 hour (Exps. 268, 270) in Tyrode medium containing 200 micrograms/ml. of papain with cysteine and were histologically examined. Metachromasia had largely or completely disappeared from the matrix of those incubated for 30 minutes and had entirely gone from all of those incubated for 1 hour (Fig. 1 b); many mitoses in all phases were present in the cartilage.

Six rudiments fixed after 30 minutes' incubation in Tyrode containing 200 micrograms/ml. of papain without cysteine (Exp. 252) were much less affected than those incubated for 30 minutes in papain and cysteine; and metachromasia had disappeared from the terminal cartilage only. Five rudiments incubated for 1 hour in papain without cysteine (Exp. 270) all contained some metachromatic material, though less than those fixed after the shorter period.

The eight rudiments fixed after 1 hour's incubation in Tyrode containing 100 I.U./ml. of vitamin A (Exps. 268, 270) were unchanged, as were the 8 controls incubated in Tyrode alone (Exps. 268, 270) (Fig. 1 a) and the 5 rudiments in Tyrode-containing cysteine (Exp. 270).

In 2 experiments, 8 pairs of rudiments were incubated in Tyrode containing papain and

cysteine for 30 minutes (Exps. 254) or 1 hour (Exp. 268); one of each pair was fixed immediately after incubation (Fig. 1 b) (see above) and the other was transferred to normal medium and cultivated for 2 or 4 days before fixation (Fig. 1 c).

TABLE I

Experiments on a Single Exposure of 7-Day Chick Bone Rudiments to Papain and/or Vitamin A in Various Solutions in Tyrode Solution

Experiment	No. pairs	Incubation			Subsequent treatment		
		Solution		Incubation period	Immediate fixation	Cultivated	Culture period
		Set a	Set b				
							days
237	1 H, F, T.	PP + CSH	Tyrode	30 min.	a, b	—	—
240	1 H, F, T, R, U.	" "		"	a	—	—
252	1 H, F, T.	PP − CHS	PP − CSH	"	a	b	2
	"	" "	" "	"	"	"	4
254	"	PP + CSH	PP + CSH	"	a	b	2
	"	" "	" "	"	"	"	4
268	1 H, T.	" "	" "	1 hr.	a	b	"
	1, H, F, T.	APP + CSH	APP + CSH	"	"	"	"
	"	A	A	"	"	"	"
	"	Tyrode	Tyrode	"	"	"	"
270	1 H, F, T, R, U.	PP + CSH	PP − CSH	"	a, b	—	—
	"	APP − CSH	A	"	"	—	—
	"	Tyrode + CSH	Tyrode	"	"	—	—

H, humerus; F, femur; T, tibia; R, radius, U, ulna. PP, papain protease, 200 γ/ml.; CSH cysteine; A, vitamin A, 100 I.U./ml.; APP, papain protease + vitamin A; set a and set b, rudiments from opposite sides of the same embryo.

The papain-treated rudiments rapidly recovered in culture; by the end of the 2nd day the metachromatic material had been largely restored, and after 4 days the matrix appeared normal and the explants had more than doubled their original length. In rudiments treated with papain without cysteine and then grown for 2 days (Exp. 252) the terminal cartilage recovered all or most of its metachromasia. Explants pretreated with vitamin A or Tyrode (Exp. 268) behaved normally during subsequent cultivation.

TABLE II

Experiments on 7-Day Chick Bone Rudiments Grown in Medium Containing Papain (γ/Ml.), Vitamin A (I.U./Ml.) or both

Experiment	No. pairs	Culture medium		Culture period	Subsequent treatment		
		Set a	Set b		Immediate fixation	To normal medium	Culture period
				days			*days*
251	2 HFT	PP, 150 γ	Control	2	a, b	—	—
257	1 "	A, 15 I.U.	"	6	a, b	—	—
261	1 "	A, 10 I.U.	"	4	a, b	—	—
	1 "	" " "	"	6	" "	—	—
262	2 "	PP, 150 γ	"	8	a, b	—	—
	2 "	A, 15 I.U.	A, 15 I.U. + PP, 150 γ	8	" "	—	—
265	2 "	A, 10 I.U.	Control	8	a, b	—	—
	2 "	PP, 75 γ	A, 10 I.U. + PP, 75 γ	8	" "	—	—
267	2 "	PP, 75 γ	Control	8	a, b	—	—
	2 "	A, 10 I.U.	1, 10 I.U. + PP, 75 γ	8	" "	—	—
269	2 "	PP, 150 γ	PP, 150 γ	6	a	b	6
	2 "	A, 15 I.U. + PP, 150 γ	A, 15 I.U. + PP, 150 γ	6	a	b	6
277	2 "	PP, 15 γ	Control	8	a, b	—	—
	2 "	PP, 1.5 γ	"	8	"		

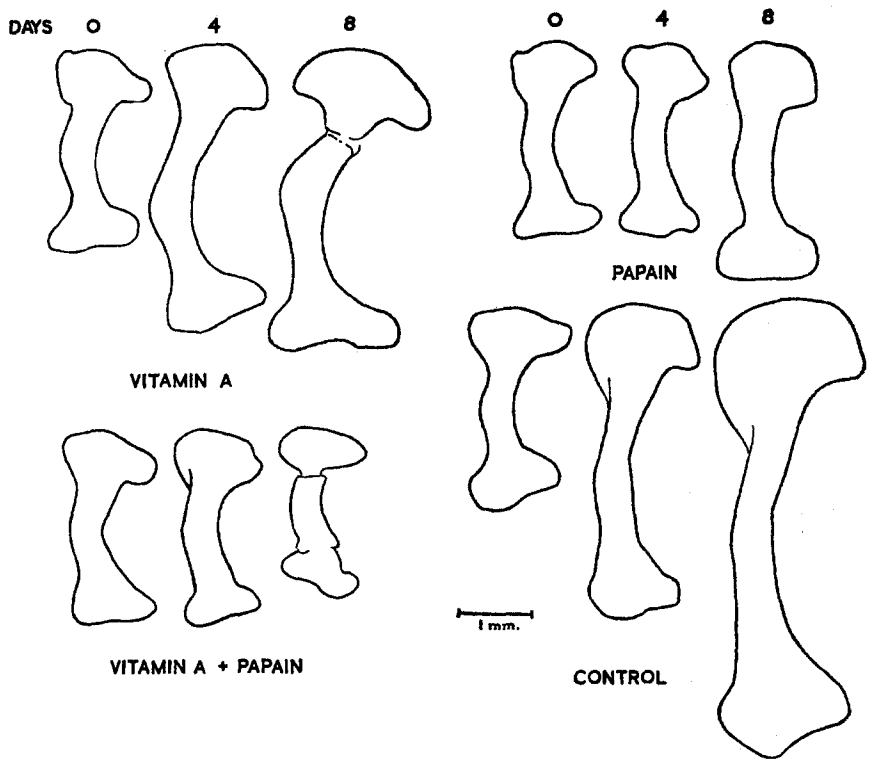
Lettering as for Table I. Papain protease + cysteine was used for all experiments except No. 251 in which cysteine was omitted.

B. Comparison of the Effects on Limb Bone Rudiments of Cultivation in Media Containing Papain and Excess Vitamin A

1. Seven-Day Chick Rudiments (Table II). The femur, tibia, and humerus were cultivated; their structure at explantation has been described in section A. Camera lucida drawings were made at 2-day intervals of all the explants except in Exps. 257, 261.

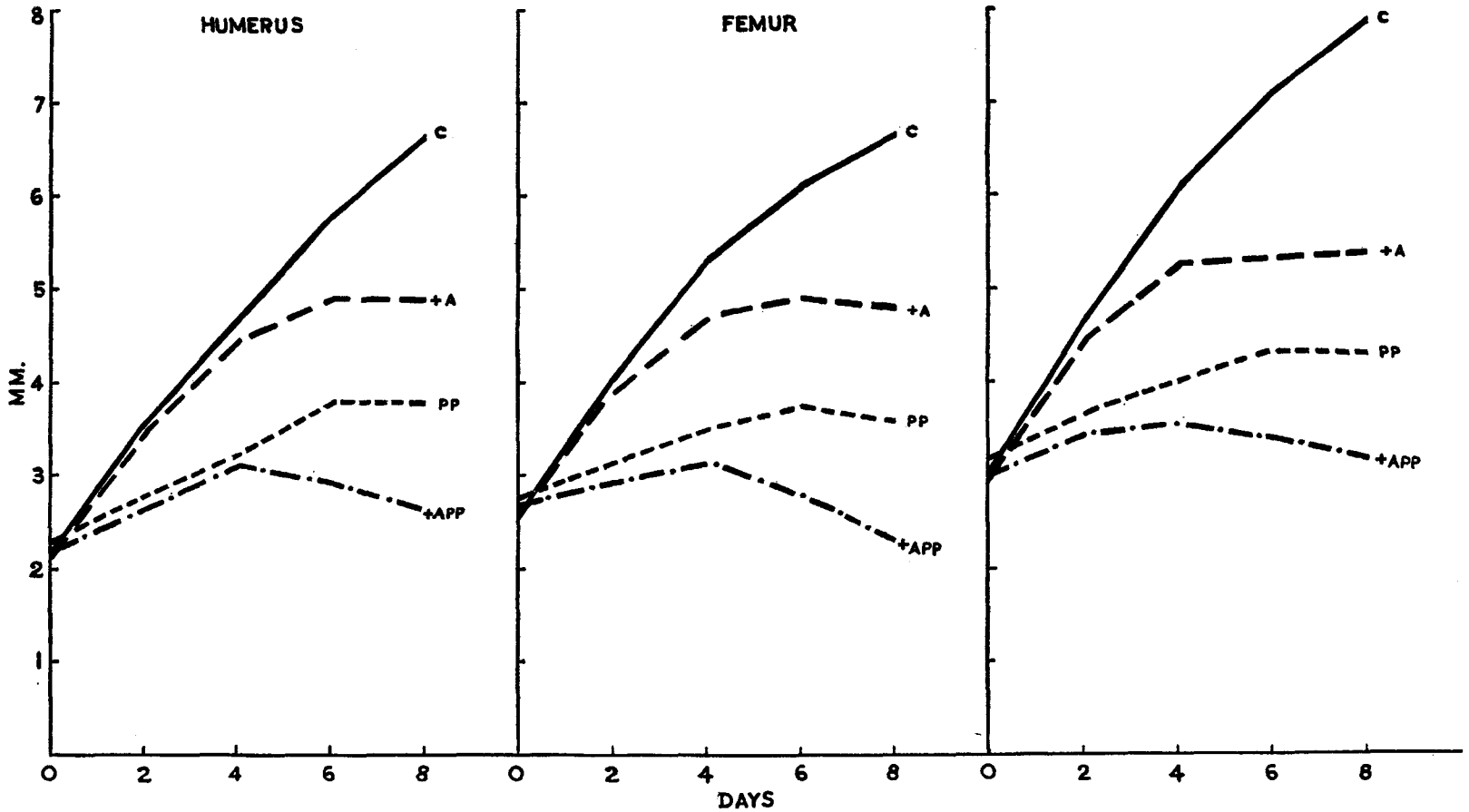
The 33 controls grown in normal medium enlarged rapidly (Text-figs. 1 and 2); by the 2nd day they had elongated by about 50 per cent and had nearly trebled their length by the 8th

day. There was a corresponding increase in the width of the epiphyses. When examined under the dissecting microscope they appeared transparent and very refractile, and the thin layer of periosteal bone originally present in most of the explants had thickened considerably during cultivation. The cartilage was enclosed in a capsule of organized connective tissue from the margin of which there was a diffuse outgrowth of ameboid fibroblasts and macrophages.



TEXT-FIG. 1. Serial camera lucida drawings of 4 living humeri in culture, from Exp. 262. Explants from 7-day chick embryos. Doses: 15 I.U./ml. of vitamin A; 150 γ/ml. of papain; control grown in normal medium. Vitamin A, the humerus is smaller than the control and there is a fracture at the proximal end of the shaft. Papain, the protease has inhibited growth more than vitamin A, but its effect is uniform throughout the rudiment. Vitamin A and papain, the two agents have a potent additive effect; there is a fracture at both ends of the shaft, and the explant is much smaller than either the vitamin A– or papain-treated rudiments.

When examined histologically after 8 days in culture, the controls appeared healthy (Fig. 2). They showed the normal differentiation into small-celled epiphyses each separated by a zone of flattened cells from the region of vacuolated, hypertrophic cells composing the middle segment of the shaft. In sections stained with toluidine blue, the cells were separated by broad partitions of intensely metachromatic matrix. On the surface of the hypertrophic cartilage there was a thick layer of young periosteal bone which also stained metachromatically in varying degrees, though far less intensely than the cartilage. In sections stained with PAS, the



TEXT-FIG. 2. Curves showing the effects of vitamin A, papain, and vitamin A with papain on the growth in length of the humerus, femur, and tibia in culture (Exps. 265, 267). Explants from 7-day chick embryos. Doses, 10 I.U./ml. of vitamin A; 75 γ/ml. of papain; controls in normal medium.

Each of the 12 curves represents the average growth of 4 explants. Papain reduces growth more than vitamin A, and vitamin A and papain more than either agent alone.

matrix was pale pink; with Van Gieson's stain it was almost colorless and the bone a vivid scarlet.

There were striking differences between the control rudiments grown in normal medium, and those taken from the opposite side of the same embryos and cultivated for 8 days in medium containing papain (Exps. 262, 267, 277) (Text-figs. 1 and 2). Some of the 18 rudiments grown in the presence of 150 micrograms/ml. of papain with cysteine (Exps. 262, 269) enlarged slightly during 6 to 8 days in culture, but others remained almost the same size or even diminished a little. The 12 explants in medium containing 75 micrograms/ml. of papain with cysteine (Exps. 265, 267) enlarged slowly until the 6th day when growth ceased. When the living cultures were observed at daily intervals under the dissecting microscope, a gradual change in the cartilage was noted. It became less refractile, the epiphyses acquired a yellowish tinge by direct illumination, the diaphysis appeared increasingly grey and opaque, and the chondroblasts more closely packed together. The humeri were the least and the tibiae the most affected by the enzyme. After each subculture, the soft tissue attached to the rudiments formed a broad zone of outgrowth. Changes seen in the 6 explants exposed to papain without cysteine (Exp. 251) were similar but much less pronounced; this was at least partly owing to the fact that the rudiments were fixed after only 2 days in culture.

The histological structure of the explants grown for 8 days in medium containing papain with cysteine presented a great contrast to that of the corresponding controls in normal medium (*cf.* Figs. 2 and 3). In the papain-treated rudiments (Fig. 9 a) there were no zones of flattened cells as in the controls, so that the hypertrophic cartilage of the shaft merged gradually with the small-celled epiphyses. The hypertrophic cells had the same vacuolated structure as in the controls but a more regular, rounded outline. The amount of intercellular material was drastically reduced throughout the rudiments, the chondroblasts being separated by extremely thin partitions which were particularly tenuous in the hypertrophic region. In sections stained with toluidine blue, metachromasia had almost disappeared from the cartilage matrix and had completely gone from the fairly thick layer of periosteal bone. In adjacent sections stained with PAS, however, the delicate intercellular septa were a deeper red than the matrix of the controls, and with Van Gieson's stain a similar but even greater difference was seen. This result showed that one component of the matrix was not removed by the enzyme and probably became compressed by the general shrinkage of the rudiment caused by the removal of other materials. The glycogen content of the cells of the papain-treated cartilage was similar to that in the controls.

Six explants were grown in medium containing 75 micrograms/ml. of papain without cysteine (Exp. 251). Although the rudiments were cultivated for 2 days only, a definite effect was observed; the intercellular partitions were much narrower than in the controls grown in normal medium, and metachromasia though still present was reduced, showing that the addition of cysteine was not necessary for the reaction.

One experiment (Exp. 277) was made with far lower concentrations of papain. With 15 micrograms/ml. of medium the explants were considerably smaller than their controls and the intercellular partitions were much narrower, so that the cartilage cells were more closely packed together; even after 8 days' cultivation, however, the matrix remained metachromatic although the staining was less intense than in the controls. A concentration of 1.5 micrograms/ml. produced no obvious effect.

The capacity of the rudiments to recover from the effects of papain was tested in Exp. 269.

Six pairs of explants were grown for 6 days in medium containing 150 micrograms/ml. of papain with cysteine after which one from each pair was fixed and sectioned and the other

transferred to normal medium for a further 6 days. During the period in the medium with papain, there was little increase in size and the rudiments examined after 6 days in culture showed almost complete loss of metachromasia and close approximation of the chondroblasts (Fig. 9 a). As soon as the rudiments were transferred to normal medium they began to enlarge (Text-fig. 3); the shaft did not grow very much but the ends increased to several times their previous size and the cartilage resumed its normal, glassy appearance. The rudiments became surrounded by a curious thick, rather hyaline capsule which felt hard when touched with the knife.

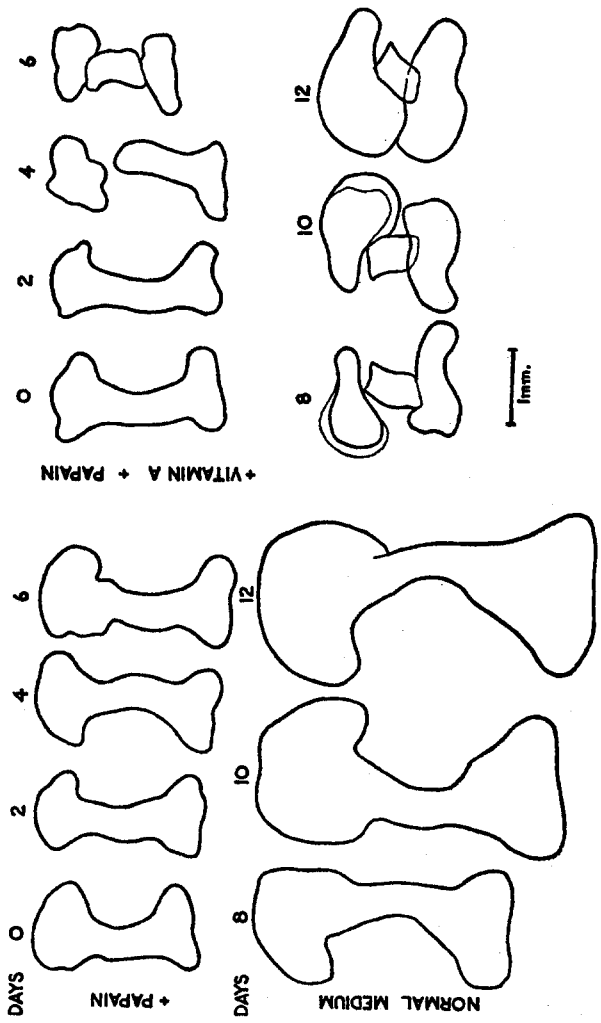
When these explants were studied histologically (Fig. 9 b), broad intensely metachromatic intercellular partitions were seen throughout the rudiment. An unexpected result was the spread into, and sometimes throughout the connective tissue capsule, of typical cartilage and of a strange diffuse, highly metachromatic chondroid tissue. In some explants the inner osteoblastic layer of the periosteum had been completely transformed into hypertrophic cartilage and the outerfibroblastic coat into the metachromatic chondroid tissues mentioned above. There was profuse mitosis throughout the capsule. This remarkable histological picture is not understood and is being investigated further.

The 27 explants grown in medium to which excess vitamin A had been added (Exps. 257, 261, 262, 265), behaved differently in several respects from those treated with papain. (Text-Figs. 1 and 2).

During the first 2 days the vitamin A-treated rudiments grew much more rapidly than did those exposed to papain, but thereafter growth declined and by the 6th day many had begun to shrink; at the end of the culture period they were much smaller than the controls in normal medium but still considerably larger than rudiments exposed to papain in the same experiment. The vitamin A–treated explants, unlike those grown in medium containing papain, usually became bent and distorted, and as previously described (Fell and Mellanby, 1952) most of them developed spontaneous fractures near one or both ends of the shaft. The surrounding soft tissue grew copiously to form a broad outgrowth round the cartilage.

Histological comparison revealed interesting resemblances and differences in structure between the vitamin A and the papain-treated explants. Both showed loss of metachromasia from the cartilage, but whereas in the papain-treated rudiments the matrix was affected fairly evenly throughout, in those cultured in excess vitamin A (Fig. 4), some areas were always altered earlier and more drastically than others; this explained why the shape of the explants which remained almost normal in the medium containing papain was much distorted in the presence of excess vitamin A. As noted in earlier work, (Fell and Mellanby 1952; Fell, Mellanby and Pelc, 1956), the most sensitive part of the bone-rudiment is the region where the zone of flattened cells is becoming transformed into hypertrophic cartilage. There, the metachromasia rapidly disappeared and usually the cells became closely squashed together, very flattened, and separated only by fibers which stained bright red with PAS and Van Gieson. Meanwhile the 2 ends of the rudiment continued to enlarge, the interior of the epiphyses remaining metachromatic for some days although metachromasia continued to disappear from an ever widening peripheral zone. As a result of this continued terminal expansion, the strongly affected, rapidly shrinking region of young hypertrophic cartilage was first distorted and then disrupted so that the end was broken off the shaft; this process was usually assisted by invasion of the proliferative zone by connective tissue cells.

In the older hypertrophic cartilage in the vitamin A-treated explants, the matrix underwent changes very similar to those produced by papain; *i.e.*, the intercellular partitions became very narrow, lost metachromasia, and acquired an increased staining reaction with Van Gieson and PAS; the effect progressed from the surface inwards. The behavior of the hypertrophic cells,



TEXT-FIG. 3. Serial camera lucida drawings of 2 living humeri in culture, from Exp. 269. Explants from 7-day chick embryos. Doses: 15 I.U./ml. of vitamin A; 150 γ/ml. of papain.

One explant was grown for 6 days in papain medium and then transferred to normal medium for a further 6 days; in the absence of papain, the explant immediately began to enlarge.

The other rudiment was cultivated for 6 days in medium containing both vitamin A and papain, and was then transplanted to normal medium for a further 6 days; after transfer the ends enlarged, but the shaft did not recover.

however, differed from that seen in the papain-treated cultures, in which the cells remained swollen and vacuolated as in the controls. With vitamin A, however, the cells lost their vacuolated appearance and diminished in size; the cytoplasm became basophilic and very irregular in outline and, as already recorded, mitotic figures were seen (Fell and Mellanby, 1952; Herbertson, 1955). Mitosis is extremely rare in normal hypertrophic cartilage and was not observed in that of papain-treated explants.

Another striking difference between the rudiments cultured in excess vitamin A and those grown in medium with or without papain was the almost complete disappearance of glycogen from the cells.

TABLE III

Experiments on 13-Day Chick Bone Rudiments Grown in Medium Containing Papain (γ/ml.), Vitamin A (i.e./ml), or Both

Experiment	No. pairs	Culture medium		Culture period
		Set a	Set b	
				days
248	3 MT; 1 D2; P 1, 2, 3, 4; 1 U; R; MC 2	PP, 150 γ	Control	6
257	" ; " ; "	A, 15 I.U.	Control	8
261	3 MT	A, 10 I.U.	Control	6
	1 D2; P 1, 2, 3, 4; 1 U; R; MC 2	", " "	"	8
263	1 D2; P 1, 2, 3, 4; 1 U; R; MC 2–3.	A, 15 I.U.	Control	"
	"	PP, 150 γ	A, 15 I.U. + PP, 150 γ	"

Papain protease + cysteine was used for all experiments. MT, metatarsal; D 2, 2nd digit of foot; P 1, 2, 3, 4, 1st to 4th phalanges of 3rd digit of foot. U, ulna; R, radius; MC 2–3, 2nd and 3rd metacarpals. Lettering as for Table I.

2. Thirteen-Day Chick Rudiments (Table III). The following bones were grown:

Hind-limb: the 2nd, 3rd, and 4th metatarsals, the 2nd digit less the terminal phalanx, all 4 phalanges (separated) of the 3rd digit; fore-limb: radius, ulna, 2nd and 3rd metacarpals.

At explantation the larger bones, *i.e.* the ulna, radius, metacarpals, and metatarsals, consisted of small-celled terminal cartilage (either epiphyses, or fused tarsal, or carpal elements), a proliferative zone, and hypertrophic cartilage. A thick tube of trabecular periosteal bone had been formed and the cartilage had been cleanly excavated from the middle segment of the bony shaft and replaced by marrow (there is no endochondral ossification in chicken bones at this stage). In the phalanges of the foot, erosion of the cartilage had usually begun in the 2nd digit and in the 2 proximal phalanges of the 3rd, but a continuous narrow cavity had not yet been formed within the sheath of periosteal bone. In all the rudiments the cartilage matrix was much denser and more plentiful than at the 7th day, and the intercellular partitions were much wider.

The controls in normal medium enlarged considerably, Camera lucida drawings made in Exp. 263 showed that during 8 days' cultivation the explants increased in length as follows: ulna, 31 per cent; radius, 38 per cent; 2nd and 3rd metacarpals, 35 per cent; 2nd digit (2 phalanges), 45 per cent; and the 4 isolated phalanges of the 3rd digit by 38, 73, 43, and 24 per cent respectively; there was a corresponding increase in width.

The 14 controls histologically examined after 6 days' cultivation (Exps. 248, 261) appeared quite healthy, although in the larger bones the hemopoietic elements had degenerated and the marrow cavity was occupied by a network of reticulum cells. The cartilage matrix was very dense and intensely metachromatic. The bone was usually healthy and periosteal ossification was progressing actively. The larger of the 28 control bones fixed after 8 days in culture (Exps. 257, 261, 263) displayed more internal degeneration than those grown for 6 days only, but some of the smaller rudiments, *e.g.* the metacarpal (Fig. 6) remained in very good condition.

The 20 explants grown on medium containing 150 micrograms/ml. of papain (Exps. 248, 263) did not elongate during 6 to 8 days' cultivation, and measurements of camera lucida drawings (Exp. 263) showed they either remained the same length or diminished slightly; the width of the cartilaginous ends also changed little. In sections stained with toluidine blue (Fig. 7), the metachromatic material was seen to have been almost completely removed from the cartilage matrix. Although the intercellular partitions were much narrower than in the corresponding controls, they were much broader than in the papain-treated 7-day rudiments; they stained a brighter red with PAS and Van Gieson than those of the controls. As in the preceding experiments, the effect of the papain was produced evenly throughout the cartilage, and the flattened cells were much less compressed than in the controls, so that the proliferative zones were not clearly demarcated from either the epiphyses on the one side or the hypertrophic cartilage on the other. In the explants fixed after 6 days, most of the cartilage cells appeared normal, but, as in the controls, there was more degeneration after 8 days.

Although the chondroblasts and the fibroblasts of the surrounding connective tissue seemed unaffected by the papain during the period of the experiment, there was degeneration of many osteoblasts and little sign of osteogenic activity. There was no obvious effect on the existing bone.

From the camera lucida drawings of Exp. 263, it was seen that the bones in medium containing vitamin A elongated slightly during the first 2 days; growth then ceased and soon the bones began to diminish in both length and width, though the shrinkage was not great.

Unlike the 7-day rudiments in excess vitamin A, the 13-day bones did not become distorted in form, so that the histological changes produced by the vitamin A were very clear in sections.

Of the 20 explants treated with 15 I.U./ml. of the vitamin (Exps. 257, 263), 12 showed the following effect (Fig. 8). Metachromasia had disappeared from a peripheral zone of the epiphyses, but though much reduced it persisted in the interior. Where each zone of flattened cells merged with the hypertrophic cartilage, a broad band of matrix had completely lost its metachromasia, acquired a fibrillar structure and a capacity for staining brightly with PAS and Van Gieson, while the cells were extremely compressed; this appearance contrasted with that of the papain-treated rudiments in which the cells of this region had largely lost their normal flattened structure. Most of the metachromasia had gone from the hypertrophic cartilage except inside the capsules of the chondroblasts where the strands of intercellular material bridging the gap between the cytoplasm and the capsular wall had broken down into deeply stained, granular debris. The hypertrophic cells, unlike those in the papain-treated explants, lost their vacuolated structure and became increasingly irregular in outline. The existing bone was not greatly affected, but there was little or no osteogenesis in progress. The 3rd metacarpal, per-

haps because of its small size, showed the most severe effect, and in one epiphysis the matrix had almost gone, leaving a dense mass of naked chondroblasts.

In 5 rudiments, metachromasia had disappeared from the epiphyses; there was no broad band of metachromatically negative matrix at the junctions of the proliferative and hypertrophic zones, but metachromasia had begun to disappear from the periphery of these regions. The 3 metatarsals and one ulna remained metachromatic throughout, but the amount and the staining capacity of the matrix were less than in the controls.

Of the 11 rudiments exposed to 10 I.U./ml. of vitamin A (Exp. 261), 4 showed the first pattern of effect described above, and in the other 7 the amount of cartilage matrix was diminished, but there were no areas free from metachromasia.

3. Late Fetal Mouse Bones (Table IV). The radius, ulna, and tibia were cultivated, and all were drawn at 2-day intervals with the aid of a camera lucida.

At explantation the bones consisted of large, intensely metachromatic cartilaginous ends, each comprising an epiphysis, a proliferative zone of flattened cells, and a hypertrophic region, and a stout shaft of trabecular bone surrounding a narrow cavity; in the hypertrophic cartilage adjoining the marrow cavity, endochondral ossification was in progress and the marrow contained spicules of endochondral bone enclosing the highly metachromatic remains of the cartilage matrix on which it had been deposited.

Forty-eight controls were grown in normal medium. They elongated by 12 to 15 per cent during a 6- to 8-day culture period and the ends increased in width by a similar amount (Text-figs. 4 and 5). In general, the shape of the bones was well preserved, but by the 6th or 8th day the terminal cartilage had sometimes become slightly bent at an angle to the shaft. Sections (Fig. 10) showed that during cultivation the cartilage matrix increased, and the periosteal bone was thickened by the deposition of new bone by the osteoblastic layer of the periosteum. Endochondral ossification, however, did not advance although excavation of the cartilage continued; this weakened the rudiment at the junction of the cartilage and the marrow cavity and probably accounted for the slight displacement of the cartilaginous ends referred to above. Throughout the cartilage, the chondroblasts were extremely vacuolated (Fig. 14). In preparations of 4-day explants stained with PAS, these vacuoles were found to contain glycogen in the epiphyses and proliferative zones, but some of those in the hypertrophic region were clear; glycogen was also plentiful in the osteoblasts, fibroblasts, and reticulum cells, and much lay freely in the marrow cavity. It was less abundant in the 6-day explants.

The 30 bones grown in the presence of 75 and 150 micrograms/ml. of papain all behaved in essentially the same way. In Exp. 253, 6 bones (one radius, ulna, and tibia from each of 2 embryos) were cultivated in medium to which had been added 150 micrograms/ml. of papain without cysteine and 6 in medium containing 75 micrograms/ml.; the effect of the higher dose was only slightly greater than that of the lower. In Exps. 259 and 264, 75 micrograms/ml. of papain with cysteine was used. The fetuses used in Exp. 253 were slightly younger than those in Exps. 259 and 264, and their bones correspondingly smaller. As these differences in dosage and size of explant, and the presence or absence of cysteine did not materially affect the results, the bones of all 3 experiments will be described together.

Measurement of the camera lucida drawings (Text-fig. 5) showed that after 2 days the papain-treated bones were about the same size as when explanted, although the controls had already begun to elongate, and when viewed under a dissecting microscope the cartilage appeared more opaque than in the normal cultures. After 4 days (Text-fig. 4) the length had sometimes diminished slightly while the terminal cartilage had shrunk somewhat and increased in opacity. These changes progressed slowly until the explants were fixed on the 6th or 8th day,

but even with a dose of 150 micrograms/ml. of papain the length only declined by about 8 per cent. This diminution was due entirely to shrinkage of the cartilage and the bone seemed little affected by the enzyme. The unorganized outgrowth from the surrounding soft tissue was often increased as compared with that in the controls.

TABLE IV

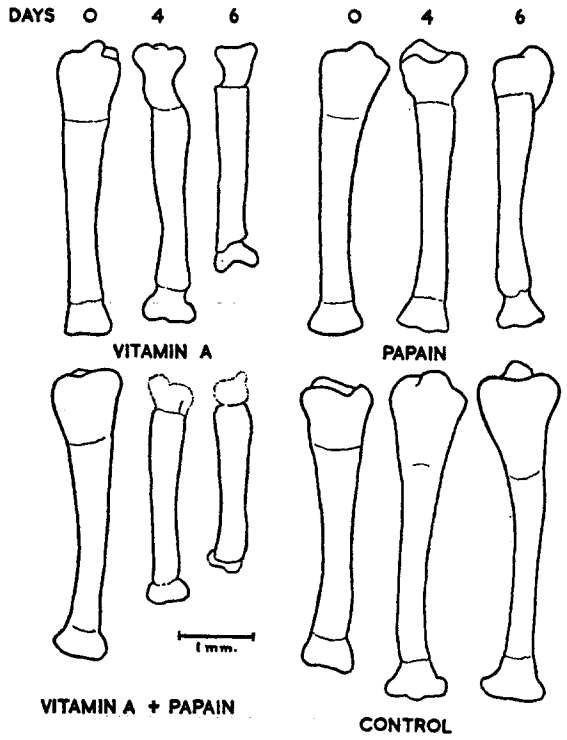
Experiments on Late Fetal Mouse Bones Grown in Medium Containing Papain (γ/ml.), Vitamin A (I.U./ml.) or Both

Experiment	No. pairs	Culture medium		Culture period	Subsequent treatment		
		Set a	Set b		Immediate fixation	To normal medium	Culture period
				days			*days*
253	1 R, U, T	PP, 75 γ	Control	4	a, b	—	—
	1 "	" "	"	6	" "	—	—
	1 "	PP, 150 γ	"	4	" "	—	—
	1 "	" "	"	6	" "	—	—
259	1 "	PP, 75 γ	"	4	a, b	—	—
	1 "	" "	"	8	" "	—	—
	1 "	A, 10 I.U.	"	4	" "	—	—
	1 "	" " "	"	8	" "	—	—
264	1 "	" " "	"	4	a, b	—	—
	1 "	" " "	"	6	" "	—	—
	1 "	PP, 75 γ	A, 10 I.U. + PP, 75 γ	4	" "	—	—
	1 "	" "	" "	6	" "	—	—
266	1 "	A, 10 I.U.	A, 10 I.U. + PP, 75 γ	4	a, b	—	—
	1 "	" "	" "	6	" "	—	—
	1 "	PP, 75 γ	Control	4	" "	—	—
	1 "	" "	"	6	" "	—	—
276	2 "	PP, 15 γ	Control	6	a, b	—	—
	2 "	1, 5 γ	"	6	" "	—	—

Papain protease + cysteine was used for all experiments except No. 253 from which the cysteine was omitted. Lettering as for Table I.

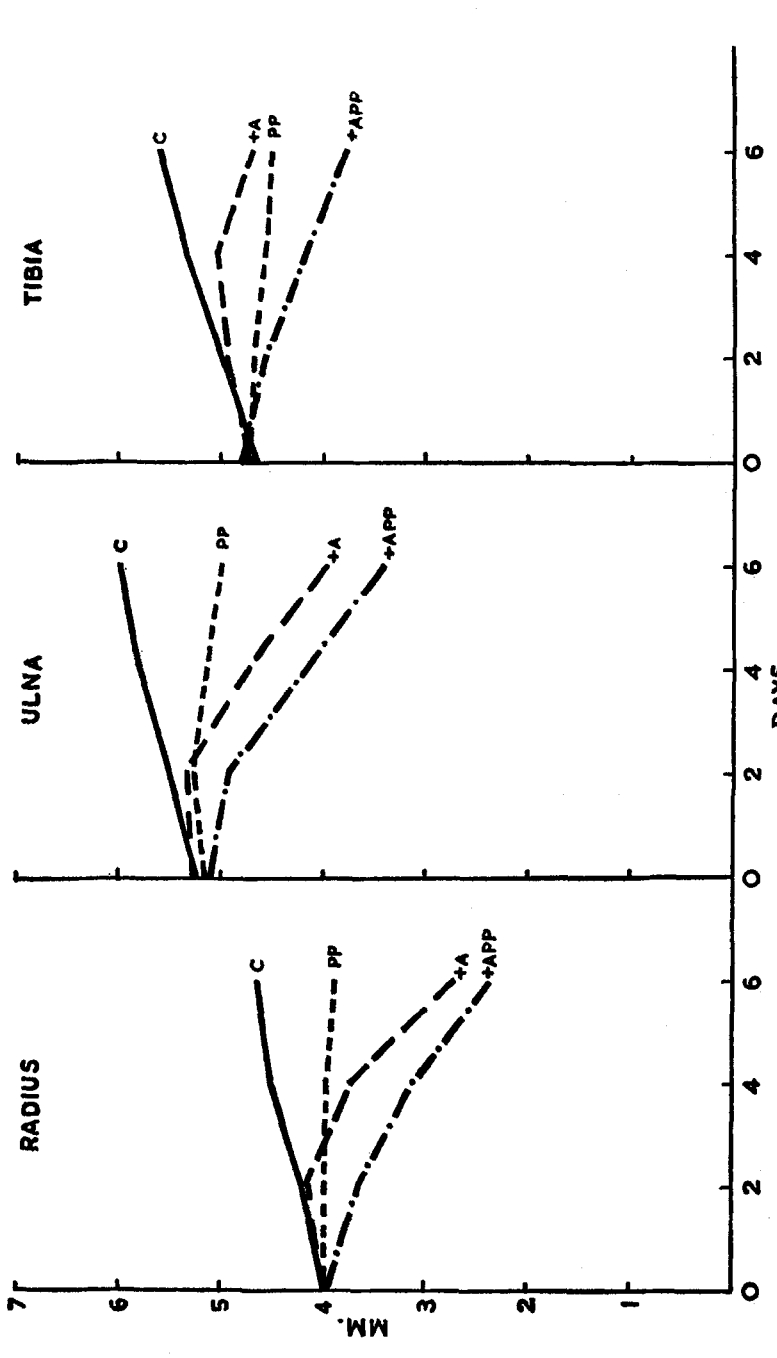
When the bones were examined histologically after 4 days in culture, the intercellular partitions of the cartilage were seen to be greatly reduced in both width and metachromasia, whereas their staining capacity with Van Gieson was greater than in the controls and with PAS was about the same as in the untreated explants. In the articular cartilage and in the proliferative and hypertrophic zones of the papain-treated rudiments, metachromasia had completely disappeared but a little remained in the interior of the epiphyses; the columnar arrangement of the cells of the growing zone, characteristic of mammalian ossifying cartilage,

was much less distinct than in the bones grown in normal medium. The chondroblasts appeared almost unaffected, although those in the interior of the cartilage contained rather less glycogen than in the controls. The periosteal bone also seemed unchanged except that the metachromatic staining sometimes present in the distal part of the shaft of the controls was



TEXT-FIG. 4. Serial camera lucida drawings of 4 living radii in culture, from Exp. 264. Explants from mouse fetuses near term. Doses: 10 I.U./ml. of vitamin A; 75 γ/ml. of papain; control in normal medium. Vitamin A, the terminal cartilage shrinks greatly and the bony shaft becomes shorter (there was also extensive rarifaction of the bone, but this is not shown in the outline drawing). Papain shrinkage of the cartilage is less than with A, and there is no obvious effect on the bony shaft. Vitamin A and papain: there is a marked additive effect of the two agents on the terminal cartilage, but the effect of vitamin A on the bony shaft is not appreciably enhanced by papain.

absent, but there was no evidence of increased resorption; the osteocytes and osteoblasts contained plenty of glycogen, many of the osteoblasts were in mitosis and osteogenesis was in progress beneath the periosteum. In the region of endochondral ossification within the marrow cavity, the metachromatic staining inside the spicules of endochrondral bone was completely unchanged by papain, so that the spicules were strikingly conspicuous in preparations stained with toluidine blue whereas the rest of the skeletal tissue was almost colourless. That this absence of effect was not due to the presence of an impermeable layer of bone deposited on the surface of the cartilaginous core, was shown by the fact that the metachromasia was equally



TEXT-FIG. 5. Curves showing the effects of vitamin A, papain, and vitamin A with papain on the growth in length of the radius, ulna, and tibia in culture (Exps. 264 and 266). Explants from late mouse fetuses. Doses: 10 I.U./ml. of vitamin A; γ/ml. of papain; controls in normal medium.

Each curve represents the average growth of 4 bones up to the 4th day when half the number of bones were fixed. Vitamin A: there is a rapid shrinkage of the radius and ulna, due partly to diminution of the cartilage and partly to resorption of bone. This effect appears more slowly in the larger and stouter tibia. Papain: the lengths of the radius and ulna are reduced much less than with vitamin A. At first the effect on the tibia is greater than that of vitamin A, but by the 6th day the vitamin A–treated tibia are shrinking at a faster rate. Vitamin A and papain: the two agents together are more effective than either alone, but the difference between the effects of vitamin A and of vitamin A and papain is relatively much less than in the wholly cartilaginous chick rudiments (*cf.* Text-fig. 2); this is because papain acts on cartilage but not appreciably on bone, whereas vitamin A affects both.

intense in spicules from which the bone had been resorbed and where cells were in direct contact with the metachromatic material.

In the older 6- to 8-day papain-treated explants (Figs. 12 and 15) the remaining metachromasia of the epiphysical matrix had almost gone, although that of the endochondral bone was still normal. The general appearance of the explants had not greatly changed.

One experiment (Exp. 276) was made with much lower concentrations of papain: 15 and 1.5 micrograms/ml. of culture medium respectively. The changes produced by 15 micrograms/ml. differed little from those described above for the higher concentrations. The effect on the cartilage of 1.5 micrograms/ml. was also quite severe, and qualitatively resembled that of the larger doses; the changes appeared more slowly, however, and after 6 days, removal of the metachromatic material was more incomplete than with the higher levels of protease.

There was a striking difference in behaviour between the mouse bones grown in medium containing 10 I.U./ml. (Exps. 259, 264, 266) of added vitamin A and those treated with papain.

When the living cultures were observed after 4 days in medium containing excess vitamin A (Text-figs. 4 and 5) the cartilage was seen to have shrunk considerably more than in the papain-treated explants and there were areas of resorption in the bone. As previously described (Fell and Mellanby, 1952), the diameter of the dwindling proliferative zone became less than that of the bony shaft, so that the cartilaginous end looked like a head and neck protruding from a too large collar; in the cultures in papain this effect was either very slight or absent. These changes progressed rapidly, and by the 6th day only a small nodule of cartilage remained at either end and the bone had a very "moth-eaten" appearance. After 8 days' cultivation (Exp. 259) little remained of the vitamin A–treated rudiments, whereas in the papain-treated explants, the bony shaft was almost unaffected and the cartilaginous ends, though reduced in size, were still intact. In the bones cultured in vitamin A, there was a profuse outgrowth of cells from the surrounding soft tissue.

In histological preparations, the fetal mouse bones, like those of the embryonic chick, showed a pronounced regional effect of vitamin A (Figs. 11 and 16). Thus in the cartilage of bones fixed after 4 days, there was a steep gradient of change from the surface inwards, unlike a more evenly distributed effect of papain. In the interior of the cartilage of the vitamin A–treated explants, there was usually an area of strongly metachromatic matrix, while at the surface there was a compact layer of chondroblasts which, even in sections stained with PAS or Van Gieson, appeared completely devoid of intercellular material. Between these two extremes was a broad zone in which the matrix resembled that of the papain-treated rudiments: the intercellular partitions had become narrow and lost their metachromasia but stained bright red with Van Gieson or PAS. The cells of this zone, however, differed from those of the explants exposed to papain. In the latter, the vacuolated cells were almost indistinguishable from those of the controls and contained much glycogen, but in the chondroblasts of the cultures in vitamin A the large vacuoles had gone and the cytoplasm had become smaller, more basophilic and had lost nearly all its glycogen; the basophilia increased towards the surface and was intense in the layer of free chondroblasts. The cells in the still metachromatic interior retained their normal appearance and high glycogen content.

The intense metachromasia inside the endochondral bone trabeculae was unaffected by the vitamin as it was by papain, even in areas where the trabeculae were being actively resorbed. The marrow cavity was much more thickly populated with cells, many of which were dividing, than in either the corresponding controls or the papain-treated cultures, and usually more osteoclasts were present. The periosteal bone was fragmentary, but the osteoblasts were abundant, healthy, and many were in mitosis. All the cells of the shaft contained much less glyco-

gen than did those of the controls or the papain-treated explants, but there was plenty of free glycogen in the marrow cavity.

After 6 days in the presence of excess vitamin A, most of the cartilage had disintegrated into a mass of free chondroblasts; some matrix remained in the interior of these masses, however, although greatly reduced both in amount and metachromatic staining. The glycogen content of the cells remained much lower than in control and papain-treated explants of the same age. Three bones were fixed after 8 days (Exp. 259); in these the cartilage had disappeared completely from the ulna and radius, but a little remained at one end of the tibia; only spicules of bone persisted in the now almost completely cellular shaft.

C. The Combined Effects of Papain and Vitamin A

1. Seven-Day Chick Rudiments.—(*a*) Effects of a single exposure to papain and vitamin A. (Table I, Exp. 270). As described above, when 7-day rudiments were incubated for 1 hour in a solution of papain in Tyrode without the addition of cysteine, the metachromatic material was incompletely removed from the matrix. An experiment was made to determine whether the effect of the papain could be increased by the addition of vitamin A.

Five rudiments (femur, tibia, humerus, radius, and ulna) were incubated for 1 hour in Tyrode containing 200 micrograms/ml. of papain without cysteine and 100 I.U./ml. of vitamin A; they were then fixed and sectioned.

A comparison of the histological preparations with those of similar rudiments incubated in papain without either cysteine or vitamin A, showed that the vitamin had not enhanced the effect of the enzyme.

(*b*) Cultivation in medium containing both vitamin A and papain (Table II). The two agents together had a pronounced additive effect (Exps. 262, 265, 267, 269).

Before the 2nd day the rudiments so treated were usually indistinguishable from those grown in the presence of papain alone, though they were much smaller than the explants in excess vitamin A. After this stage, however, they became shorter and narrower than the papain-treated rudiments (Text-figs. 1 and 2) and by the 6th day a fracture developed at one or both ends at the junction of the proliferative and hypertrophic zones, as described above in the vitamin A–treated cultures; rudiments from smaller embryos sometimes showed this effect as early as the 4th day. During further cultivation the explants became fragmentary and much distorted. There was a profuse outgrowth of the surrounding soft tissue throughout the period of culture.

Explants fixed and sectioned after 6 to 8 days in medium containing vitamin A and papain showed much greater histological changes than those exposed to either agent alone (Figs. 5 and 18 a). There was a thin film of slightly metachromatic matrix over the chondroblasts in the interior of the epiphyses, but towards the surface the intercellular substance had vanished; in some explants the epiphyses consisted almost entirely of a compact mass of naked chondroblasts. Metachromasia had completely gone from the shaft which was separated from the epiphyses by a dense mass of cells with no intercellular partitions; the hypertrophic chondroblasts had undergone the changes typical of vitamin A described above and many were in mitosis. In Exp. 262 cell division was very active throughout the explants. Like the vitamin A–treated explants, the rudiments grown in the presence of vitamin A and papain contained much less glycogen than those grown in papain alone, but it was curious that in 3 explants

(Exp. 269) in which the cartilage matrix had almost completely disintegrated the chondroblasts contained more glycogen than was seen in any of the cultures in vitamin A.

An experiment (269) was made to see how far the explants were capable of recovery following culture in medium containing papain and vitamin A.

Six pairs of rudiments were grown in medium containing 150 micrograms/ml. papain and 15 I.U./ml. of vitamin A; after 6 days one of each pair was fixed and sectioned and the other transferred to normal medium for a further 6 days (Fig. 18). The terminal cartilage soon began to enlarge (Text-fig. 3) and to resume its normal hyaline appearance, but the detached fragment of shaft remained unaltered. As in the papain-treated cultures, the capsule of connective tissue surrounding the cartilage became organized and refractile and helped to obscure the already confusing morphology of the explants.

On histological examination, it was found that as in the papain cultures transferred to normal medium, the terminal cartilage had regenerated large quantities of metachromatic matrix, but the matrix of the shafts, unlike that in papain cultures, remained unchanged. In some explants the shaft fragment was compressed between the expanding terminal cartilage and enclosed by dense capsular tissue so that it was largely degenerate, but in others the now basophil cells appeared healthy although they had failed to synthesize chondroitin sulfate. The connective tissue capsule presented a chaotic mixture of true cartilage, sometimes both small-celled and hypertrophic, diffuse chondroid tissue like that seen in the regenerating papain treated rudiments, and in several explants large areas of osteoid tissue spreading outwards from the shaft fragment. Glycogen was plentiful in both terminal cartilage and capsular cells, but none was seen in the cells of the shaft. There was profuse mitosis throughout the explants.

2. Thirteen-Day Chick Bones. (Table III).—

Nine bones from a 13-day embryo were grown for 8 days in medium containing 150 micrograms/ml. of papain and 15 I.U./ml. of vitamin A, and compared with similar rudiments cultivated on a clot containing papain or vitamin A only (Exp. 263). At first there was little difference between either group of explants, though both were considerably smaller than the controls. Of the larger bones, the ulna changed little throughout the culture period, but by the 8th day the terminal cartilages of the radius and of the 2nd metacarpal had greatly diminished in size as compared with those of the corresponding bones treated with papain or vitamin A. They had also acquired well marked constrictions at the junctions of the epiphyses and proliferative zones; in the slender 3rd metacarpal this effect was seen as early as the 4th day and by the 8th day one end had almost disintegrated. Similar constrictions appeared on the 8th day at one or both ends of the phalangeal explants from the 2nd and 3rd digit of the foot, and were accompanied by a greater shrinkage of the cartilage than was seen in either the papain or vitamin A rudiments.

The histological changes were more advanced than in the papain or vitamin A–treated bones. All the rudiments had completely lost their metachromasia with toluidine blue. Near the articular surfaces and in the constrictions described above, the matrix had entirely disappeared, leaving a dense mass of free chondroblasts; this was particularly conspicuous in the 3rd metacarpal and the phalanges. The hypertrophic cartilage cells had the irregular shape and very basophilic cytoplasm characteristic of these cells in the explants in vitamin A or papain of the previous experiments; sometimes several cells occupied one capsule and mitotic figures were found amongst them. The periosteal bone was largely necrotic but there was no evidence that it was being resorbed, and there was no deposition of new bone.

3. Late Fetal Mouse Bones (Table IV, Exps. 264, 266).—

Twelve fetal mouse bones were grown on a clot containing 75 micrograms/ml. of papain and 10 I.U./ml. of vitamin A; 6 were fixed after 4 days', and 6 after 6 days', cultivation.

The combined effects of the two agents on the terminal cartilage was much greater than that of either alone (Text-figs. 4 and 5). As early as the 2nd day the ends of the rudiments treated with both papain and vitamin A were smaller than those of the corresponding explants grown in either agent alone, and by the 6th day the cartilage had almost completely disappeared in the radius and ulna and was much reduced in the larger tibia. There was no evidence, however, that the drastic effect of vitamin A on the bone was significantly increased by the presence of papain.

In histological preparations of the explants fixed after 4 days, a very faint metachromasia sometimes remained in the interior of the cartilage but the matrix had completely gone from the peripheral region, liberating the chondroblasts, some of which were in mitosis. Metachromasia remained intense within the spicules of endochondral bone. By the 6th day (Figs. 13 and 17) the cartilage had almost and sometimes entirely disappeared. There was intense resorption of the periosteal bone, but the sections confirmed the observations on the living cultures, and indicated that resorption was no greater than in the explants grown in vitamin A alone.

DISCUSSION

Before the implications of these results are discussed, it is necessary to consider how the changes produced by vitamin A in explanted skeletal tissue *in vitro* compare with those produced in the intact skeleton of young animals.

The administration of large amounts of vitamin A to young rats or guinea pigs results in bony abnormalities characterized by spontaneous fractures, remodelling of bone, excessive osteoclasis and ossification of the epiphyseal plates (Wolbach, 1947). These changes have been interpreted by Wolbach to be the result of acceleration of all phases of normal bone growth, but this concept is not supported by any direct evidence, and does not adequately explain the observed changes in bone.

The changes produced by excess vitamin A in the fetal mouse bones in culture are similar to but more severe than those seen in the skeleton of a hypervitaminotic animal. Both *in vitro* and *in vivo*, vitamin A causes an abnormally rapid erosion of epiphyseal plate cartilage from the diaphyseal surface, and intense resorption of bone (*cf.* Wolbach, 1947; Barnicot and Datta, 1956). In the rabbit loss of the cartilage matrix in epiphyseal, articular and tracheal cartilage also occurs (Thomas, McCluskey, Potter, and Weissmann, 1960). In the explants, however, these processes end in the complete dissolution of both cartilage and bone, an extreme effect that could not occur *in vivo* because the animal would die before this stage was reached. It is probable that the greater speed of the resorptive processes in the cultures is correlated with the age of the bones. *In vivo* the younger the animal, the more susceptible is its skeleton to excess vitamin A (Moore, 1957) and in the present study the explants were obtained from late fetuses; in earlier organ-culture experiments

(Fell and Mellanby, 1952) it was found that bone rudiments from younger embryos disappeared more rapidly in vitamin A–containing medium than did those from fetuses near term. There may be other contributory factors to the rapid destruction of the mouse bones in culture. Thus vitamin A added directly to the plasma of the culture medium is more potent than the same concentration of vitamin A introduced "naturally" into the plasma by feeding the donor cock on a high vitamin A diet (Fell and Mellanby, 1952); it is possible that the vitamin is more readily available to bones by direct contact with a medium high in vitamin A, than it is *via* the circulation of an animal with hypervitaminosis A.

It is less easy to compare the effects of excess vitamin A on the cartilaginous rudiments from 7-day chick embryos with those produced in the limb skeleton of young hatched chicks (Wolbach and Hegsted, 1952), because the normal histological structure of the earlier embryonic limb skeleton is so different from that of the hatched chick. The changes caused by vitamin A in the hypertrophic chondroblasts of both the 7- and 13-day explants seem to be very similar to those described by Wolbach and Hegsted in the postembryonic chick bones, although these authors do not record mitosis in the hypertrophic cells of vitamin A–treated chicks. Loss of metachromasia from the cartilage matrix of the rudiments occurred in culture; metachromasia was not studied by Wolbach and Hegsted in the cartilage of the hypervitaminotic chicks. In the 13-day rudiments, which possess trabecular periosteal bone enclosing a marrow cavity, the bone does not undergo rapid resorption in medium containing excess vitamin A, unlike fetal mouse bones. Wolbach and Hegsted (1952) found, however, that the bones of young chicks also differed from those of mammals in their response to excess vitamin A; instead of becoming rarified and developing fractures, the cortical bone rapidly became "exceedingly dense" owing to the speedy replacement of cancellous by compact bone.

A comparison of the effects of papain and of vitamin A on the explants reveals interesting similarities and differences. Both agents remove chondroitin sulfate from the cartilage matrix, but whereas papain does so fairly uniformly, vitamin A has a well marked regional effect. In the explants, treated with vitamin A, loss of metachromasia occurs first and is most advanced in the peripheral cartilage (mouse and chick rudiments) and in the proliferative zones of flattened cells (chick).

Mouse cartilage is more susceptible to papain than that of the 7-day chick; thus, a low concentration of papain (1.5 microgram/ml.) produces a distinct effect in the mouse cartilage but no visible change in the 7-day chick rudiments. This is probably due to the fact that the rate of synthesis of chondroitin sulfate in culture is greater in 7-day chick cartilage than in fetal mouse cartilage, so that a small depletion is readily made good in the former but not in the latter. Experiments with minimal concentrations of vitamin A have not been made,

but with those used (10 and 15 I.U./ml.) a more rapid and drastic effect is produced on the mouse than on the chick cartilage.

Histologically, papain appears to affect only the matrix, and produces no obvious change in the chondroblasts, whereas in the rudiments treated with vitamin A the cartilage cells show disappearance of glycogen, reduction of cytoplasmic vacuoles and consequently of size, increased basophilia, and the presence of mitosis in the altered hypertrophic chrondroblasts. In the intact rabbit, however, the changes seen in cartilage cells following administration of papain or of vitamin A were similar and consisted of slight shrinkage, and loss of PAS-positive material and basophilia.

The effects of vitamin A and papain in combination on embryonic chick and mouse cartilage are very striking. In the 7-day chick rudiments, the rapid, uniform removal of metachromasia characteristic of papain is seen, but this is associated with the regional action of vitamin A as evinced by the early dissolution of the zones of proliferative cells, which usually causes a double fracture, and the complete disappearance of the peripheral matrix from the epiphyseal cartilage; the result of the combined action is a spectacular shrinkage of the whole rudiment. The cellular changes (loss of vacuoles and glycogen, and presence of mitosis in the hypertrophic cells) are those seen in vitamin A–treated cartilage. As described above, these severe effects are reversible in the epiphyses but not in the diaphyses.

It is interesting that papain preparations from which cysteine has been removed by dialysis were nearly or quite as effective as fully activated preparations when added to the culture medium in the more prolonged experiments. This was not so when the rudiments were incubated in papain solutions in Tyrode for periods of only 30 minutes or 1 hour; under these conditions the papain without cysteine produced much less change than the activated enzyme, which removed all or most of the metachromasia from the cartilage. The effect of papain without cysteine was not enhanced by the addition of a large concentration of vitamin A to the solution in these short term experiments.

A major question to be considered is whether papain and vitamin A produce their effects on cartilage matrix by a similar mechanism. Theoretically, vitamin A might cause chondroitin sulfate to disappear in two ways: (*a*) by inhibiting synthesis, so that chrondroitin sulfate lost from the matrix during the normal turnover would not be replaced and (*b*) by the active breakdown of one or more components of the matrix. It is very unlikely, however, that the disappearance of the chondroitin sulfate is due to lack of synthesis alone. Thus in the rabbit experiments, Thomas, McCluskey, Potter, and Weissmann (1960) showed that S^{35} previously incorporated in the cartilage was released after treatment with vitamin A, but not in the controls receiving corn oil; further, increased levels of chrondroitin sulfate appeared in the blood coincidently with the decrease of this component in the cartilage matrix. In autoradiographic

studies on organ cultures (Fell, Mellanby and Pelc, 1956), the limb bone rudiments of 6-day chick embryos were grown either in normal medium or in medium containing excess vitamin A and were treated with $S^{35}O_4$ at various stages. In the medium with excess vitamin A, the cartilage first failed to incorporate new sulfate, indicating that synthesis of chrondroitin sulfate had been inhibited, and when the explants were grown for a further period in medium containing excess vitamin A and no S^{35}, the radioactive material already present in the matrix was lost at the same time as the metachromasia disappeared. Since under the same conditions S^{35} remained in the matrix of labelled controls transferred to unlabelled normal medium for the same length of time, its loss from the vitamin A–treated cartilage could not be due solely to ordinary turnover in the absence of synthesis. The effects of excess vitamin A on cartilage *in vivo* and in culture are compatible with a hydrolytic action on chondromucoprotein similar to that of papain. It is possible that chondroitin sulfate can also be removed from the matrix of living cartilage by enzymes other than proteases. Thus Paff and Seifter (1950) report the loss of basophilia and diminution of intercellular material in embryonic chick limb cartilage incubated in a solution of hyaluronidase in Tyrode.

Despite the similarities of the effects of papain and vitamin A on cartilage, these two agents differ considerably in their action on fetal mouse bone in culture, in that the bone is almost or completely unaffected by the protease, but disintegrates with remarkable speed in the presence of excess vitamin A. This indicates that the bony changes produced by vitamin A are not simply the result of activation of a proteolytic enzyme resembling papain. However, it cannot be assumed that all of the effects of hypervitaminosis A are mediated by a single mechanism.

As a tentative working hypothesis it is suggested that vitamin A may enhance the activity of a number of cellular enzymes, one of which resembles papain in its effect; such an enhanced hydrolytic activity might be due to the activation of enzymes, to their greater production or to their increased liberation through an increased permeability of the cells or their organelles.

SUMMARY

The effects of papain protease and of vitamin A on explanted limb bone rudiments from 7- and 13-day chick embryos and fetal mice have been studied and compared.

The incubation of cartilaginous rudiments from 7-day chick embryos in a solution containing papain and cysteine resulted in complete loss of the metachromasia of the cartilage matrix within 1 hour; explants treated in this fashion recovered normal metachromatic staining properties when grown in normal medium for 4 days.

The incubation of 7-day chick cartilage rudiments in a solution containing

papain without cysteine resulted in partial loss of metachromasia from cartilage within 1 hour; the addition of vitamin A to the solution did not enhance the effect of papain during this period.

The addition of papain to the culture medium in which 7-day chick embryo cartilage rudiments were grown resulted in uniform loss of the metachromasia of the cartilage matrix; similar explants grown in the presence of excess vitamin A also showed loss of the metachromasia of cartilage, but certain regions of the cartilage were affected earlier and more severely than others. Changes in cartilage cells, including loss of glycogen, occurred when the rudiment was grown in medium containing excess vitamin A, but not when it was grown in the presence of papain.

Bone rudiments from 13-day chick embryos showed changes in cartilage similar to those seen in 7-day chick embryo rudiments when grown in the presence of papain or of excess vitamin A; the existing bone was not affected under these conditions. When grown in the presence of papain or excess vitamin A, the cartilage of late fetal mouse bone underwent changes similar to those already described in chick embryo rudiments.

In contrast to the chick embryo rudiments, those from the fetal mouse showed rapid resorption of bone when grown in the presence of excess vitamin A. Papain had no effect on bone from either source.

The changes seen in cartilage of explants grown in the presence of vitamin A and papain together were greater than those seen with either agent alone. The changes seen in fetal mouse bone grown in the presence of vitamin A were not enhanced by the additional presence of papain.

On the basis of these observations, it is suggested that the changes in cartilage seen in experimental hypervitaminosis A may be the result of activation of a proteolytic enzyme or enzymes with properties similar to papain.

The authors wish to thank Mr. L. J. King for his expert technical assistance and in particular for perfecting the method of sterilizing papain and dissolving it in plasma; they are indebted to Mr. J. T. Dingle for valuable criticism.

BIBLIOGRAPHY

Barnicot, N. A., and Datta, S. R., Vitamin A and bone, *in* The Biochemistry and Physiology of Bone, (J. H. Bourne, editor), New York, Academic Press, Inc., 1956, 507.

Fell, H. B., and Mellanby, E., The effect of Hypervitaminosis A on embryonic limb-bones cultivated *in vitro*, *J. Physiol.*, 1952, **116**, 320.

Fell, H. B., and Mellanby, E., The biological action of thyroxine on embryonic bones grown in tissue culture, *J. Physiol.*, 1955, **127**, 427.

Fell, H. B., Mellanby, E., and Pelc, S. R. Influence of excess vitamin A on the sulphate metabolism of bone rudiments grown *in vitro*, *J. Physiol.*, 1956, **134**, 179.

Fell, H. B., and Robison, R., The growth, development, and phosphatase activity of

embryonic avian femora and limb-buds cultivated *in vitro*, *Biochem. J.*, 1929, **23**, 767.

Herbertson, M. A., The reversibility of the effect of hypervitaminosis A on embryonic limb bones cultivated *in vitro*, *J. Embryol. and Exp. Morphol.*, 1955, **3**, 355.

McCluskey, R. T., and Thomas, L., The removal of cartilage matrix, *in vivo*, by papain. Identification of crystalline papain protease as the cause of the phenomenon, *J. Exp. Med.*, 1958, **108**, 371.

Moore, T., Vitamin A, Amsterdam, Elsevier Publishing Company, 1957.

Paff, G. H., and Seifter, J., The effect of hyaluronidase on bone growth *in vitro*, *Anat. Rec.*, 1950, **106**, 525.

Thomas, L., Reversible collapse of rabbit ears after intravenous papain and prevention of recovery by cortisone, *J. Exp. Med.*, 1956, **104**, 245.

Thomas, L., McCluskey, R. T., Potter, J. L., and Weissmann, G., Comparison of the effects of papain and vitamin A on cartilage and bone. I. The effects in rabbits, *J. Exp. Med.*, 1960, 111, 705.

Wolbach, S. R., Vitamin A deficiency and excess in relation to skeletal growth, *J. Bone and Joint Surg.*, 1947, **29**, 171.

Wolbach, S. B., and Hegsted, D. M., Hypervitaminosis A and the skeleton of growing chicks, *Arch. Path.*, 1952, **54**, 30.

EXPLANATION OF PLATES

Photography by Mr. V. C. Norfield, Strangeways Research Laboratory. All sections were stained with toluidine blue.

PLATE 58

FIG. 1. Three humeri from 7-day chick embryos (Exp. 268). Fig. 1 a, control fixed after 1 hour's incubation in Tyrode. Fig. 1 b, humerus fixed after 1 hour's incubation in Tyrode containing 200 γ/ml. of papain + cysteine; the metachromatic material has been completely removed (*cf.* Fig. 1 a). Fig. 1 c, opposite humerus from the same chick as in Fig. 1 b incubated for 1 hr. in Tyrode + 200 γ/ml. of papain and cysteine and then cultivated for 4 days on a normal plasma: embryo extract clot; the metachromatic material has been restored. × 15.

FIG. 2. Control femur from a 7-day chick embryo grown for 8 days on a normal plasma: embryo extract clot (Exp. 262). × 15.

FIG. 3. Similar femur grown for 8 days in medium containing 150 γ/ml. of papain (exp. 262). The cartilage is uniformly devoid of metachromatic material; note the thick layer of periosteal bone on the surface of the cartilaginous shaft. × 15.

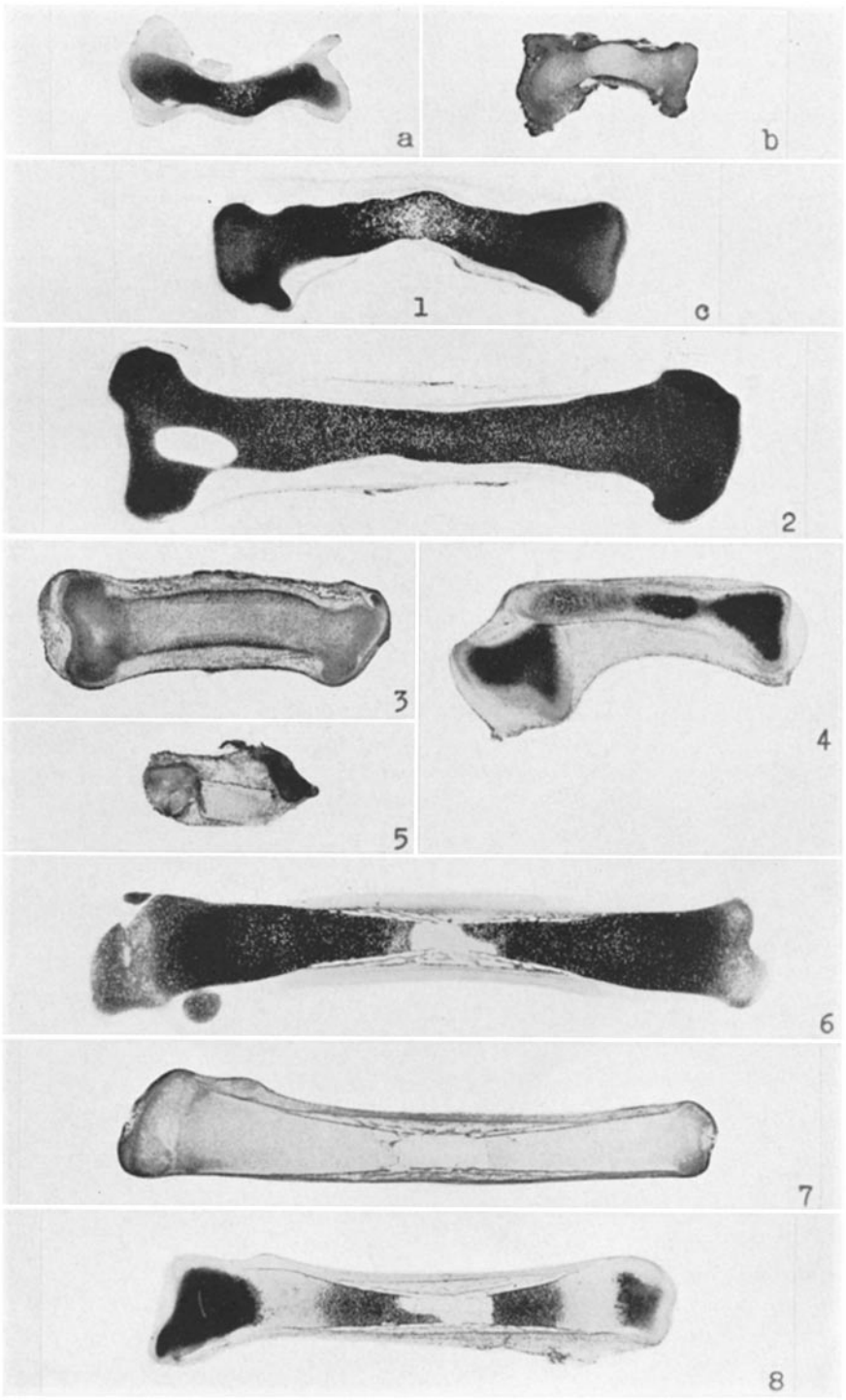
FIG. 4. Femur from 7-day chick embryo grown for 8 days in medium containing 15 I.U./ml. of vitamin A (Exp. 262). The condylar end is fractured, and metachromasia has disappeared from the periphery of the rudiment and is reduced throughout the cartilage. × 15.

FIG. 5. Femur from the same chick as the explant shown in Fig. 3, fixed after 8 days' cultivation in medium containing both vitamin A (15 I.U./ml.) and papain (150 γ/ml.) (Exp. 262). Both ends are detached from the shaft. Metachromasia has almost gone and the rudiment is far smaller than the femur treated with either papain (Fig. 3) or vitamin A (Fig. 4 alone). × 15.

FIG. 6. Control 2nd metacarpal from a 13-day embryo grown for 8 days on a normal plasma: embryo extract clot. (Exp. 263). A thick layer of trabecular bone and a small marrow cavity are present. × 15.

FIG. 7. Second metacarpal from the same experiment (Exp. 263) as the preceding bone, fixed after 8 days' growth in medium containing 150 γ/ml. of papain. Note the uniform disappearance of metachromasia throughout the cartilage. × 15.

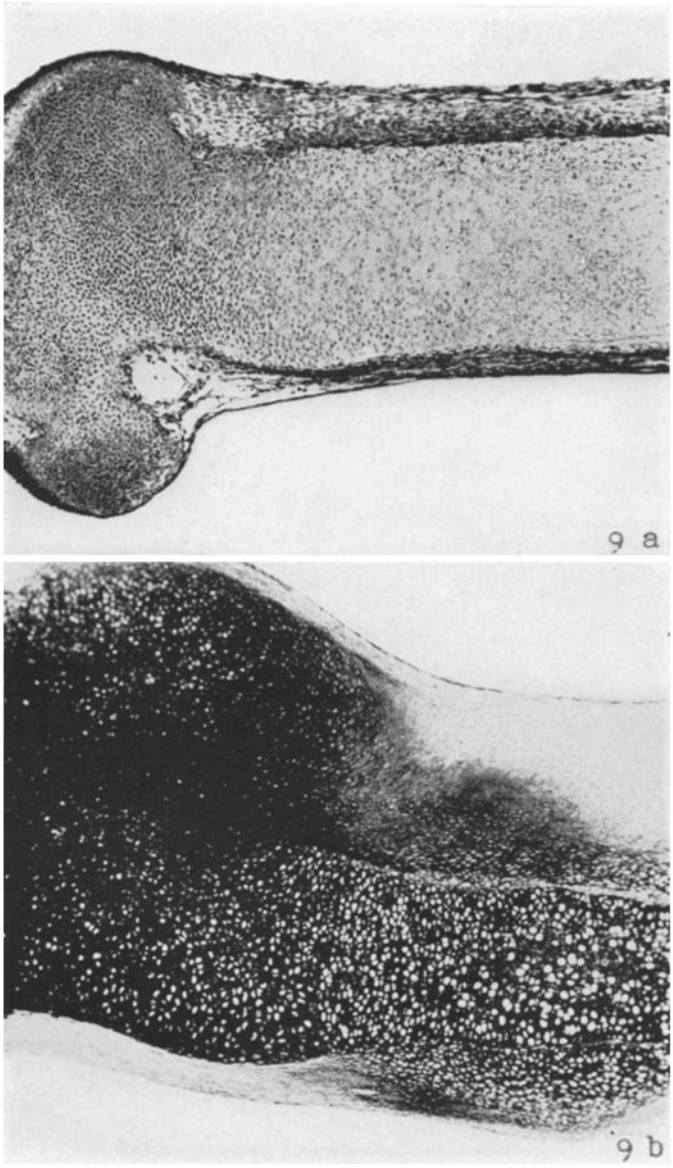
FIG. 8. The opposite 2nd metacarpal from the same 13-day embryo as the explant shown in Fig. 6, after 8 days' cultivation in medium containing 15 I.U./ml. of vitamin A. (Exp. 263). The explant is much smaller than its control in Fig. 6 and metachromasia has disappeared from the proliferative zones of flattened cells and from the periphery of the epiphyses. × 15.



(Fell and Thomas: Effects of papain and vitamin A on cartilage. II)

PLATE 59

FIG. 9. Femora from the same 7-day chick embryo, both grown for 6 days in medium containing 150 γ/ml. papain. (Exp. 269). Fig. 9 a was fixed immediately after removal from the papain medium; Fig. 9 b was transferred from papain to normal medium and cultivated for a further 6 days. In Fig. 9 a note the disappearance of metachromasia from the matrix and narrowing of the intercellular partitions; in Fig. 9 b the metachromatic material has been restored, the periosteum has become completely chondrified and chondrification is spreading diffusely into the surrounding connective tissue. $\times$ 70.



(Fell and Thomas: Effects of papain and vitamin A on cartilage. II)

PLATE 60

FIG. 10. Control radius from a mouse fetus near term, after 6 days' cultivation in normal medium (Exp. 264). Note the large, intensely metachromatic cartilaginous ends and bony shaft enclosing a large marrow cavity. × 15.

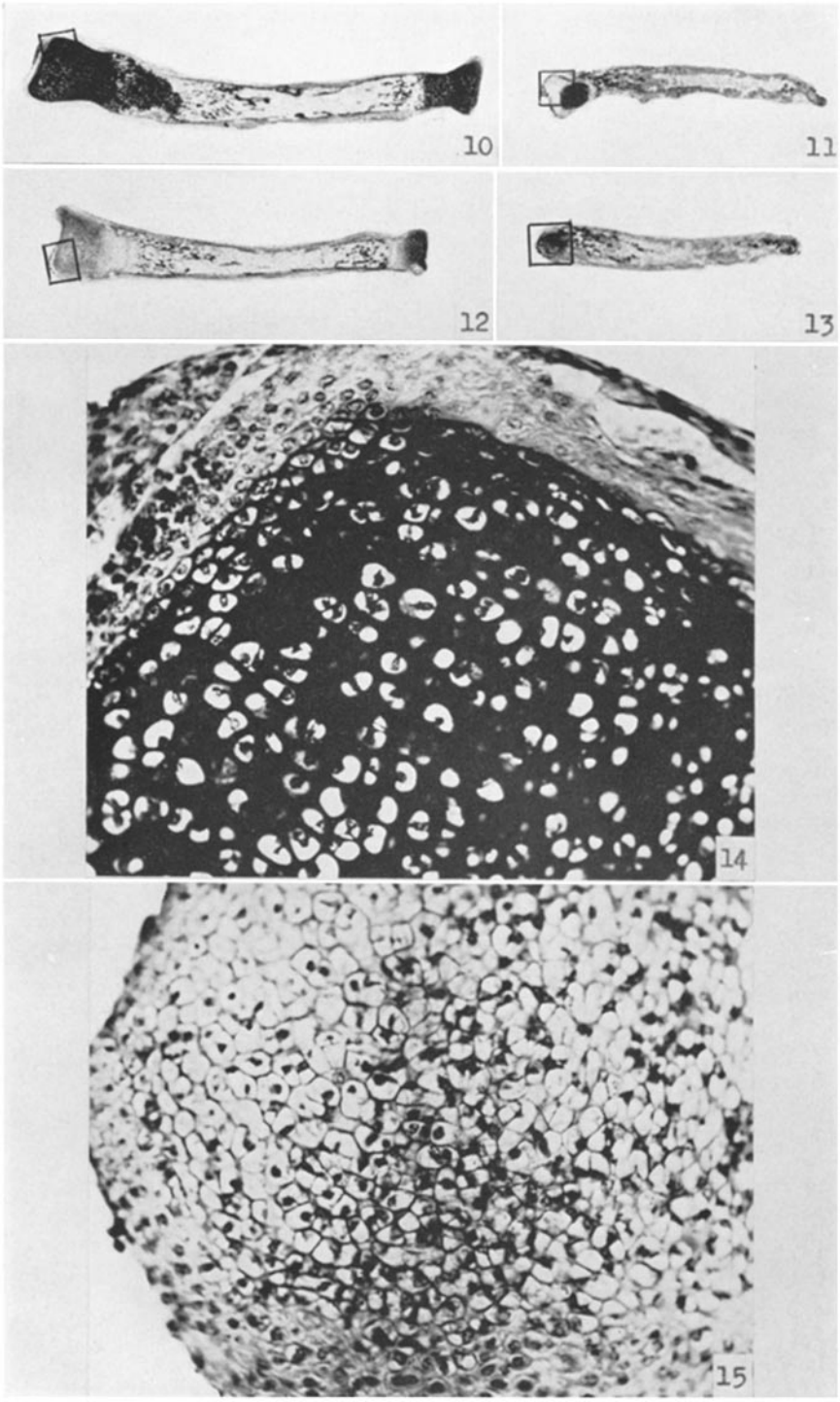
FIG. 11. The opposite radius from the same mouse fetus as the explant shown in Fig. 10, after 6 days' cultivation in medium containing 10 I.U./ml. of vitamin A (Exp. 264). Note extensive resorption of both bone and cartilage and loss of metachromasia from the peripheral cartilaginous matrix. × 15.

FIG. 12. Radius from a litter mate of the fetus that provided the explants shown in Figs. 10 and 11, after 6 days' cultivation in medium containing 75 γ/ml. of papain (Exp. 264). Most of the metachromatic material has been extracted fairly uniformly from the cartilage, but the bone is almost unaffected. × 15.

FIG. 13. The opposite radius from the same fetus as the explant shown in Fig. 12, after 6 days' cultivation in medium containing both vitamin A (10 I.U./ml.) and papain (75 γ/ml.) (Exp. 264). The two agents have had a drastic additive effect on the cartilage but not on the bone which is similar to that of the vitamin A–treated radius (*cf.* Fig. 11). × 15.

FIG. 14. Control cartilage from the marked area in Fig. 10. Note the broad, intensely metachromatic intercellular partitions and the very vacuolated chondroblasts. (In PAS-stained sections, these vacuoles are seen to contain glycogen.) × 300.

FIG. 15. Papain-treated cartilage from the marked area in Fig. 12. The cells retain their vacuoles (and as shown by PAS-stained sections their high glycogen content); the matrix is reduced to thin intercellular partitions which have largely lost their metachromasia, but the superficial chondroblasts are not free as in the vitamin A explant shown in Fig. 16. × 300.

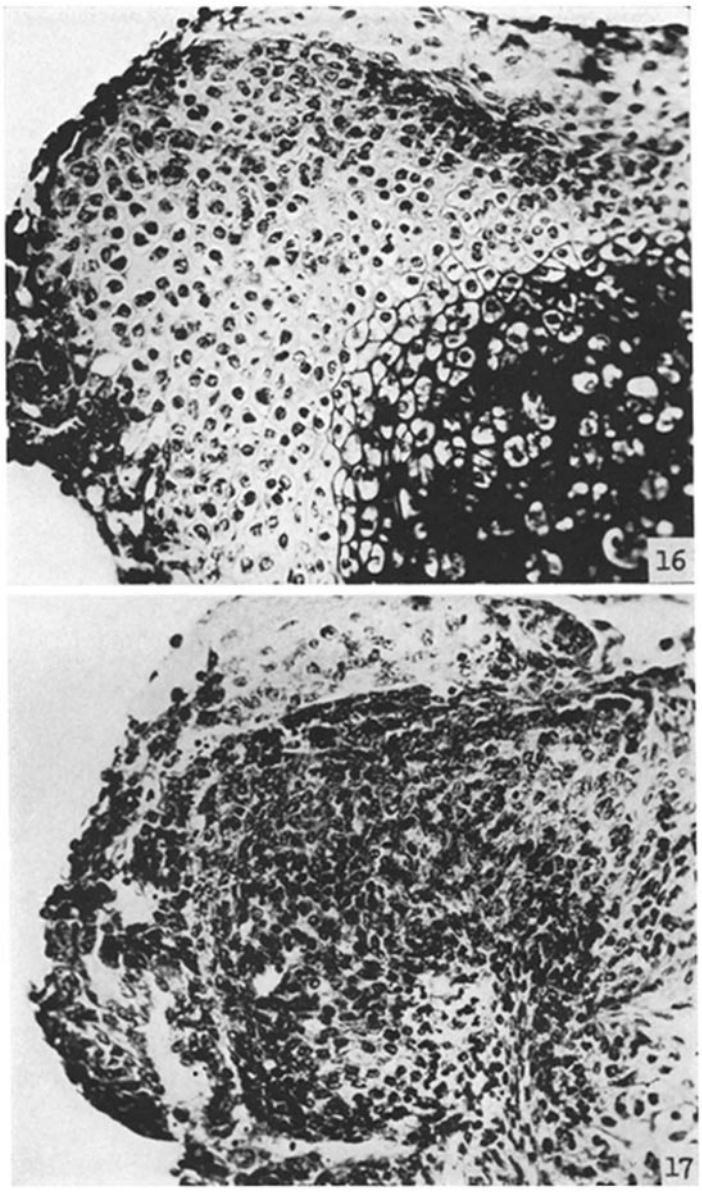


(Fell and Thomas: Effects of papain and vitamin A on cartilage. II)

PLATE 61

FIG. 16. Vitamin A–treated cartilage from the marked area of Fig. 11. The cells are smaller, much closer together than in the control (*cf.* Fig. 14) and have lost their (glycogen-containing) vacuoles. The cartilaginous matrix in the interior is reduced in amount but still metachromatic; beyond this region it has completely lost its metachromasia and near the surface has entirely disappeared, leaving the chondroblasts free. × 300.

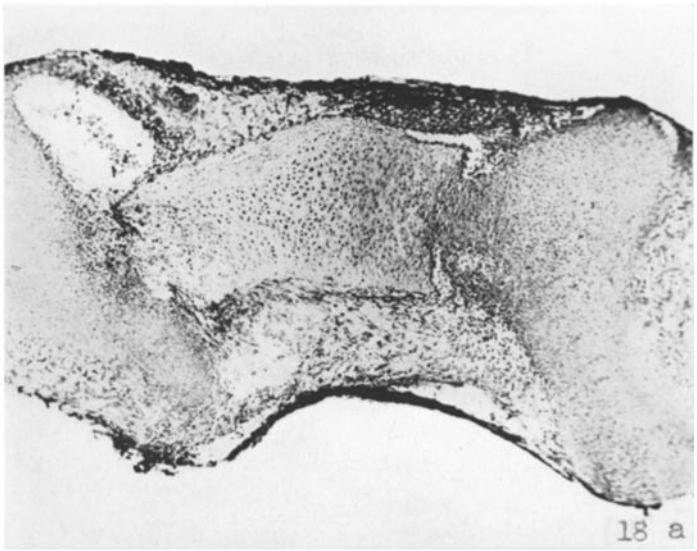
FIG. 17. Vitamin A– and papain–treated cartilage from the marked area in Fig. 13. The cartilage cells have lost their vacuoles as in Fig. 15, and the intercellular material has completely disappeared. × 300.

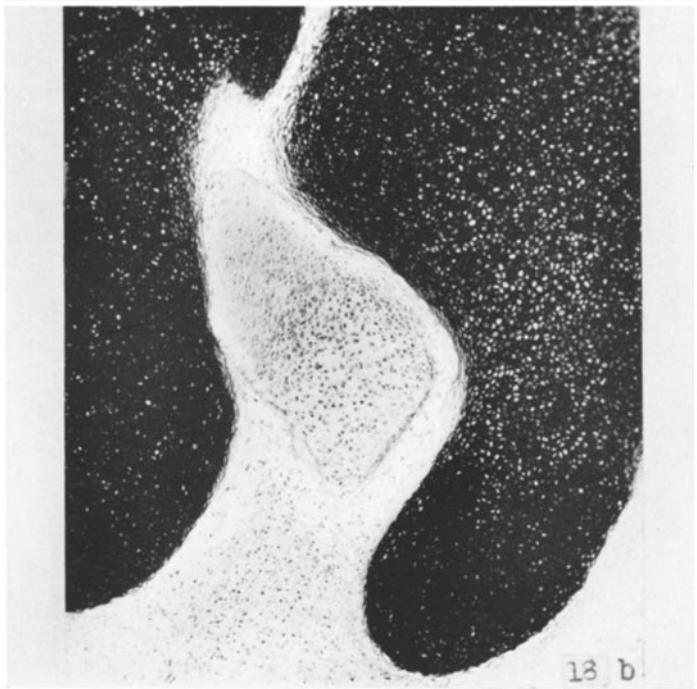


(Fell and Thomas: Effects of papain and vitamin A on cartilage. II)

PLATE 62

FIG. 18. Humeri from the same 7-day chick embryo (Exp. 269), grown for 6 days in medium containing both vitamin A (15 I.U./ml.) and papain (140 γ/ml.). Fig. 18 a was fixed immediately after removal from the papain medium; Fig. 18 b was transferred to normal medium and grown for a further 6 days. In Fig. 18 a metachromasia has gone and in the epiphyses the peripheral matrix has disappeared completely, liberating the chondroblasts; both ends are detached from the shaft. In Fig. 18 b metachromatic matrix has been restored in the epiphyses but not in the detached remnant of the shaft although the diaphyseal chondroblasts are not necrotic. $\times$ 70.





(Fell and Thomas: Effects of papain and vitamin A on cartilage. II)