# Studies on the Mode of Action of Excess of Vitamin A

## 6. LYSOSOMAL PROTEASE AND THE DEGRADATION OF CARTILAGE MATRIX*

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Changes in the matrix of cartilaginous limb-bone rudiments after cultivation *in vitro* in the presence of excess of vitamin A were first demonstrated by Fell & Mellanby (1952). The possible relationship of these changes, which include cellular alterations as well as loss of extracellular material, to the liberation of lysosomal enzymes was postulated by Lucy, Dingle & Fell (1961) and by Dingle (1961). It was considered that release of a proteolytic enzyme from the lysosomal particles was responsible for the degradation of the protein–polysaccharide complex of the matrix, and that liberation of other lysosomal hydrolytic enzymes might cause the cellular changes observed in cartilage cultivated in the presence of the vitamin; this hypothesis was supported by observations on the specificity of the action of the vitamin, both in organ culture and on the isolated lysosomes (Fell, Dingle & Webb, 1962). Recent experiments on the effect of the vitamin on the cell membrane of erythrocytes (Dingle & Lucy, 1962), and on mitochondria (J. A. Lucy, M. Luscombe & J. T. Dingle, unpublished work), provide further evidence that one site of vitamin A action is at the lipoprotein membranes of cells and their organelles.

Though the evidence so far obtained favoured this hypothesis concerning the mode of action of vitamin A on cartilage, three important pieces of information were still lacking. (1) Although normal cartilaginous rudiments had been found to contain a protease, with an acid pH optimum, that when freed by treatment with hypo-osmotic solutions can degrade the matrix (Lucy *et al.* 1961), and though there was increased lysis of the plasma clot by cartilaginous rudiments grown in the presence of excess of vitamin A (Dingle, Lucy & Fell, 1961), the liberation of an acid protease by the vitamin A-treated explants had not been clearly demonstrated. (2) It was not known whether the vitamin could release the enzymes from intracellular particles isolated from cartilage, though it had been shown to liberate a protease from lysosomes isolated from rat liver (Dingle, 1961). (3) It was not known whether acid hydrolases from isolated intracellular particles could degrade cartilage rudiments. These three points have now been investigated.

* Part 5: Dingle & Lucy (1962).

## MATERIAL AND METHODS

*Experiments in organ culture.* The humeri, femora and tibiae of 6- and 7-day-old chick embryos were grown by the watch-glass method (Fell & Mellanby, 1952); the medium and conditions of culture were those described by Dingle *et al.* (1961). Vitamin A (10 i.u. or 3·3 $\mu$g./ml.) dissolved in ethanol was added to the plasma; the controls received ethanol alone. At the end of the cultivation period the rudiments with their surrounding capsule of connective tissue were removed from the plasma clot, washed twice in Tyrode's solution, once in synthetic medium, and then placed on a grid in a watch-glass containing 1·6 ml. of synthetic medium. The explants were incubated for up to 29 hr. at 38° under an atmosphere of oxygen + nitrogen + carbon dioxide (20:75:5). The rudiments were then removed from the watch-glass and weighed.

The following chemically defined media were used: for experiments on the rate of enzyme release from vitamin A-treated explants, medium BGJ (Biggers, Gwatkin & Heyner, 1961); for Expts. 358, 359 and 362, medium BL1 (Biggers & Lucy, 1960); for Expt. 377, Eagle's (1959) medium, without tyrosine; for Expts. 360 and 373, Parker 199 medium (Morgan, Morton & Parker, 1950).

*Protease assay.* The cartilaginous rudiments were homogenized in 2 ml. of a solution of a neutral detergent, 0·1% BRIJ 35 (Atlas Powder Co., Wilmington, Del., U.S.A.), a motor-driven homogenizer being used (Aldridge, Emery & Street, 1960), and the suspension was kept at 5° until it was assayed. Immediately before assay, 0·5 ml. of the homogenate was diluted with 0·5 ml. of 0·1 M-acetate buffer, pH 4·0. The synthetic culture medium (1·6 ml.) was adjusted to pH 4·0 with 0·5 N-HCl and then diluted to 4·0 ml. by the addition of 0·1 M-acetate buffer, pH 4·0. Samples (1·0 ml.) of both the solutions to be assayed were added to 1·0 ml. of 4·0% (w/v) haemoglobin dissolved in 0·1 M-acetate buffer, pH 4·0, and incubated at 37° for 60 min. in stoppered tubes. A duplicate sample of each solution was prepared and 5·0 ml. of ice-cold 4·7% (w/v) trichloroacetic acid added immediately without incubation. At the end of the incubation period the reaction was stopped by the addition of trichloroacetic acid, and the acid-soluble 'tyrosine' was measured in the filtrate by the method of Anson (1938). The proteolytic activity is expressed as the increase in $\mu$g. of acid-soluble 'tyrosine'/hr.

*Preparation of a particulate fraction from embryonic cartilage.* Cartilage was obtained from limb-bone rudiments from 10-day-old chick embryos and also from two 59-day-old bovine embryos. The cartilaginous rudiments were dissected in the usual way, but it was necessary to remove the area of calcification that was present in the middle of the shaft of the long bones from the bovine embryos.

The cartilage was homogenized in 0·25 M-sucrose, centrifuged at 600*g* for 5 min. to remove debris, and then at 10 000*g* for 20 min. Protease activity was determined in the supernatant fluid and in the particulate fractions. The 'free activity' is expressed as a percentage of free to total activity [free × 100/(free + bound)]. The action of vitamin A was tested as described for rat-liver lysosomes by Dingle (1961). The enzyme activity released by the vitamin is expressed as a percentage of the bound activity.

*Preparation of a crude protease from a particulate fraction of rat liver.* All manipulations were done in the cold room at 4°. A portion (50 g.) of rat liver was homogenized in 500 ml. of 0·25 M-sucrose, the nuclei and debris were removed by centrifuging at 600*g* for 5 min., and the mitochondrial and lysosomal pellet was obtained after centrifuging at 10 000*g* for 20 min. The pellet was resuspended in 100 ml. of 0·1% BRIJ 35, allowed to stand in the cold for 30 min. and then recentrifuged. The pellet was washed twice with BRIJ 35 and the washings were added to the first supernatant. Sodium chloride was added to give a final concentration of 0·9% and the pH was adjusted to pH 4·6. The solution was allowed to stand in the cold for 30 min. and the precipitate removed by centrifuging. Saturated ammonium sulphate (5 vol.) was then added and the solution kept in the cold room for 3 hr. The precipitate was removed by centrifuging and dissolved in about 25 ml. of water. This solution was dialysed overnight against water, the precipitate removed by centrifuging and the solution freeze-dried. The yield was 280 mg. of dry powder with a proteolytic activity under the above conditions of assay of 17 400 μg. of 'tyrosine'/hr. This crude protease preparation was dissolved in 10 ml. of 0·9% sodium chloride.

*Action of the protease preparation on limb-bone rudiments from chick embryos.* The femora from 9-day-old chick embryos were dissected free from muscle and connective tissue and were kept at room temperature, in a mixture of equal parts of 0·9% sodium chloride and amniotic fluid, until the beginning of the experiment. The rudiments were washed in 0·9% sodium chloride and then placed in Hanks solution (Hanks & Wallace, 1949), or in 0·9% sodium chloride made 0·1 M with respect to phosphate buffer of the required pH. The enzyme, dissolved in 0·9% sodium chloride, was added and the rudiments were incubated at 37°. In some experiments an equal amount of enzyme that had been inactivated by heating at 100° for 3 min. was added to the controls, and in other experiments the controls received an albumin solution. Single rudiments were removed at intervals and fixed for histological observation, and samples of the suspending fluid taken for the determination of polysaccharide by the turbidimetric method of Di Ferranti (1956).

*Histology.* The limb-bone rudiments were fixed in Zenker's fluid containing 3% (w/v) of acetic acid for 30 min., followed by Zenker's fluid without acetic acid for not less than 1 hr. After being washed for some hours in tap water, the rudiments were dehydrated, cleared in cedar-wood oil and embedded in paraffin wax. Sections were stained for 30 min. in toluidine blue [0·5% (w/v) in 5% (v/v) ethanol] and mounted in Depex (G. T. Gurr Ltd., London).

## RESULTS

*Release of a protease from vitamin A-treated limb-bone rudiments.* Previous observations on the lysis of the plasma clot during the growth of limb-bone rudiments *in vitro* indicated the possible release of proteolytic enzymes during cultivation in the presence of vitamin A (Dingle *et al.* 1961). Initially, therefore, attempts were made to determine the proteolytic activity of the 'natural' medium (embryo extract–plasma clot). Though this proved possible and though the vitamin-treated cultures usually showed a higher proteolytic activity than the controls, the results were variable and difficult to reproduce. At the beginning of the culture period there was substantial proteolytic activity in the medium, probably originating in the embryo extract, and this activity decreased during incubation. The presence of this unstable enzyme made it difficult to obtain an accurate measure of enzyme liberation from the explants. It was advisable therefore to grow the rudiments on natural medium until the desired degree of the vitamin A effect had been obtained, as shown by microscopic observations of the living explants, and then to transfer the rudiments to a fully synthetic medium to study the liberation of protease. Since vitamin A rapidly breaks down in the absence of protein, it was not added to the chemically defined medium.

The first experiment was made to investigate the influence of the 6 days' pretreatment with vitamin A on the rate of release of protease into the chemically defined medium; the result was expressed as a percentage of the activity liberated by paired control rudiments grown for the same period in normal medium. After 4 hr. in synthetic medium, the vitamin A-treated explants released 60% more protease than the controls, and after 29 hr. 275% more (Fig. 1).

For reasons that are not properly understood, the rate at which the vitamin acted varied in different experiments. The release of enzyme, however, was correlated with the severity of the morphological changes produced: the more severe the gross anatomical effect and the more numerous the fractures in the treated explants, the greater was the liberation of enzyme (Table 1). For example, in Expt. 358, in which the rudiments were cultured for 4 days on natural medium before transfer to synthetic medium, there was only a slight action of the vitamin and no increase in enzyme release could be found in the vitamin A-treated cultures as compared with the controls. On the other hand, in Expt. 339, which was also of 4 days' duration, there was a marked effect of the vitamin and an increased liberation of protease into the medium. The greatest effect as indicated by the number of bones fractured and the difference in weight between the hypervitaminotic explants and their controls was seen in Expt. 362 (Table 1): in this experiment the liberation of enzyme was about 7 times the control value.

The total amount of protease present in the

control cultures (explant plus medium) after 5 days in culture (i.e. 4 days on natural medium followed by 1 day on synthetic medium) was sufficient to liberate 137–139 μg. of 'tyrosine'/hr. in each of three experiments. Prolonging the culture period in natural medium to 6 or 8 days (Expts. 360 and 362) caused a small increase in the total amount of enzyme in the controls (156–159 μg./hr.), but a substantial increase in the total enzyme content of the severely affected vitamin A-treated cultures (187–257 μg./hr.).

Although more enzyme was released into the medium from the vitamin-treated cartilage, the treated rudiments themselves contained at least as much as, and often more than, the controls at the end of the experimental period.

The pH optimum, with haemoglobin as substrate, of the protease liberated into the synthetic medium by the vitamin-treated explants was very similar to that of the enzyme in an aqueous extract of normal embryonic cartilage, and to that of the enzymes released from a particular fraction of the normal rudiments (Fig. 2).

*Action of the vitamin on intracellular particles.* The centrifuging of homogenates of bovine and avian embryonic cartilage yielded a particulate fraction containing an acid protease. Protease activity was also found in the supernatant fraction; the percentage of free activity in the mammalian cartilage

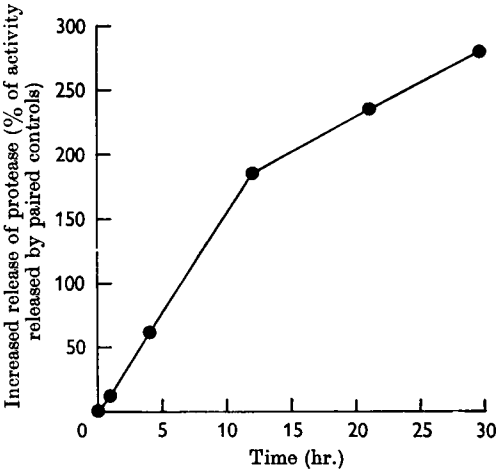


Fig. 1. Release of protease from vitamin A-treated chick-limb-bone rudiments. Sixteen rudiments were grown on natural medium in the presence of 10 i.u. of vitamin A for 6 days and then transferred to a synthetic medium. Control paired rudiments were treated in a similar manner. Samples of the synthetic medium were removed and their proteolytic activity was measured. The effect of the vitamin on enzyme release is expressed as a percentage of the enzyme activity released by the controls at each time.

Table 1. *Release of protease from vitamin A-treated chick-limb-bone rudiments*

In each experiment 16 rudiments (12 in Expt. 373) were grown on natural medium containing 10 i.u. of vitamin A/ml. for 4–8 days, then transferred to synthetic medium for 21–23 hr. at 38°. The gas phase was $O_2 + N_2 + CO_2$ (20:75:5). At the end of the incubation period the rudiments were removed, weighed and homogenized in 0·1% BRIJ 35, and the protease activity was measured. Control cultures were treated in the same manner. The protease was also measured in the medium.

| Expt. no. | Treatment | Time in natural medium (days) | No. of rudiments with fractures | Time in synthetic medium (hr.) | Total wet wt. (mg.) | Protease activity (μg. of 'tyrosine'/hr.) | | | Sp. activity of total protease (μg. of 'tyrosine'/hr./mg. wet wt. of tissue) |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | In medium | In rudiments | Total | |
| 358 | Vitamin A | 4 | 0/16 | 21 | — | 6·4 | 123·2 | 129·6 | — |
| | Control | 4 | 0/16 | 21 | — | 14·8 | 121·9 | 136·7 | — |
| 362 | Vitamin A | 4 | 0/16 | 23 | 33·5 | 21·2 | 171·7 | 192·9 | 5·8 |
| | Control | 4 | 0/16 | 23 | 49·9 | 9·5 | 128·3 | 137·8 | 2·8 |
| 373 | Vitamin A | 8 | 1/12 | 21 | 34·4 | 41·3 | 72·3 | 113·6 | 3·3 |
| | Control | 8 | 0/12 | 21 | 77·2 | 23·4 | 97·6 | 121·0 | 1·6 |
| 359 | Vitamin A | 4 | 8/16 | 21 | — | 62·6 | 142·6 | 205·2 | — |
| | Control | 4 | 0/16 | 21 | — | 38·2 | 100·7 | 138·9 | — |
| 360 | Vitamin A | 6 | 11/16 | 23 | 24·3 | 38·2 | 148·4 | 186·6 | 7·8 |
| | Control | 6 | 0/16 | 23 | 61·1 | 21·2 | 134·6 | 155·8 | 2·5 |
| 362 | Vitamin A | 8 | 16/16 | 23 | 25·0 | 103·9 | 152·6 | 256·5 | 10·3 |
| | Control | 8 | 0/16 | 23 | 104·9 | 14·8 | 144·2 | 159·0 | 1·4 |

(46 %) was similar to that in the avian (49 %). The high activity in the supernatant fraction from the mammalian tissue may have been due to an unavoidable delay of about 2 hr. between the killing of the animal and the homogenization of the dissected cartilage. The release of bound proteolytic activity by vitamin A (100 $\mu$g./ml.) added *in vitro* was greater with the particles isolated from bovine (61 %) than from chick cartilage (40 %).

*Degradation of normal cartilage matrix by lysosomal protease.* Experiments were made on the disappearance of metachromatic material from limb-bone rudiments, from 9-day-old chick embryos, incubated for 6 hr. in phosphate-buffered 0·9 % sodium chloride containing lysosomal protease obtained from rat liver. Fig. 3 shows the action of the enzyme on normal rudiments at both pH 6 and pH 7. At pH 7 the metachromasia was greatly decreased, whereas at pH 6·0 it was entirely absent; the controls showed little loss of metachromasia. This experiment indicated that the crude enzyme preparation was active in degrading the matrix of the cartilaginous rudiments and that it was effective at neutral pH.

Further experiments indicated that this disappearance of metachromasia was associated with considerable loss of polymeric chondroitin sulphate from the rudiments (Fig. 4). The controls, which in this experiment had been treated with enzyme that had been heated to 100° for 3 min., lost some polysaccharide; since this heating of the enzyme solution destroyed about 95 % of its activity, it seems probable that the loss of chondroitin sulphate in the controls was due to the release of intracellular enzymes in the cartilage itself. This action of the lysosomal extract in liberating chondroitin sulphate and causing disappearance of metachromasia was dependent on temperature (Table 2). Thus although there was rapid loss of chondroitin sulphate and of metachromasia at 37° there was little or none at 5°.

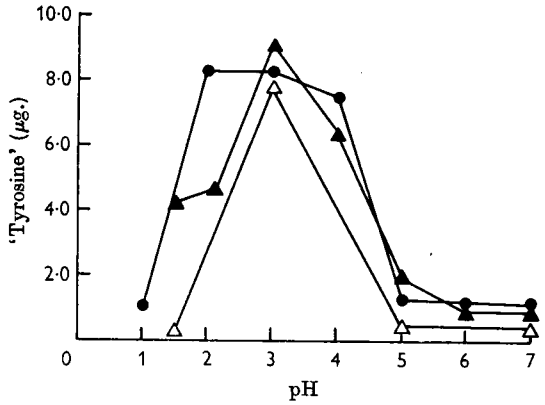


Fig. 2. Effect of pH on the protease from chick-limb-bone rudiments. The enzyme was released from a particulate fraction (obtained after centrifuging an homogenate of the rudiments at 10 000*g* for 20 min.) by treatment with 0·1 % BRIJ 35. The enzyme liberated into the synthetic medium was measured after adjustment of the pH with HCl to approximately the correct value; each sample was then diluted with an equal volume of 0·1 M-acetate buffer of the appropriate pH. The rudiments were treated with glass-distilled water at 4° for 1 hr. to obtain the aqueous extract. The measurements of protease activity were made with haemoglobin (2%, w/v) as substrate, and acid-soluble 'tyrosine' was measured as indicated in the text. ●, Aqueous extract of rudiments; ▲, synthetic medium; △, particulate fraction of rudiments.

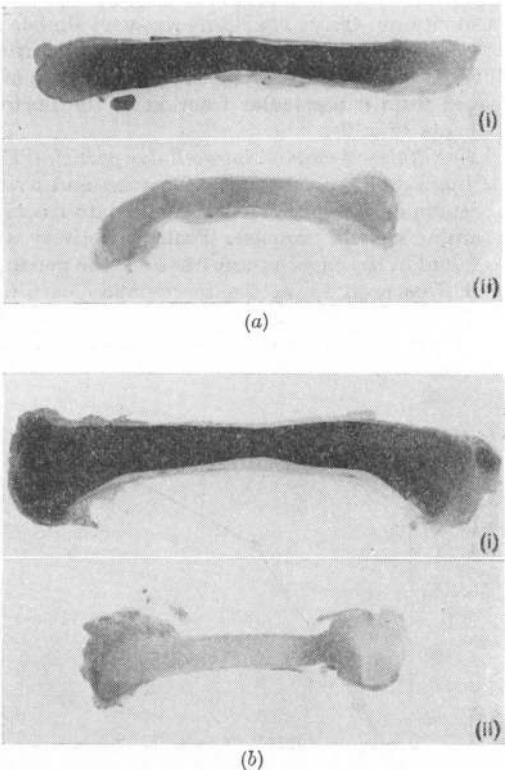


Fig. 3. Normal cartilaginous long-bone rudiments from a 9-day chick embryo, incubated for 6 hr. in phosphate-buffered 0·9 % sodium chloride with and without the addition of lysosomal extract. Sections were stained with toluidine blue. Magnification × 12. (*a*) (i) Control femur incubated at pH 7·0: little or no effect on metachromasia; (ii) femur incubated at pH 7·0 in the presence of lysosomal extract: metachromasia is greatly reduced, but some remains. (*b*) (i) Control tibia incubated in buffer at pH 6·0 without lysosomal extract: little or no effect on metachromasia; (ii) tibia incubated at pH 6·0 in the presence of lysosomal extract: metachromasia is absent.

## DISCUSSION

The present studies show that, after cultivation in the presence of excess of vitamin A (3·3 μg./ml.), the cartilaginous limb-bone rudiments from chick embryos release an acid protease into the culture medium. The control rudiments also liberate some proteolytic enzyme into the medium, but much less than explants showing advanced resorption in response to vitamin A. The significance of the enzymic activity liberated by the controls is not yet known, but if it is a feature of normal cell activity, possibly connected with digestion of the nutritive medium, it may provide a clue concerning the physiological function of the lysosomal protease. Carrel & Baker (1926) demonstrated a proteolytic enzyme, in embryo extract, that was ineffective at neutral pH but became increasingly active between pH 6·2 and 5·0. They suggested that this enzyme, acting at the cell surface, might hydrolyse proteins to proteases which would then be absorbed and utilized by the tissues.

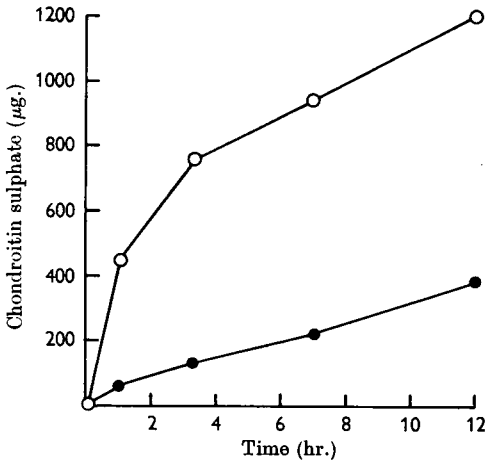


Fig. 4. Release of chondroitin sulphate from chick-limb-bone rudiments by lysosomal protease. Normal rudiments from 10-day-old chick embryos were incubated at 37° in 0·1 M-phosphate buffer, pH 7·0, containing the lysosomal extract. The controls contained enzyme that had been heated at 100° for 3 min. The liberation of polysaccharide was measured by the method of Di Ferranti (1956) on samples taken at intervals. ○, Treated with enzyme; ●, treated with boiled enzyme (control).

Since the amount of enzyme present in the vitamin A-treated explants at the end of the experimental period was often higher than in the larger control rudiments, the increased release of protease appears to be correlated with either an increased synthesis or an activation of the enzyme. This is especially striking in Expt. 362, in which the morphological changes were severe; the wet weight was only 25 % of the controls and yet the total enzyme content was almost twice that of the controls.

The optimum pH of the protease released by the explants is similar to that of the enzyme liberated from normal rudiments by treatment with hypo-osmotic solutions (Lucy *et al.* 1961) and also to that released by vitamin A from particles isolated from homogenates of normal cartilage in the present experiments. It seems likely, therefore, that the same protease, or group of proteases, is involved in all three experimental systems, and hence that the proteolytic enzymes of chick cartilage are present in the cell in a particle-bound form. Though it is probable that the protease is situated in lysosomal granules, further experiments are needed to see whether it is associated with other typical lysosomal enzymes (de Duve, 1959) before a firm conclusion can be reached.

The crude preparation of protease from rat-liver lysosomes proved to be very effective in degrading

Table 2. *Loss of metachromasia and chondroitin sulphate from enzyme-treated chick-limb-bone rudiments*

Normal rudiments were incubated at 5° and 37° in a lysosomal extract buffered at pH 7·0 with 0·1 M-phosphate buffer. Samples of the solution were taken at intervals for measurement of polysaccharide and single rudiments were removed and fixed for histological observation. Histological changes are indicated as follows: −, no change; ±, some loss of metachromasia from superficial cartilage of epiphyses; +, substantial loss from epiphyses; + +, substantial loss from epiphyses and zone of flattened cells; + + +, substantial loss from whole rudiments.

| | Temperature of incubation | | | |
|---|---|---|---|---|
| | 5° | | 37° | |
| Incubation period (min.) | Metachromasia loss | Chondroitin sulphate released (μg.) | Metachromasia loss | Chondroitin sulphate released (μg.) |
| 30 | − | 100 | ± | 140 |
| 60 | − | 155 | + | 220 |
| 100 | − | 170 | | 400 |
| 180 | − | 180 | + + | 540 |
| 270 | ± | 220 | + + + | 1040 |

the extracellular material of the cartilage even at neutral pH. In previous work on cartilaginous rudiments (pretreated with hypo-osmotic solutions) incubated in buffer at acid pH for 2 hr., the loss of metachromasia from the matrix was paralleled by the loss of polymeric chondroitin sulphate and by degradation of protein (Lucy *et al.* 1961). In the present experiments with the lysosomal protease of rat liver, the disappearance of metachromasia was again correlated with a release of chondroitin sulphate. It would have been preferable to have prepared the enzyme from particles obtained from chick cartilage, but since the limb-bone rudiments weighed only 2–3 mg. (wet wt.) it was impossible to obtain enough enzyme from this material. The results show, however, that a protease with an acid pH optimum, similar to that released by vitamin A from chick cartilage, can break down cartilage matrix at a neutral pH, though more slowly than at the acid optimum pH.

On the basis of a comparison of the effect of the proteolytic enzyme papain and of vitamin A on embryonic skeletal tissues, Fell & Thomas (1960) proposed, as a tentative working hypothesis, that vitamin A might enhance the activity of a number of cellular enzymes, one of which resembled papain in its effects. They further suggested that such an enhanced hydrolytic activity might be due to activation of the enzymes, to their greater production, or to their increased liberation through an increased permeability of cells or their organelles; this theory was supported by the observation by Dingle (1961) that isolated lysosomal particles released a bound proteolytic enzyme on the addition of vitamin A. The present results provide further evidence that the vitamin, when added in excess of normal requirements, causes a change in the permeability or stability of intracellular particles. It is possible that all the effects of vitamin A observed in cartilage, including the changes in metabolism, tissue composition (Dingle *et al.* 1961) and degradation of the extracellular material (Lucy *et al.* 1961), were due to alterations in the composition or stability of the membrane systems of the cells and their organelles, including lysosomes and mitochondria. This view is supported by the work of Dingle & Lucy (1962) on the instability of the erythrocyte membrane under the influence of the vitamin.

In all these systems, including the erythrocytes, the lysosomes and the cartilaginous explants, the specificity for the action of the vitamin is the same as that for reversing the effect of hypovitaminosis A in the intact animal. It may well be that one of the physiological actions of vitamin A is the control of membrane structure; if this were so it would help to explain the wide range of biological effects produced by both hypo- and hyper-vitaminosis A.

## SUMMARY

1. Cartilaginous rudiments grown in the presence of 3·3 $\mu$g. of vitamin A/ml. release an acid protease into the culture medium. The pH optimum of this enzyme is similar to that of a protease obtained from a particulate fraction of the rudiments.

2. A crude protease preparation derived from rat-liver lysosomes proved to be effective in degrading the extracellular material of cartilage, even at neutral pH.

3. Vitamin A released a protease from a particulate preparation obtained from bovine and chick cartilage.

4. The relationship of these studies to the physiological action of vitamin A in tissues is discussed.

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