Acta orthop. scand. 41, 44–56, 1970

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EFFECT OF INSULIN ON BONE IN TISSUE CULTURE

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Received 22.v.68

In the course of investigating the influence of various environmental factors on the growth and regeneration of osteogenic cells following trauma *in vitro,* it has been observed that the presence of insulin in culture stimulates the proliferation of cartilage cells at the fracture site in embryonic chick bones. L. W. Gorham (1965) showed marked differentiation of embryonic cartilage and bone in mouse, cultured in a medium containing insulin. Similarly *in vivo* an increased rate of endochondral ossification during healing of fractured bones under the influence of insulin has also been demonstrated by Udupa & Singh (1965).

To support the above-mentioned observations (Prasad & Reynold 1968), degradation of cartilage was produced by the method described by Fell & Thomas (1960). These workers were able to produce the degradation of cartilage by the use of an enzyme, Papain, in culture explants. In the present experiment a newly introduced proteolytic enzyme called 'Annanase' obtained from the pineapple plant *(Annana coccus)* has been used which is claimed to be less toxic than papain. In addition, the effect of insulin on the degraded cartilage (produced by Annanase) has been studied. This paper deals with some of the histological, histochemical, and biochemical changes in the bone as well as in the medium employed. Microautoradiography with labelled sulphur was also done. In addition, increase in length and weight of the bones under study were also recorded.

METHODS AND MATERIALS

The tibiae of fourteen-day-old chick embryos consisting of a calcified shaft of the bone with haemopoitic tissue and large cartilaginous area were used. The bones

This work has been carried out under a grant given by Wellcome Trust, London.

with intact periosteum were isolated from the soft tissues under a dissecting microscope and were kept immersed in Tyrode solution during dissection. The bones were kept on a shallow stainless steel grid table in the culture dish. The culture chambers were the same as described by Fell & Weiss (1965); they were incubated at 38.5° C for six to eight days. The experiments were carried out in groups. In the first group, bones were grown in the control medium; in the second, paired intact bones were cultivated in the Annanase medium. In the third group bones were cultured for 1–4 days in Annanase and then transferred into insulin medium and grown for a further 2 days.

The chemically defined medium was prepared as described by Prasad & Reynold (1968). Annanase was added to the medium at different concentrations, i.e. 10 μg, 20 μg, 30 μg, and 50 μg per millilitre of the medium. In other experiments, insulin was added at the concentration of 500 micro-units/ml. The crystalline insulin (24.5 unit/mg) used in this experiment was received from Allen & Hanbury, London, and a concentrated solution was prepared in 0.001 N HCl. This was diluted into the medium. The medium was changed every second day.

The lengths of the explants were measured every alternate day by means of a calibrated eye-piece grid. The wet weights were determined by blotting the bones on filter paper and weighing them in stoppered tubes. The explants were then dried for 48 hours *in vacuo* over phosphorus pentoxide and re-weighed. Two bones from each group were harvested at different intervals and were fixed in acetic Zenker's solution, decalcified with formal nitric acid, and embedded in paraffin wax. The serial sections were cut six microns thick and stained with Delafield's haemotoxylin and chromotrope and toluidine blue staining. The cultured bones and the used media of the different groups were biochemically analysed. The hydroxyproline contents of the explants were determined by the method of Prockop & Udenfiend (1960) and the collagen content was obtained by multiplying the hydroxyproline content by 7.46. The amount of hexosamine in the explants and in the used media was estimated by the method of Boas (1963). The sialic acid concentration in the used medium was measured as per methods described by L. Svenverholom (1957) and glucoronic acid was determined by the method of Glicks (1958).

In addition, microautoradiography of the tissues under study was done with labelled sulphur sodium. Two bones from each group were incubated for 4 hrs after adding radioactive sulphur in the dose of 10/μ curies. The tissue was fixed in acid alcohol, decalcified, and embedded in paraffin. Serial sections six microns thick were cut. Autoradiography was done by putting these slides into close contact with Kodak A R-10 stripping film. This whole procedure was carried out at 20–21° C. The slides were kept in a light-tight boxes and left for exposure at 10° C for six weeks. Exposed films were developed in Kodak 19 B developer and stained with haematoxylin and eosin. The staining procedures were carried out at 17° C.

RESULTS

Examination of the living explants at different intervals during the culture period led to the following general observations. Bones cultivated in the presence of Annanase (50 μg/ml of the medium) showed

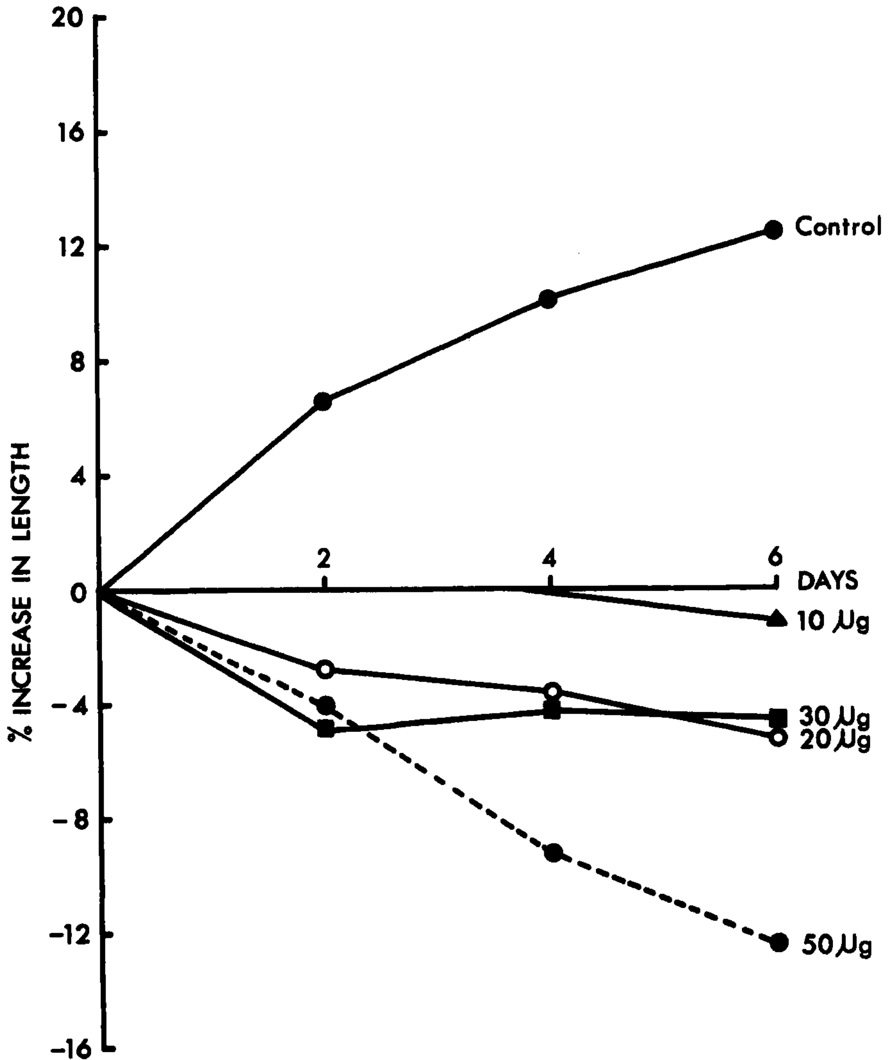


Figure 1. Percentage of increase or decrease in length of explants grown 6 days in various concentrations of Annanase. Note the marked decrease in length at the concentration of 50 μg/ml.

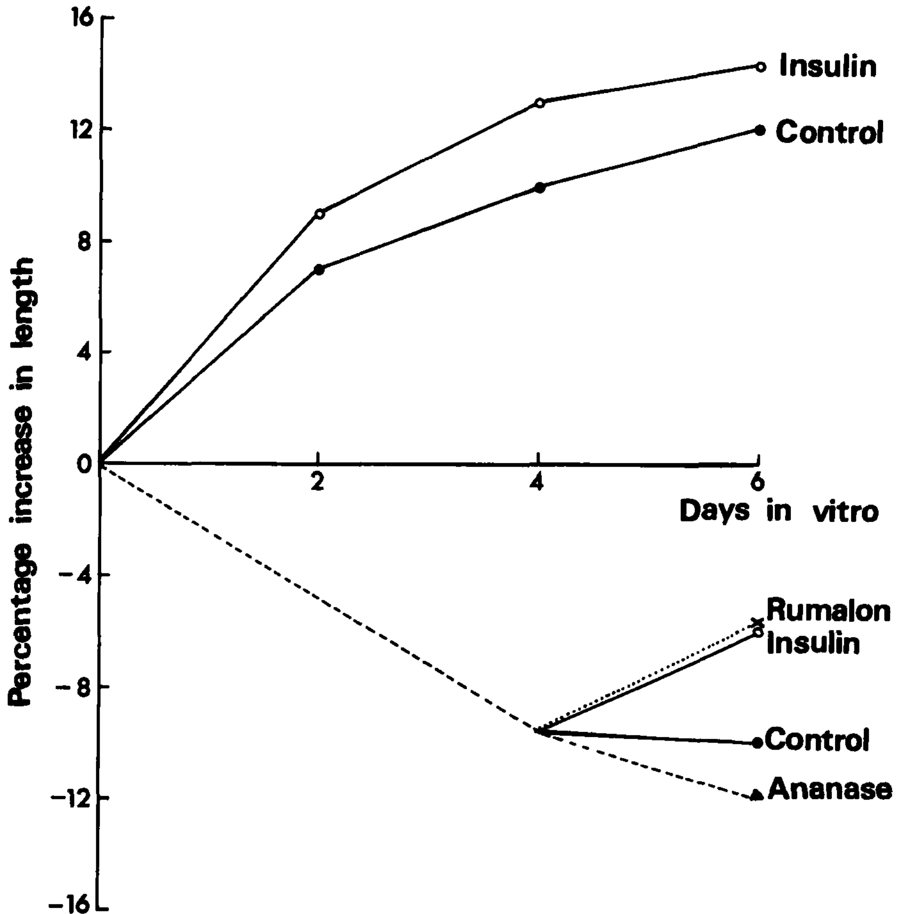


Figure 2. Comparative study of bones cultured in different media. Statistically there is no difference between control and insulin-treated explants: both show continuous increase in length, whereas bones cultured in Annanase show a decrease in length. If the Annanase-treated explants, after 4 days of culture, are transferred to the insulin or rumalon medium, growth resumes.

a resorption and softening of the cartilaginous end. In control series there was continuous longitudinal with anatomical differentiation.

In the first series of experiments the explants were cultured in various concentrations of Annanase. This enzyme showed optimum activity at the dose of 50 μg/ml in the medium (Figure 1). In the control series a gradual and continuous increase in the length of the bone was noted. The increase in the length of the bone cultured in

Annanase, insulin, and control medium were measured and compared with one another (Figure 2). The explants grown in the control medium and insulin medium yielded continuous growth up to six days but without much difference between the two, whereas the bones cultured in Annanase medium showed a gradual decline in length up to six days. If similar explants were grown 4 days in Annanase medium and then transferred into the media containing insulin or into the control medium and cultured for a further two days, there was an increase in length of the bones grown in insulin medium whereas the bones cultured in control medium did not show any longitudinal increase nor further diminution in length (Figure 2). The dry weight of the bones grown in the Annanase medium for six days was significantly lower, i.e. 5.50 mg ($\pm$ 0.46 $p = 0.05$), in comparison with the control, i.e. 8.7 mg ($\pm$ 1.47 $p = 0.001$), whereas the dry weight of the bones cultured for two days in Annanase medium and then transferred and grown for a further four days in insulin medium was 6.625 mg ($\pm$ 1.92 $p = 0.005$) (Table 1). The dry weight/wet weight ratios of the bones grown in different media are given in Table 1, which shows no significant difference between control and the insulin series. On the other hand, the bones treated with Annanase showed marked decrease in the dry weight/wet weight ratio.

The explants grown in various media were analysed for hydroxyproline and hexosamine in order to measure the mucopolysaccharide and collagen contents. The hexosamine content of bones treated with annanase was much lower than the control, followed by increased hexosamine content of the bones if the same explants were transferred into the control or insulin medium (Figure 3). Moreover, the amount was significantly greater in insulin-treated bone in comparison with control. The estimation of hydroxyproline in the bones treated with Annanase for six days yielded 15.3 mg% (statistically not significant). When the bones were transferred and grown for a further four days after treatment with Annanase, an increase in hydroxyproline content was noticed (Figure 4). Statistically there was not much difference.

Samples of the used medium were collected and analysed for hexosamine, glucoronic acid, and sialic acid contents. The hexosamine content (Annanase-treated series) was maximum, i.e. 231.12 μg, after two days of culture followed by a gradual decrease with further culturing. The further breakdown of hexosamine was stopped if the Annanase-treated explants were transferred to the control or insulin medium (Table 2). Similarly the breakdown of glucoronic acid was

Table 1. Statistical analysis of the gain in dry weight and dry/wet weight ratio of the bones treated with insulin and Annanase (mean of 10 bones). There is not much difference between control and insulin but the difference between control and Annanase and insulin is statistically significant.

	Dry weight (mg)	t	P	$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100$ (mg)	t	P	
Control	8.7 ± 1.47	4.8	<0.001	14.63 ± 1.61	3.26	<0.01	Control vs. Annanase
Annanase	5.50 ± 0.46	1.64	>0.05	12.70 ± 0.46	0.20	>0.05	Control vs. insulin
Insulin	6.625 ± 1.92	1.61	>0.05	14.475 ± 1.30	3.64	<0.01	Annanase vs. insulin

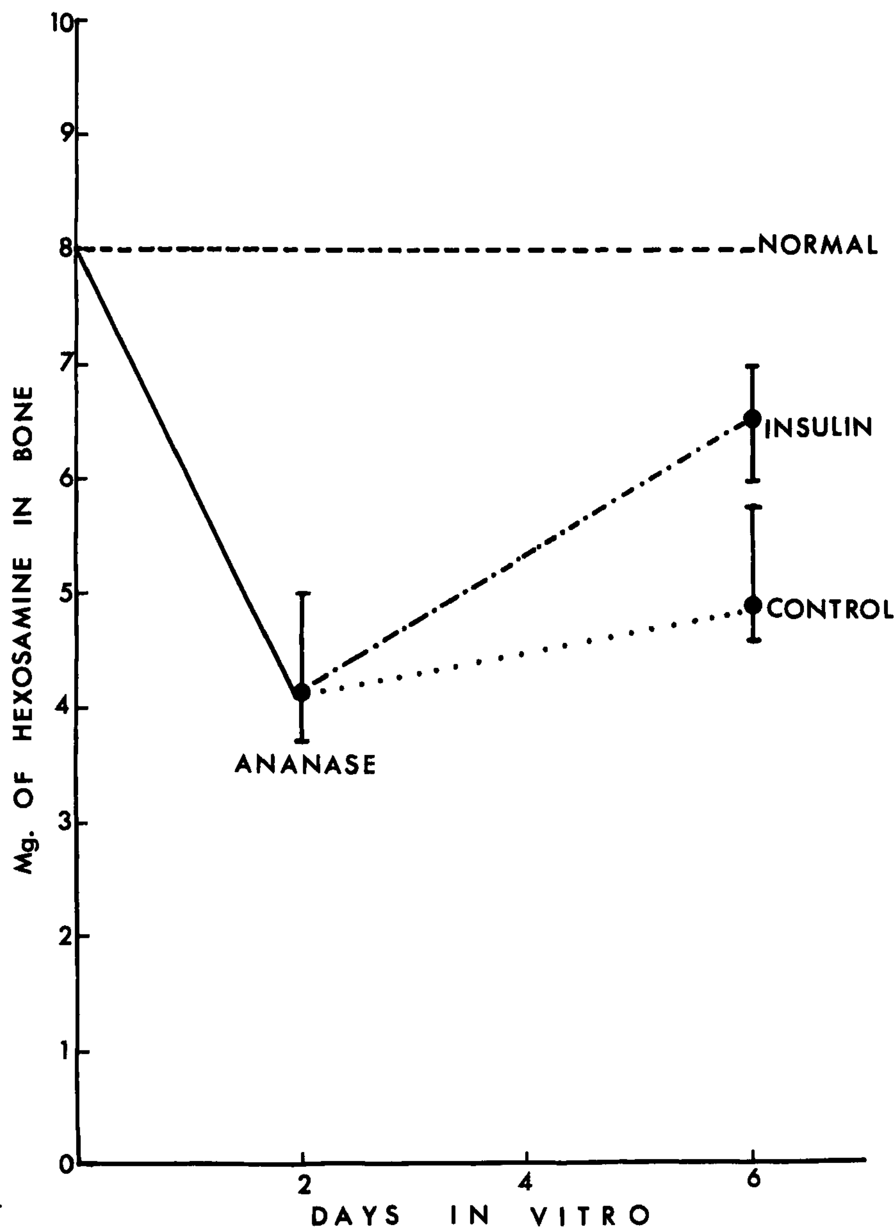


Figure 3. Hexosamine content of bones cultured in the various media. Bones cultured in Annanase lose approximately half of their hexosamine content. In the control medium, bone show less loss of hexosamine, and in the insulin medium the bones have almost normal levels.

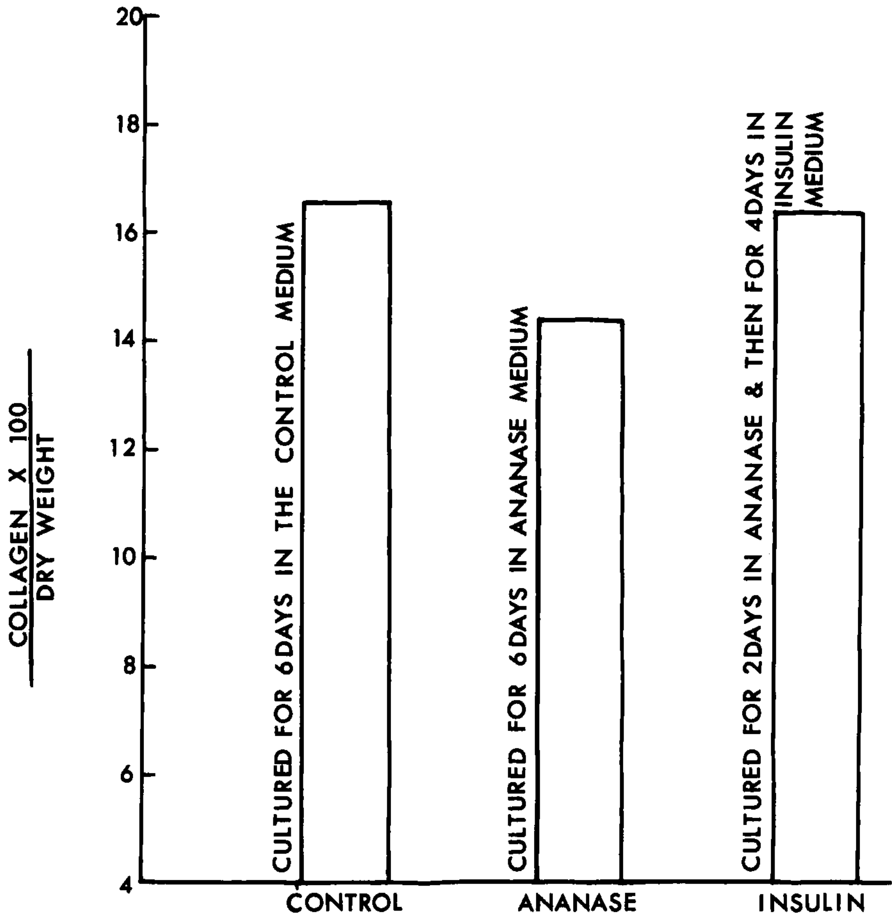


Figure 4. Collagen content of bones grown in the various media. Statistically there is little difference between the control, Annanase, and insulin-treated series.

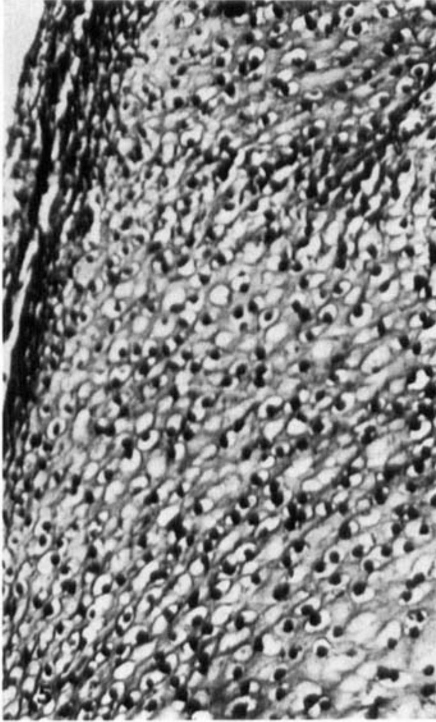
greater in the Annanase-treated series, i.e. 103.69 μg ± 19.33. In subsequent days the release of glucoronic acid was significantly decreased when the explants were cultured in the insulin medium in comparison with the control medium. The determination of sialic acid also showed a greater release of the substance into the medium if the culture was treated with Annanase. The breakdown of sialic acid was stopped by insulin, whereas it continued up to six days in the control medium (Table 2).

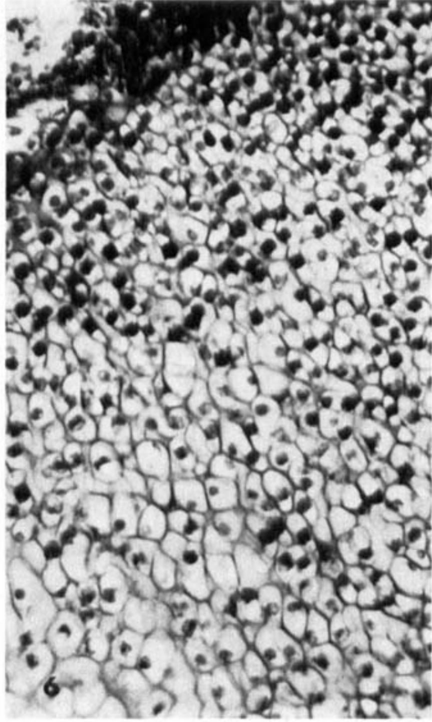
The histological (Delafield, haematoxylin, and chromotrope) and histochemical (toluidine blue) staining showed marked degradation of

Table 2. Statistical analysis of the biochemical analysis (glucoronic acid, hexosamine, and sialic acid) of the used medium (means of 10 samples).

	Biochemical analysis of the media employed								
	I	II	III	I vs. II		I vs. III		II vs. III	
				t	P	t	P	t	P
Glucoronic acid (μg)	103.69±19.33	69.17±4.23	42.56±9.62	5.24	<0.001	8.01	<0.001	7.54	>0.01
Hexosamine (μg)	231.12±34.85	56.05±7.69	13.42±4.54	12.02	<0.001	13.75	<0.001	10.86	<0.01
Sialic acid (μg)	63.44± 5.24	35.42±5.71	none	9.53	<0.001	–	highly signif.	–	highly signif.

I: Cultured for 2 days in Annanase.
II: Cultured for 4 days in the control medium after preliminary culture in Annanase.
III: Cultured for 4 days in the insulin medium after preliminary culture in Annanase.





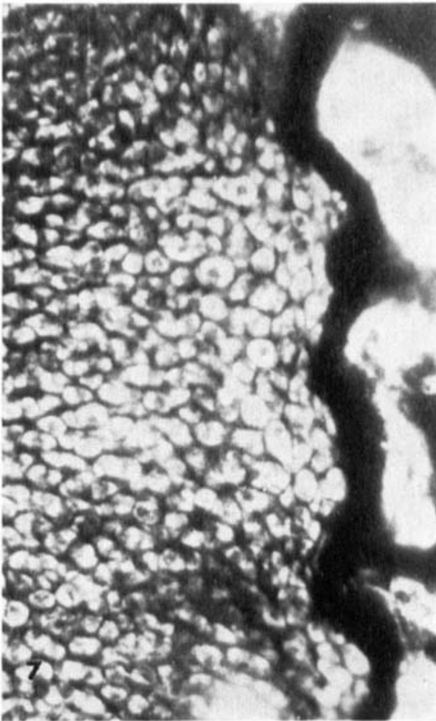


Figure 5. Histological picture of a control bone stained with toluidine blue, normal metachromatic staining. × 460.

Figure 6. Histological picture of a bone cultured for 4 days in the Annanase medium. Complete loss of metachromasia and dilated cartilage cells. Toluidine blue staining. × 460.

Figure 7. Histological picture of a bone cultured for 2 days in insulin medium after 4 days' culture in the Annanase medium. Reappearance of metachromatic staining with toluidine blue. × 460.

cartilage cells, and some of them converted into fibroblasts with complete loss of metachromasia in Annanase-treated bones (Figure 6) in comparison with control (Figure 5), whereas they regained metachromatic staining (toluidine blue) in insulin-treated bone (Figure 7).

The mucopolysaccharide synthesis was also studied by microautoradiography with radioactive sulphur. Generally there was a fair amount of deposition of silver grains, possibly sulphated mucopolysaccharide, whereas the explants treated with Annanase showed significantly lesser amounts of sulphated material in the cartilaginous area. Again if the same explants were transferred and grown in the presence of insulin, they yielded deposition of radioactive materials.

DISCUSSION

The results described in the present paper indicate that this new proteolytic enzyme Annanase produces degradation of the cartilaginous area of long bones. It confirms the findings of Fell & Thomas (1960) regarding papain. This enzyme is used orally in clinical cases of arthritis and also in various types of oedema in order to reduce the swelling. In the present study it has been observed that dry weight wet ratio of Annanase-treated bones decreases in comparison with the controls, indicating its dehydrating action on the cells. The decrease in dry weight suggests that it might also effect the mineral contents of the bone. The biochemical observations suggest that it produces a breakdown of the cellular membranes and release of the intracellular substances. Annanase also produces breakdown of protein and hexosamine; however it has little effect on hydroxyproline content.

The effect of insulin on normal and Annanase-treated bone suggests that it might be one of the main factor responsible for the proliferation of cartilage cells and increase of the mucopolysaccharide synthesis. This also confirms our earlier observations (Prasad & Reynold 1969) on the effect of insulin on bone repair *in vitro*. Hay (1958) demonstrated the effect of insulin on embryonic chick femurs *in vitro* and suggested that insulin at first stimulates the growth which is then followed by a diminution in length. The increase in length and weight of the bones treated with insulin as observed in our experiments suggests that it has little effect on normal growing explants (chick embryonic tibiae) in comparison with the control medium. The increase in length and gain in dry weight was greater in insulin-treated bone than in those treated with Annanase. This suggests that insulin

may also act as a growth hormone (Lawrence et al. 1959), probably helping in the differentiation of bone and cartilage during the regenerative stage. Gorham (1965) showed marked differentiation of embryonic cartilage and bone of the mouse cultured in the chemically defined medium.

The biochemical findings suggest that insulin might prevent further breakdown of mucopolysaccharide and at the same time increase its synthesis. These findings also correlate with the observations of Schiller & Dorfman (1957).

SUMMARY

1. A new proteolytic enzyme, Annanase, obtained from the *Annana coccus* plant has been studied for its effect on chick embryonic tibiae growth rate. The optimum level of action of this enzyme (50 μg/ml of the medium) has been determined. This new enzyme produces degradation of the cartilaginous end of the bone and breakdown of hexosamine, glucoronic acid, and sialic acid. There was little effect on the breakdown of collagen.

2. The effect of crystalline insulin (below the physiological level) was studied on the normal chick embryonic tibia and also on bones treated with Annanase. An increase of mucopolysaccharide synthesis and regeneration of cartilage cells in bones treated with insulin was observed. An increase in length and weight of the bones was also noted.

ACKNOWLEDGEMENT

We are indebted to Professor Dr. K. N. Udupa, MS, FRCS, FACS, FAMS, Director of the Surgical Research Laboratory, and Professor Dame Honor, B. Fell, D. Sc., FRS Director, Strangeways Research Laboratory, for their valuable advice and encouragement throughout this experiment. We are also grateful to G. C. Saxena, S. K. Mishra, and T. H. Azmi for their help during this work.

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