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EFFECT OF HEPARIN AND SYNTHETIC ANTICOAGULANTS ON AMPHIBIAN AND AVIAN DEVELOPMENT

BY

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The long-term administration of the synthetic heparin substitutes dextran sulphate and laminarin sulphate to rabbits, guinea-pigs and rats induces osteoporosis and fractures (Adams, Thorpe & Glynn, 1958 ; Hint & Richter, 1958 ; Ellis, 1965). Heparin is relatively nontoxic in this respect, although mild degrees of osteoporosis and occasional fractures are produced if large enough doses are used in guinea-pigs (Ellis, 1965). Griffith, Nichols, Asher & Flanagan (1965) and Jaffe & Willis (1965) have recently reported osteoporosis and fractures in patients treated with heparin for prolonged periods. The precise mechanisms involved are unknown although it is thought the excess of natural or synthetic sulphated polysaccharide might inhibit ossification by competition with the naturally occurring sulphated polysaccharide in cartilage and bone. Chang, Witschi & Ponseti (1955) have shown that the addition of lathyrogenic factor, β-aminopropionitrile, to the water in which amphibians are kept interferes with normal limb development during metamorphosis and with the regeneration of limbs following amputation. Their observations suggested that the effects of heparin and various synthetic substitutes might be conveniently studied using a similar technique. It is also known that a number of substances introduced into the yolk-sac or on to the chorioallantoic membrane of the developing chick embryo interfere with normal skeletal growth, for example insulin (Landauer, 1945, 1947a, b), β-aminopropionitrile (Chang *et al.*, 1955) and cortisone (Karnofsky, Ridgway & Patterson, 1951), and it was therefore also decided to study the effect of dextran sulphate on the developing chick embryo.

METHODS

Compounds. These were: sodium heparin (Boots), batch No. 5657, 122 anticoagulant units per mg ; low molecular weight sodium dextran sulphate (Glaxo Laboratories), batch No. DSB 13, 13.8 anticoagulant units per mg, intrinsic viscosity of parent dextran 0.03 ; an acid hydrolysed preparation of dextran with intrinsic viscosity 0.04, prepared by Dr C. Ricketts ; low molecular weight laminarin sulphate (Boots), batch No. LM111, 5.5 anticoagulant units per mg ; and bovine sodium chondroitin sulphate (Evans), batch No. E50140.

Frog larvae (Rana temporaria). 1,058 larvae of the common frog were obtained from a local pond as ova and were used approximately 1 week after hatching. Two experiments were undertaken.

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Experiment 1. Ninety-eight larvae were allocated in batches of nineteen or twenty to five vessels of water, four with added dextran sulphate giving final concentrations of 0.016, 0.08, 0.40 and 2.0 mg/ml., respectively. The solutions were renewed weekly. Equal quantities of filamentous algae, chopped watercress or lettuce leaves were added regularly.

Experiment 2. 960 larvae were allocated in batches of twenty to a series of vessels containing heparin, dextran sulphate, laminarin sulphate, chondroitin sulphate, sodium sulphate or dextran in concentrations varying from 0.10 to 4.0 mg/ml., and observed for 15 days. Other details of management were as for Experiment 1.

The stages of development referred to are after Witschi (1956a).

Chick embryos. Thirty-six fertile eggs of White Leghorn and Light Sussex hens were selected on the fifth day of incubation and treated as follows. Eleven were injected into the yolk-sac with sterile dextran sulphate solution, individual eggs receiving from 0.01 to 10 mg. Twenty-five were injected on to the chorioallantoic membrane with dextran sulphate. Thirteen received an injection on the fifth day, eight an injection on the seventh day and four injections on the seventh and ninth days. The dose per embryo varied from 0.08 to 10 mg. These particular times were selected since it is during the fifth day that precartilaginous condensation of mesenchyme occurs at the site of the future skeleton and during the seventh day that chondrification is almost complete and ossification commences (Witschi, 1956b).

Miscellaneous. Radiographs of the surviving chick embryos were obtained on the nineteenth day of development.

Tissues were fixed in formalin and undecalcified sections stained with haematoxylin and eosin and by the V. Kóssa technique to demonstrate sites of calcification.

RESULTS

Effects on frog larvae

Experiment 1. The results are summarized in Table 1. The number of deaths increased in proportion to the dose of dextran sulphate used and with high doses (0.4 and 2.0 mg/ml.) all larvae died before reaching the stage of incipient metamorphosis.

TABLE 1

EFFECT OF DEXTRAN SULPHATE ON THE DEVELOPING FROG LARVA

(Experiment 1)

Dextran sulphate (mg/ml.)	Initial no. of larvae	Survivors at		Percentage attaining stages 30–33 of	
		Stage 26	Stages 30–33	Initial larvae	Larvae at stage 26
0	19	18	14	73·7	77·8
0·016	20	16	11	55·0	68·8
0·08	19	14	9	47·7	64·3
0·4	20	0	0	0	—
2·0	20	0	0	0	—

Approximately 60% of the larvae dying developed subcutaneous vesicles on the body and tail. Larvae surviving to the phase of incipient metamorphosis (Stages 26 to 29) continued to develop normally. There was no difference in the size and general external appearances of the Stage 33 control and dextran sulphate-treated larvae. Histological examination of these larvae showed that there was no abnormality of cartilage and bone formation or of mineralization of the skeleton.

Experiment 2. The results obtained are summarized in Table 2. Heparin and laminarin sulphate behaved like dextran sulphate and were toxic to the larvae whereas

TABLE 2

PERCENTAGE MORTALITY OF FROG LARVAE AFTER 15 DAYS IN WATER CONTAINING ADDED SULPHATED POLYSACCHARIDES

(Experiment 2)

Compound	Mortality (%) for concentration (mg/ml.)											
	4·0	2·0	1·0	0·80	0·60	0·50	0·40	0·38	0·30	0·25	0·20	0·13
Dextran sulphate	—	100	85	75	25	15	10	—	0	—	0	—
Dextran	—	0	0	0	0	0	0	—	0	—	0	—
Sodium sulphate	—	0	0	0	0	0	0	—	0	—	0	—
Heparin	100	80	80	—	—	5	—	15	—	5	—	0
Chondroitin sulphate	0	0	0	—	—	0	—	0	—	0	—	0
Laminarin sulphate	100	100	60	—	—	35	—	10	—	5	—	0

dextran, sodium sulphate and chondroitin sulphate appeared to be innocuous. Calculation of the LD50 values for the various substances, using the relationship between probit percentage mortality and log concentration (Bliss, 1934a, b), shows that heparin is the least toxic; the values at the fifteenth day are 0.69, 0.79 and 1.02 mg/ml., for dextran sulphate, laminarin sulphate and heparin, respectively.

Effects on chick embryos

The embryos injected with dextran sulphate into the yolk-sac developed normally and were killed after 19 days incubation. Their weights (mean and standard deviations 20.5 g $\pm$ 2.6) were within the normal range (Witschi, 1956c) and radiologically the skeleton was normal. Histological examination confirmed that skeletal growth and mineralization were normal.

The embryos injected with dextran sulphate onto the chlorioallantoic membrane only survived when the dose was 0.4 mg or less. The general appearances, weights, and radiological and histological appearances of the skeleton were all normal in survivors killed after 19 days incubation. Embryos receiving 1 to 10 mg of dextran sulphate died, usually within 24 to 48 hr, but were normal in size and skeletal development at the time of death. Those given 0.5 mg survived to the thirteenth or fourteenth day of incubation and at death appeared to be normal in size for their age. Dead embryos were congested and in many there were widespread haemorrhages.

DISCUSSION

Sodium dextran sulphate added to the water in high concentration is toxic for frog larvae and kills the animals if sufficient is given. Animals receiving sublethal doses and surviving to the stage of metamorphosis may undergo this process in a normal manner. There is no effect on the development of the frog skeleton and osteoporosis and fractures do not occur. Laminarin sulphate and heparin resemble dextran sulphate in this toxicity whereas dextran, chondroitin sulphate and sodium sulphate are without effect. It appears that the sulphate content of the material used is important only when combined with a polysaccharide since inorganic sodium sulphate and dextran are without effect.

The mechanism of cutaneous vesicle formation in the dextran sulphate-treated frog larvae is not known. Since the particular dextran sulphate used is of low molecular size it is unlikely that the vesicles are induced by a disturbance of osmo-regulatory

mechanisms. The dextran sulphate may become incorporated in the ground substance with resultant faulty adhesive properties of the intercellular cement substance so that the skin becomes separated at the sites of minor trauma.

In the doses used dextran sulphate injected into the yolk-sac does not interfere with skeletal development or general growth of the developing chick embryo. The dose of 10 mg given would have been fatal if applied to the chorioallantoic membrane, so presumably the dextran sulphate fails to gain access to the embryo when injected into the yolk-sac. Substances in the yolk-sac do not normally enter the embryo through the vitello-intestinal duct but are absorbed by way of the vitelline veins after " digestion " by the inner surface of the sac. It is therefore probable that dextran sulphate is rendered innocuous by enzymatic degradation before absorption occurs. When dextran is injected on to the chorioallantoic membrane in doses greater than 0.4 mg the embryo is rapidly killed, whereas smaller doses are without effect and the embryos develop normally.

These experiments indicate that dextran sulphate does not behave like β-amino-propionitrile or insulin on the developing frog or chick embryo and there is no growth inhibiting effect as there is with cortisone. Furthermore, ossification in the developing frog larvae and chick embryo does not appear to be impaired by dextran sulphate as it is in the rabbit, guinea-pig and rat. In these mammals the maximum effects are observed at the growing ends of the long bones where there are independent epiphyseal centres of ossification. Such epiphyseal centres do not occur in birds and amphibians and this may in part explain the discrepancy.

SUMMARY

1. Concentrations of 0.4 mg/ml. or more of heparin, laminarin sulphate or dextran sulphate prove fatal to young developing frog larvae before they reach the phase of incipient metamorphosis. With sublethal doses metamorphosis and skeletal development are normal. Chondroitin sulphate and dextran are without effect.

2. Dextran sulphate in doses of 1 mg kills developing chick embryos when injected on to the chorioallantoic membrane. With sublethal doses development is normal.

3. The lack of skeletal abnormalities in these animals contrasts with the occurrence of osteoporosis and fractures in rabbits, rats and guinea-pigs treated with synthetic heparin substitutes.

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