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THE EFFECTS OF HEPARIN AND DEXTRAN SULPHATE ON CULTURED MOUSE LIMB BONES

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SUMMARY.—Heparin and the synthetic substitute dextran sulphate induce osteoporisis following prolonged administration to man or experimental animals. The possibility that this is brought about by a direct toxic effect on bone has been studied in tissue culture using explants of mouse radii, ulnae and tibiae. Fifty-three bones from new born or day old mice were cultured as controls, 73 with added heparin and 74 with added dextran sulphate at concentrations 0·1, 1·0 and 5·0 mg. per ml. Cultures were continued for 6 days. Control bones increased in length by approximately 22 per cent during this period and although little endochondral ossification occurred there was considerable periosteal and endosteal new bone formation.

When heparin or dextran sulphate was added to the culture medium there was progressive impairment of linear growth with increasing concentrations of these substances. Thus at a concentration of 0·1 mg. per ml. there was little impairment of linear growth but at 5·0 mg. per ml. the bones increased in length by only approximately 15 per cent.

With 1·0 and 5·0 mg. per ml. of heparin or dextran sulphate there was increased resorption of bone and impaired new bone formation. At the highest concentration of 5 mg. per ml. both substances almost completely inhibited new bone formation. Undecalcified sections showed no loss of mineral from the remaining diaphyseal bone and there was no impairment of alkaline phosphatase activity demonstrated histochemically.

The concentrations of heparin or dextran sulphate required in the present tissue culture experiments to produce bone changes were higher than those achieved in the blood and tissue fluids of experimental animals even after prolonged administration. For the *in vivo* changes to be brought about by a direct effect of heparin or dextran sulphate on bone it would be necessary to postulate a selective accumulation of these substances in bone tissues.

THE synthetic heparin substitute, dextran sulphate, induces osteoporosis and fractures after prolonged administration to rabbits, guinea-pigs and rats (Hint and Richter, 1958; Ellis, 1965). Similar changes have been reported with another synthetic sulphated polysaccharide, laminarin sulphate (Adams, Thorpe and Glynn, 1958). Heparin itself is relatively less toxic in this respect, large anticoagulant doses being necessary to induce osteoporosis and fractures in animals (Gillman and Naidoo, 1957; Coleman, cited by Regelson and Holland, 1958; Ellis, 1965). Similar changes have also been described in patients treated with heparin for prolonged periods (Dimond, 1964; Griffith, Nichols, Asher and Flanagan, 1965; Jaffe and Willis, 1965).

The precise mechanism of these changes is unknown. An excess of certain sulphated polysaccharides might interfere with the normal process of ossification

by competition with the naturally occurring sulphated polysaccharides in bone and cartilage. Sulphated polysaccharides possess calcium binding properties and it has been suggested (Hahnemann, 1965) that heparin may decrease the concentration of ionised calcium in the plasma causing secondary hyperparathyroidism and osteoporosis. Against this explanation is the observation (Ellis, 1965) that calcium salts of heparin and dextran sulphate are just as effective in inducing osteoporosis as are the corresponding sodium salts. Attempts to elucidate the problem in laboratory animals are hindered by the occurrence of non-specific side effects causing impaired appetite and inanition. Since inanition can cause osteoporosis (Follis, 1958) it is essential to eliminate its effect from other direct or indirect toxic actions which heparin and dextran sulphate might have on bone and cartilage. Using the pair-fed control technique, it has been shown that inanition alone probably does not account wholly for the bony abnormalities in dextran sulphate treated animals (Ellis, 1965). Inanition nevertheless has some effect since there is an impaired metabolic uptake of radioactive sulphur by the tissues from pair-fed control as well as from dextran sulphate-treated guinea-pigs (Ellis and Lawrence, 1966).

In the present series of experiments the possibility that the bone changes are due to direct or indirect toxic effects of heparin and dextran sulphate has been further investigated by observing the behaviour of mouse limb bones cultured in media containing these substances.

MATERIALS AND METHODS

Dextran sulphates.—Two samples of low molecular weight (7000–10,000) sodium dextran sulphate, supplied by Glaxo Laboratories Ltd., were used (Batch DSB 13, potency 13·8 anticoagulant units per mg. with 17·1 per cent sulphur, intrinsic viscosity of parent dextran 0·03 and Batch PDS 153/A, Lab. Ref. BAU 9/314 potency 14·0 anticoagulant units per mg.).

Heparin.—Sodium heparin supplied by Boots Pure Drug Co. Ltd. (Batch No. 5657) was used. This contained 122 anticoagulant units per mg.

The heparin and dextran sulphate were dissolved prior to use in the chemically defined component of the culture medium and sterilised by filtration through a Seitz pad using a Hemming's filter assembly. Concentrations used varied from 0·1–5 mg. per ml. of final culture medium.

Initially a series of experiments with 214 explants of mouse bone and cartilage derived from ribs, sterna, thoracic vertebrae and limb bones of near term or day old mice of inbred strains C57 and A/Gr_b was undertaken in order to investigate the effects of various culture media and gas phases (Ellis and Tiplady, unpublished). Inferior results were obtained with Trowell's (1959) T8 medium and medium 199 (Burroughs Wellcome and Co., Code No. TC 20), used alone or supplemented with horse serum and chick embryo extract. Oxygen (95 per cent) with 5 per cent carbon dioxide produced bone resorption, as noted by others (Goldhaber, 1958). Optimal bone preservation and new growth occurred with air and 5 per cent CO_2 and the culture medium BGJ of Biggers, Gwatkins and Heyner (1961), supplemented with sodium acetate (5 mg. per 100 ml.), ascorbic acid (150 μg. per ml.) as recommended by Fell (1966, personal communication) and 25 per cent horse serum previously heated to remove any inhibitory effect (Fell and Weiss, 1965). In the main group of experiments reported this was the medium and gas phase adopted.

Limb bones (radius, ulna and tibia) were dissected from new born or 1–2 day old mice of strains BALB/C and A/Gr_b and cultured for 6 days on lens tissue supported on a titanium grid in an aluminium Trowell's Type II culture chamber. A total of 200 limb bones was used, comprising 53 control, 73 heparin and 74 dextran sulphate cultures. Individual bones were cultured for 6 days, 3 on each grid in 5 ml. of supplemented BGJ medium alone or with added heparin or dextran sulphate to give final concentrations of 0·1, 1·0 and 5 mg. per ml. The medium was renewed after 3 days. The lengths of individual bones were measured before and after culture using a stereoscopic microscope with a calibrated eyepiece.

Histology.—After culture limb bones were placed in Worcester fixative for 2 hr which had the combined effect of fixation and decalcification. In some experiments undecalcified frozen sections were cut using a Dittes cryostat. Paraffin sections of decalcified bones were cut at 5 μ and stained with haematoxylin and eosin or with toluidine blue. Cryostat undecalcified sections were stained by the von Kossa technique to demonstrate the state of bone mineralisation and by Burstone's method for alkaline phosphatase using naphthol AS-MX phosphate (Pearse, 1961).

RESULTS

All the limb bones at the time of explantation were at an advanced stage of development with a tube-like diaphysis of trabecular bone surrounding the marrow and enlarged terminal cartilages at either end. Within the cartilage there were zones of young, flattened proliferative cells, maturing cells and developing trabeculae of the metaphysis. A thin layer of new bone had formed on the surface of the metaphyseal cartilaginous spicules.

Control cultures

The general configuration of explanted limb bones was preserved after 6 days' culture, although the cartilaginous extremities were somewhat disproportionately enlarged relative to the diaphysis (Fig. 1A and 1B). Measurement of the total length of individual bones before and after culture (Table I) showed that there was considerable linear growth, but this was less than in limb bones growing for the same period *in vivo*. Thus the mean initial lengths for the 53 radii, ulnae and tibiae were respectively 4·1, 5·3 and 5·3 mm. and after 6 days' culture the corresponding figures were 5·1, 6·3 and 6·5 mm., whereas the mean lengths of the radii, ulnae and tibiae dissected from a series of mice aged 6–7 days were respectively 5·7, 7·1 and 7·4 mm.

Histologically, the architecture and cytology of the cartilage at the extremities of the limb were well-preserved at the 6th day of culture (Fig. 3A). Occasional mitoses were recognisable in chondrocytes and the perichondrium appeared active. There was no loss of cartilaginous inter-cellular matrix and no diminution of metachromasia with toluidine blue. The metaphyseal cartilaginous spicules with bone at their surfaces persisted but there was usually little evidence of progressive endochondral ossification. In contrast there was considerable deposition of new bone beneath the multi-layered cellular periosteum extending along the length of the diaphysis and beyond to form a " cuff " of bone around part of the cartilaginous extremities. There was some minimal resorption of the original bone of the diaphysis and small numbers of osteoclasts were often present. In addition there was endosteal new bone formation on the inner surface of the original diaphyseal bone. In the marrow there was generally good preservation of haemopoietic tissue, capillaries and erythrocytes, although some bones showed slight replacement of the marrow by stellate fibroblast-like cells. The undecalcified sections showed no loss of mineral from the original bone, the mature zone of cartilage or the cartilage spicules at the metaphysis. The newly-formed subperiosteal bone was in the non-mineralised state (osteoid). The overall appearance was one of preservation of the greater part of the original bone with active new subperiosteal and endosteal bone formation and growth of cartilage but no significant endochondral ossification.

There was an intense reaction for alkaline phosphatase in the periosteum, endosteum, perichondrium, the maturing zone of cartilage and in certain marrow

cells. In the perichondrium the reaction extended into the subjacent chondrocytes and matrix. Nearer the centre of the cartilaginous extremities the reaction was negative, but approaching the metaphysis again there was a positive reaction initially in the nuclei, then the cytoplasm and finally within the matrix surrounding the mature cells. The original bone gave a negative reaction for alkaline phosphatase as did the metaphyseal cartilaginous spicules although there was an intense positive reaction at the surface of these spicules.

Heparin cultures

At all 3 concentrations of heparin the general shape of the limb bones was preserved as in the controls. The addition of heparin did not affect the pH of the culture medium. The intensity of the reaction for alkaline phosphatase was not diminished in any of the bones cultured with heparin and the distribution resembled that in the controls. Undecalcified sections revealed normal mineralisation of the remaining original diaphyseal bone and metaphyseal spicules of cartilage (Fig. 2). None of the cultured bones showed loss of metachromasia with toluidine blue. There were differences in the growth rates (Table I) and histological appearances depending upon the concentration of heparin in the culture medium.

Heparin 0·1 *mg. per ml.*—Histologically there was little appreciable difference from the controls. In some cultures there was possibly a slight increase in the extent of resorption of the original diaphyseal bone, but there was no impairment of new bone formation and the cartilage appeared normal.

Heparin 1·0 *mg. per ml.*—There was some increased resorption of the original diaphyseal bone, but the amount of periosteal and endosteal new bone formation appeared comparable to that in the controls (Fig. 4). The " cuff " of new bone around part of the cartilaginous extremities was less conspicuous, however. The periosteum, perichondrium and cartilage were unaffected.

Heparin 5·0 *mg. per ml.*—Histologically (Fig. 3B), the perichondrium and periosteum were thinner and less active-looking, osteoblasts were inconspicuous and

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EXPLANATION OF PLATES

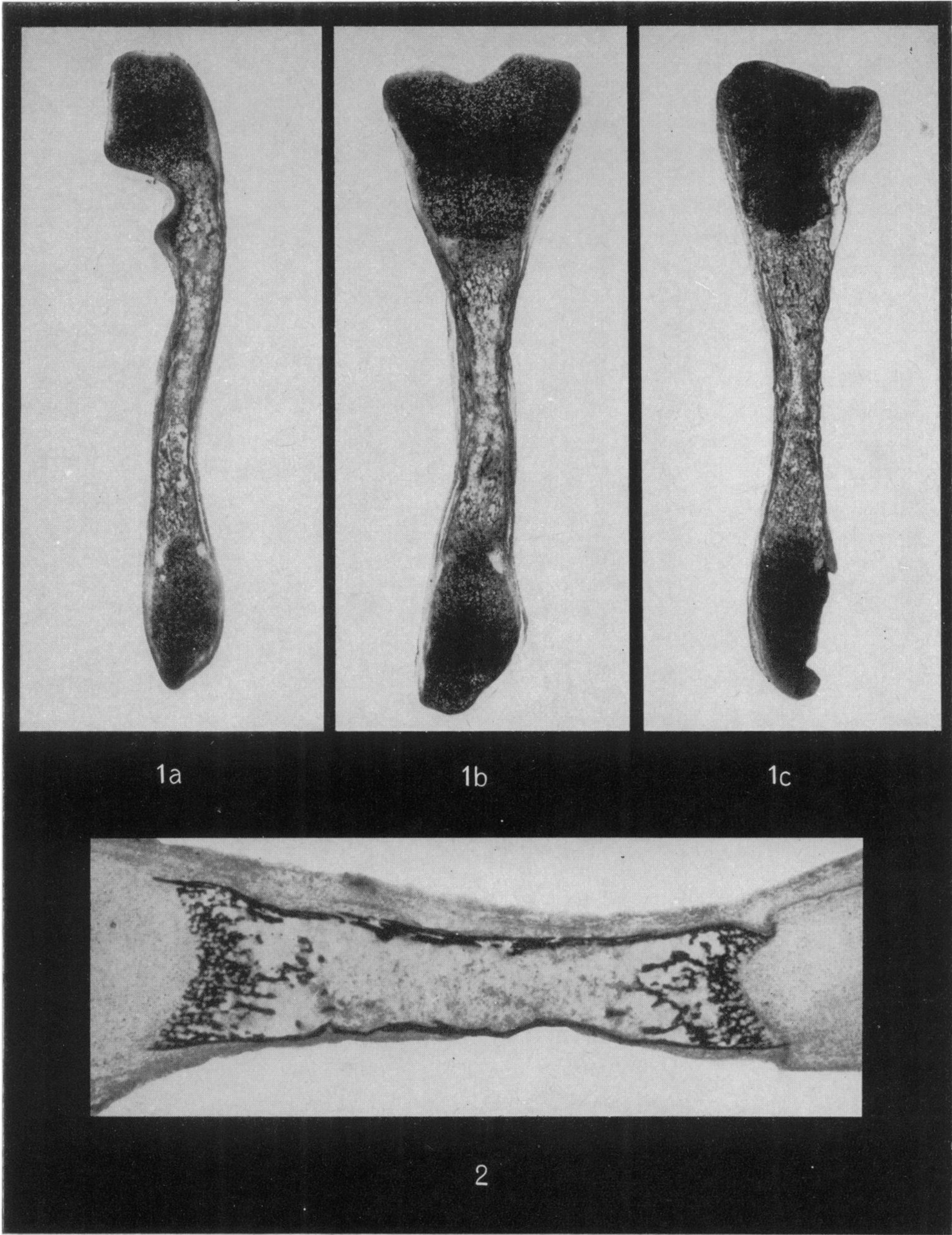
FIG. 1.—Limb bones from day-old mice cultured for 6 days in supplemented BGJ medium alone or with added dextran sulphate. Toluidine blue × 16. 1A and 1B. Control ulna and tibia, respectively, showing preservation of their normal shapes. 1C. Tibia cultured with dextran sulphate 5 mg./ml. Note the preservation of shape, ill-defined perichondrium and periosteum and some thinning of diaphyseal bone. (Tibiae 1B and 1C derived from same animal.)

FIG. 2.—Undecalcified section of tibia cultured 6 days in supplemented BGJ medium with heparin 5 mg./ml. Note the residual diaphyseal bone and metaphyseal cartilage and bone are normally mineralised. von Kossa. × 25.

FIG. 3.—Ulnae cultured 6 days in control medium alone (3A) and with added heparin (3B) or dextran sulphate (3C). Toluidine blue × 60. 3A. Control showing active perichondrium and periosteum with new bone formation and persistence of original bone. 3B. Heparin 5 mg./ml. showing some loss of original bone and considerable impairment of new bone formation. 3C. Dextran sulphate 5 mg./ml. showing more extensive resorption of bone and thinning of the perichondrium and periosteum.

FIG. 4.—Radius cultured 6 days with heparin 1 mg./ml. showing new bone formation, viable osteocytes in lacunae and numerous endosteal osteoblasts. H. and E. × 240.

FIG. 5.—Tibia cultured 6 days with dextran sulphate 5 mg./ml. showing lack of new bone formation and resorption of original bone. Some osteocytes are necrotic and the few remaining endosteal osteoblasts appear flattened. The periosteum is thin and comprises inactive-looking cells. Capillaries with erythrocytes are still preserved. H. and E. × 240.



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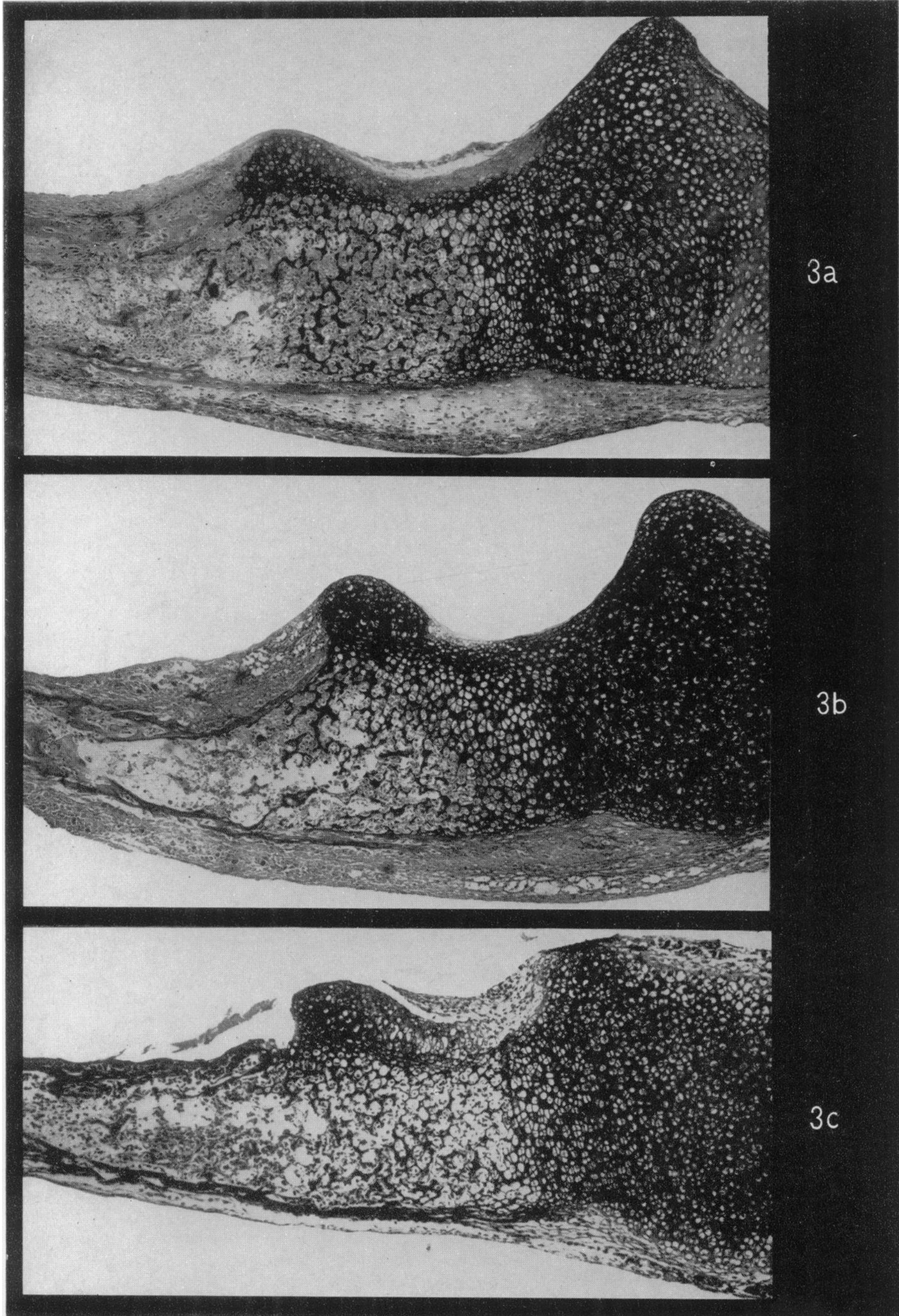

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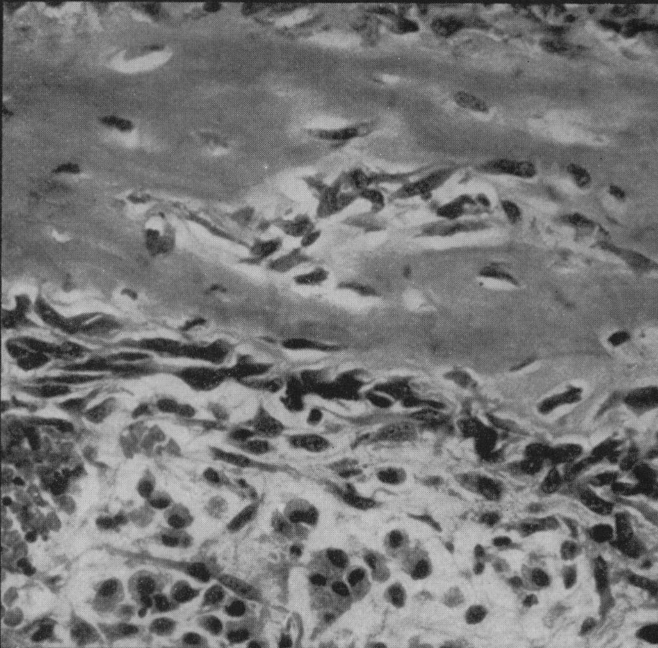
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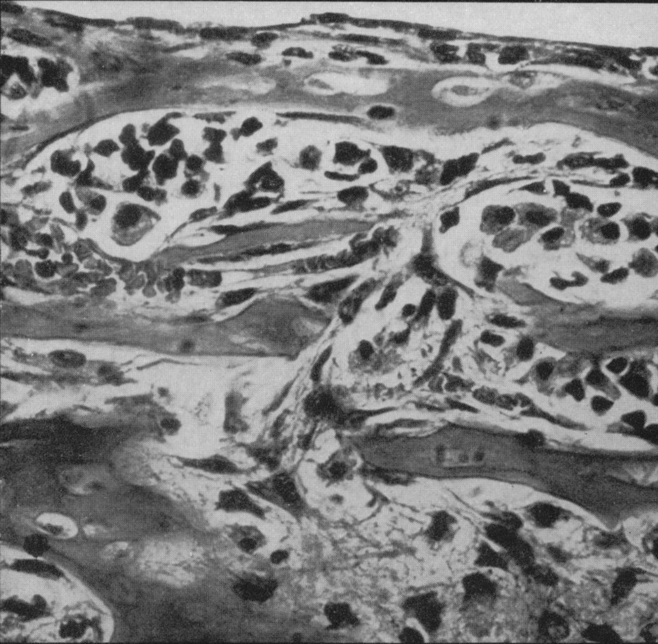
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there was virtually no new bone formation from the periosteum or endosteum. The original diaphyseal bone was irregularly resorbed by osteoclasts especially on the endosteal aspect. The cartilage cells at the bone extremities were abnormal, there being some loss of the usual columnar disposition of the proliferating chondrocytes. The mature zone appeared normal, however, and the cartilaginous spicules at the metaphysis were well-preserved. The marrow showed extensive loss of haemopoietic tissue and replacement by stellate fibroblast-like cells although erythrocytes remained normal in appearance.

Dextran sulphate cultures

The general shape of the limb bones were preserved (Fig. 1C) and the addition of dextran sulphate did not affect the pH of the culture medium. There was no alteration in the reaction for alkaline phosphatase and mineralisation of the remaining original diaphyseal bone was normal. With the higher concentration of dextran sulphate there was some alteration in the metachromatic reaction of cartilage with toluidine blue. As with heparin cultures there were differences in the rates of growth (Table I) of the explants and the histological appearances after culture, depending upon the concentration of dextran sulphate.

TABLE I.—*Lengths of Mouse Limb Bones Initially and after* 6 *Days' Culture*

		Length of limb bone (mm.)*						
			Heparin concentration (mgm./ml.)			Dextran sulphate concentration (mg./ml.)		
Bone		Control	0·1	1·0	5·0	0·1	1·0	5·0
Radius	Initial length	4·1±0·3	4·2±0·4	3·9±0·3	4·4±0·3	4·2±0·2	4·0±0·2	4·1±0·2
	Final length	5·1±0·4	5·0±0·4	4·6±0·3	4·8±0·6	4·9±0·2	4·7±0·3	4·7±0·3
Ulna	Initial length	5·3±0·3	5·4±0·5	4·9±0·4	5·4±0·6	5·3±0·1	5·1±0·3	5·4±0·2
	Final length	6·3±0·4	6·3±0·5	5·6±0·4	5·9±0·6	6·2±0·2	5·8±0·3	5·9±0·3
Tibia	Initial length	5·3±0·5	5·4±0·6	4·6±0·4	5·5±0·7	5·1±0·3	5·1±0·4	5·2±0·3
	Final length	6·5±0·6	6·7±0·5	5·8±0·4	6·6±0·1	6·4±0·3	6·1±0·3	6·2±0·3

* Mean total bone length ± S.D.

Dextran sulphate 0·1 *mg. per ml.*—Histologically, the periosteum and perichondrium were slightly thinned and the perichondrium appeared less cellular than usual with a rather reticular open stroma. The cells of the cartilaginous extremities appeared normal and the matrix showed no reduction in metachromasia with toluidine blue. At the metaphyses the cartilaginous spicules were preserved but an abnormal feature was the tendency for mature chondrocytes to persist instead of disintegrating. There was slightly more resorption of the original diaphyseal bone than in the controls but osteoclasts were not particularly prominent. There was reduction in the amount of periosteal and endosteal new bone formation. The marrow haemopoietic cells were reduced in number and the endosteal osteoblasts were relatively inconspicuous and were proliferating into the marrow as stellate fibroblast-like cells.

Dextran sulphate 1·0 *mg. per ml.*—The changes were more marked and there was little if any new bone formation in most of the explants. The periosteum was thinned and the perichondrium was oedematous in appearance. The cartilaginous

extremities and the metaphyseal spicules resembled those in explants from 0·1 mg. per ml. cultures. The original diaphyseal bone showed increased irregular resorption on the endosteal aspect. The marrow was patchily necrotic in some bones and as before was less cellular. There were pools of erythrocytes and eosinophilic necrotic marrow cells. A conspicuous feature was the presence of enlarged cells with granular cytoplasm resembling macrophages in the marrow, throughout the diaphysis and amongst the cartilaginous spicules at the metaphysis. The finely granular material stained with toluidine blue.

Dextran sulphate 5·0 mg. per ml.—The greatest abnormalities were observed in these cultures (Fig. 3C and 5). In the cartilaginous extremities there was disturbance of the usual regular arrangement of chondrocytes and the columnar arrangement of flattened cells in the proliferating zone was lost. The matrix appeared less homogeneous and less basophilic than usual. At the periphery there was some loss of metachromasia with toluidine blue. The perichondrium was ill-defined and comprised rounded cells with swollen cytoplasm separated by loose oedematous stroma giving a fenestrated appearance. The subjacent cartilage cells were similarly affected. Metaphyseal cartilage spicules were still recognisable. The attenuated periosteum resembled the perichondrium and there was almost complete cessation of periosteal and endosteal new bone formation. Normal osteoblasts were not recognisable. The original diaphyseal bone was partly necrotic and many osteocytic lacunae were empty. The bone was irregularly thinned although there was no excess of osteoclasts. In some bones there were small numbers of fibroblast-like cells forming a little fine collagen in the spaces created. The marrow was partly necrotic and its cellularity diminished. Apart from a few stellate fibroblast-like cells most of the cells were enlarged with rounded granular or vacuolated cytoplasm resembling macrophages. Some of these gave a metachromatic reaction with toluidine blue. Small groups of erythrocytes were preserved.

Comparison of the linear growth control limb bones and those cultured with heparin and dextran sulphate

Control and test limb bones from individual mice or their siblings were cultured in parallel in 19 separate experiments. In order to compare the rate of growth of bones cultured in heparin or dextran sulphate with one another and with control bones the difference between the pre- and post-culture lengths for each individual bone was calculated as a percentage of the initial length. It was then possible to compare the percentage changes in length within the test and control groups collectively. The data for 199 explanted limb bones are summarised in Table II. It will be seen that in the case of both heparin and dextran sulphate there was impaired linear growth of the explants and this increased with increase in concentration from 0·1–5·0 mg. per ml. In the case of the radius there was significantly less linear growth at the highest concentration of dextran sulphate and heparin ($P < 0{\cdot}001$). At the intermediate concentration (1·0 mg./ml.) there were also significant differences for dextran sulphate ($P < 0{\cdot}01$) and heparin ($P < 0{\cdot}05$, $> 0{\cdot}02$). At the lowest concentration (0·1 mg./ml.) there was a significant difference in the case of dextran sulphate only ($P < 0{\cdot}05$, $> 0{\cdot}02$). Growth of the ulna was significantly diminished with dextran sulphate and heparin at 5 mg. per ml. ($P < 0{\cdot}001$) and with dextran sulphate at 1 mg. per ml. ($P < 0{\cdot}02$, $> 0{\cdot}01$) and heparin ($P < 0{\cdot}05$, $> 0{\cdot}02$). The tibia was apparently least affected by heparin

TABLE II.—*Percentage Increase in Length of Cultured Mouse Limb Bones*

	Control			Sulphated polysaccharide: Heparin		Sulphated polysaccharide: Dextran sulphate	
Bone	No. of explants	Increase in* length (per cent)	Concentration (mg./ml.)	No. of explants	Increase in* length (per cent)	No. of explants	Increase in* length (per cent)
			0·1	7	19·7±4·6	8	‡18·7±2·9
Radius	19	23·1±4·9	1·0	8	‡18·9±3·9	8	†17·6±1·7
			5·0	8	†11·2±3·0	8	†13·8±4·3
			0·1	9	16·5±4·1	8	17·7±2·9
Ulna	18	18·8±4·1	1·0	8	‡16·5±4·7	8	†14·2±3·9
			5·0	8	†11·6±3·8	9	†10·6±2·1
			0·1	8	24·9±7·3	8	26·5±5·3
Tibia	15	23·9±3·7	1·0	9	28·4±9·7	8	‡20·2±3·9
			5·0	8	‡19·7±5·0	9	†19·6±2·6

* Mean percentage increase in length ± S.D.
† and ‡ Difference from controls significant at $P < 0·01$ or $0·001$ and $P < 0·05$ respectively.

and only at the 5 mg./ml. level was there significant impairment of linear growth ($P < 0·05$, $> 0·02$). Dextran sulphate produced impaired linear growth of the tibia at concentrations of 5·0 mg./ml. ($P < 0·01$) and 1·0 mg./ml. ($P < 0·05$, $> 0·02$). Comparison of the linear growth of radii and ulnae in the presence of heparin and dextran sulphate revealed no significant difference at all 3 concentrations used ($P > 0·10$).

DISCUSSION

The explanted limb bone in culture provides a model with which to study the resorption of bone and the formation of new bone. The present experiments show that at high concentrations dextran sulphate and heparin induce excess resorption of bone and inhibit new bone formation. At a concentration of 5 mg. per ml. both substances cause almost complete arrest of new subperiosteal and endosteal bone formation. Only at the higher concentrations of dextran sulphate was there any alteration in cartilage, the stroma of which appeared oedematous immediately beneath the perichondrium. Here there was also some loss of metachromasia with toluidine blue. The metachromatic material in the macrophages observed within the marrow at this concentration presumably represented dextran sulphate although one cannot exclude the possibility that some was sulphated polysaccharide liberated from altered cartilage. It was not possible to study the effects of these substances on endochondral ossification since in our cultured mouse limb bones this process shows little progress as Fell (1966, personal communication) has also observed.

Although the histopathological changes induced by dextran sulphate *in vivo* resemble those described in hypervitaminosis A (Wolbach, 1947; Ellis, 1965) in tissue culture, dextran sulphate is less effective than Vitamin A in producing resorption of limb bone explants. Excess Vitamin A rapidly induces shrinkage and resorption of the cartilaginous extremities and rarefaction of the bony diaphysis in cultured chick and mouse embryo limb bones (Fell and Mellanby, 1952; Fell, 1956). Work with Vitamin A has largely employed cartilaginous bones and it is known that the vitamin inhibits chondrocyte function directly (McElligott, 1962). Dextran

sulphate and heparin *in vivo* do not alter the morphology of cartilage matrix or chondrocytes and a vitamin A-like effect in culture would not be expected. In the present experiments there was progressive impairment of linear growth with increasing heparin or dextran sulphate concentration. The growth of the cartilaginous extremities accounts for most of the increase in length of the limb bone explants and the impaired increase in length with the highest concentration of heparin and especially dextran sulphate is in keeping with the loss of the normal proliferating zone of cartilage cells. Although the dextran sulphate and heparin induced resorption of the original diaphyseal bone this was never complete.

In dextran sulphate treated animals osteoporosis results from a negative balance between osteoblastic formation and osteoclastic resorption of bone. Histologically, this appears to result from diminished osteoblastic and possibly excessive osteoclastic activities, although the parathyroid glands do not appear to be hyperplastic (Ellis, 1965). If dextran sulphate and heparin exert a direct toxic effect on bone one would expect excessive resorption of bone and impaired new bone formation in culture and the present experiments show this to be the case. However, the concentrations of heparin and dextran sulphate necessary to produce an effect in tissue culture were much higher than those achieved in the plasma *in vivo*. Thus, a daily injection of 20 mg. dextran sulphate per kg. body weight for 5–7 weeks induces severe osteoporosis and fractures. The highest plasma level attained after a single intravenous injection of this amount of heparin or dextran sulphate would approximate to 0·5 mg. per ml. In the case of heparin this would be short-lived since the blood is rapidly cleared following a single i.v. dose. Even repeated daily injections would not produce an accumulation of heparin in the plasma to a toxic level since after clearance from the blood 60 per cent of it is excreted in the urine within 48 hr (Eiber, Danishefsky and Borelli, 1960). With large doses as much as one third might be expected to be excreted within 6 hr, half unchanged and the remainder as uroheparin (McAllister and Demis, 1966). To achieve *in vivo* the toxic level required in the present tissue culture experiments preferential retention of heparin in bone would be needed, but there is no evidence that this is so. Radioactive tracer studies have shown an apparent predilection of heparin for the liver, lungs, kidney and spleen (Eiber, Danishefsky and Borelli, 1958; Levy and Petracek, 1962; Lemaire, Picard and Gardais, 1967).

Dextran sulphate behaves somewhat differently *in vivo*. Following a single intravenous injection the blood is cleared in a similar way to heparin but subsequently there seems to be a tendency in man and rabbits for accumulation to occur in the extravascular tissues (Jeavons, Walton and Ricketts, 1956). The rate of renal excretion is less for dextran sulphate than for heparin, only 30 per cent being eliminated in 2 days and up to 50 per cent at 4 days (Ricketts, Walton and Saddington, 1954). With repeated daily injections of dextran sulphate there could well be periods in which 2 or 3 times the amount of a single daily injection could reside in the animal's tissues and blood together. On this basis and with a uniform distribution, the concentration would still fall short of the lowest concentration (0·1 mg. per ml.) used in the present culture experiments in which little toxic effect was observed on the limb bone explants.

The precise fate of injected dextran sulphate in animals is unknown (Ricketts *et al.*, 1954), although there is evidence that it is partly metabolised (Weigel and Walton, 1959). Jeavons *et al.* (1956) considered that the cumulative anticoagulant effect of dextran sulphate might be due to a lower rate of inactivation or destruc-

tion of dextran sulphate compared with heparin. From the results obtained it appears that if there is a direct toxic effect on bone there must be considerable accumulation of heparin or dextran sulphate in that tissue to achieve the necessary toxic concentration.

It seems equally possible that these substances exert their effect on bone indirectly either by competition with the naturally occurring sulphated polysaccharides or by some other means such as inhibition of enzymes, for example, those of the tricarboxylic acid cycle (Ellis, 1965). It is known that heparin and its synthetic substitutes interfere with a wide range of enzyme functions (Walton, Ellis and Taylor, 1957; Ellis and Walton, 1959). In the present experiments there was no apparent interference with alkaline phosphatase activity.

A further possibility is that heparin and dextran sulphate may potentiate the normal processes of bone resorption. In this respect the observations of Goldhaber (1965) may be relevant. He noted that heparin added to the medium in which mice calvariae were cultured had no effect on the extent of bone resorption. The addition of small amounts of heparin did, however, enhance the resorptive effect of parathyroid extract. Thus the addition of 0·2–10 units per ml. of heparin to a culture medium containing insufficient parathyroid extract to induce resorption of bone produced a resorptive effect equivalent to that usually obtained with 5 times the quantity of parathyroid extract. The synthetic sulphated polysaccharide " Treburon " (pectin sulphate) was also effective in doses of 0·01–0·1 mg. per ml. whereas chondroitin sulphate was ineffective. This is in keeping with the observation that the long term administration of chondroitin sulphate, unlike synthetic laminarin sulphate and dextran sulphate, does not produce osteoporosis and fractures in guinea-pigs (Ellis, 1965) and that chondroitin sulphate is less toxic than heparin and dextran sulphate to amphibians (Ellis, 1966). Goldhaber made the suggestion that heparin may behave as a parathyroid co-factor which stimulates bone resorption. Since this hypothesis may help to throw some light on the bone changes occurring in dextran sulphate or heparin treated animals we are currently studying the effect of parathyroidectomy on such animals to determine whether or not this will prevent the development of the bone changes. To date, our results suggest that parathyroidectomy does not protect animals from osteoporosis induced by dextran sulphate. Preliminary experiments using parathormone and heparin in tissue culture with explanted mouse limb bones have shown that there is little, if any, synergistic effect in this type of experiment.

The possibility of a non-specific toxic effect of high concentrations of heparin or dextran sulphate on cultured tissues has to be considered. Explants of mouse vas deferens, prostate, caput epididymis and tracheal slices survive and grow well even in concentrations of dextran sulphate up to 10 mg. per ml. (Ellis, unpublished). We have also found the addition of inorganic sulphur as sodium sulphate in concentrations up to 5 mg. per ml. of culture medium has little effect on bone resorption or formation in explanted mouse limb bones.

In conclusion, at high concentrations dextran sulphate and heparin appear to act directly on bone in tissue culture producing both increased resorption of bone and impaired new bone formation. At concentrations comparable with those achieved in the blood of injected animals there is little if any toxic effect in culture. If the osteoporosis in such animals is due to a direct toxic effect of heparin or dextran sulphate then it would be necessary to postulate a selective accumulation of these substances in bone tissues.

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