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Studies on the Mode of Action of Excess of Vitamin A

3. RELEASE OF A BOUND PROTEASE BY THE ACTION OF VITAMIN A

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In the two preceding papers (Dingle, Lucy & Fell, 1961; Lucy, Dingle & Fell, 1961) it was shown that excess of vitamin A causes changes in both the composition and metabolism of cartilage cells and extensive breakdown of extracellular material. Hypo-osmotic treatment of embryonic cartilage released a proteolytic enzyme and degraded the matrix in a manner that was very similar to the effects of excess of vitamin A.

It was suggested that some of the effects of vitamin A might be explained if the vitamin causes the lysosomes to release a protease which would split the protein–polysaccharide complex of the matrix.

This paper presents evidence for a direct action of vitamin A upon isolated particulate components of liver cells and demonstrates some of the conditions under which the vitamin may release a bound protease.

METHODS

Tissue fractionations. Material was obtained from the livers of young adult rats. The tissue was cooled in ice-cold 0·25 M-sucrose, weighed and a suspension (1 g. in 10 ml.) made, an all-glass motor-driven homogenizer being used. Debris and nuclei were removed by centrifuging at 600*g* for 5 min. in a Serval Superspeed centrifuge. The supernatant was either centrifuged at 10 000*g* for 25 min. to sediment all mitochondria-like particles, or else submitted to a fraction procedure similar to that of Appelmans, Wattiaux & de Duve (1955). For the latter process the extract was first spun at 10 000*g* for 5 min. to give the 'heavy' fraction, the pellet was washed twice under the same conditions and the very loosely packed material removed. This, together with the first supernatant, was spun at 10 000*g* for 25 min. to form the 'light' fraction.

All manipulations were done in a cold room at 4°, and, provided that care was taken when using the homogenizer, negligible hydrolytic activity was released from the particles.

Enzyme assay. The free protease was assayed in 0·1 M-sodium acetate buffer, pH 4, with 2% (w/v) haemoglobin as substrate. The hydrolysis of the protein, which was estimated by the method of Anson (1938), was at 37° for 20 min. Activity was expressed as μg. of acid-soluble 'tyrosine' released. The reaction was stopped by the addition of ice-cold trichloroacetic acid and the mixture kept cold until the addition of the alkali (Gianetto & de Duve, 1955). Under these conditions the presence of sucrose did not appreciably interfere with the estimation of tyrosine. 'Total' activity was measured after suspending the particles in glass-redistilled water (10 ml./g. of original tissue) and storing at 4° for 30 min.

Treatment with vitamin A. A 1:5 suspension of the particles was made in 0·25 M-sucrose and crystalline vitamin A alcohol (Roche Products Ltd.) dissolved in ethanol was added. An equal amount of ethanol was added to the control tubes, though at the maximum concentration of ethanol used (1%, v/v) there was no appreciable effect upon enzyme release. The particles were then incubated, usually at 37° for 45 min. under nitrogen, and at the end of the reaction period the suspensions were spun at 15 000*g* for 20 min. in the cold. The enzyme released was measured in the clear supernatant fluid and the amount expressed as a percentage of the total activity released by hypo-osmotic treatment.

RESULTS

Rate of release of the protease. The effect of incubation of a 'mitochondrial' suspension with increasing amounts of vitamin A has been investigated (Fig. 1). The protease activity of the controls

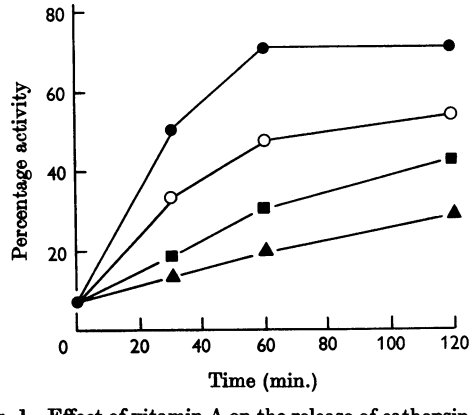

Fig. 1. Effect of vitamin A on the release of cathepsin from rat-liver lysosomes. 'Mitochondrial' fraction was suspended in 0·25 M-sucrose and incubated at 37° under nitrogen, with varying concentrations of vitamin A. At the end of the incubation period the suspension was spun at 15 000*g* for 20 min. Proteolytic activity was measured in the supernatant and the amount is expressed as a percentage of the total activity released by hypo-osmotic treatment. ▲, Control; ■, 0·5 μg. of vitamin A/mg.; ○, 1·0 μg. of vitamin A/mg.; ●, 2·0 μg. of vitamin A/mg.

increased with time, but the vitamin caused a more rapid release of the enzyme. The experiment also demonstrates that the release of enzyme is related to the concentration of vitamin, and that the maximum rate of release is in the first hour of incubation.

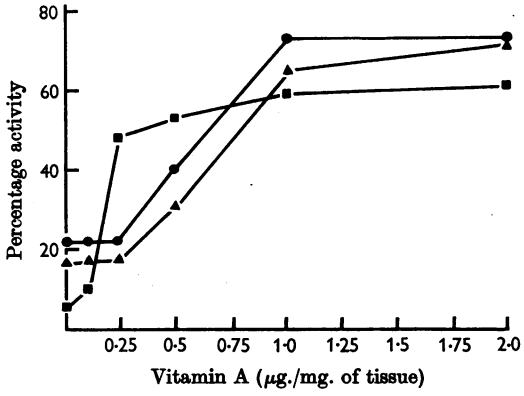

Fig. 2. Release of the protease from various mitochondrial fractions. Rat-liver mitochondria were fractionated in 0·25 M-sucrose as described in the text. The various fractions were incubated at 37° with vitamin A, for 45 min. under nitrogen. At the end of the incubation period the suspensions were spun at 15 000*g* for 20 min. and the proteolytic activity was estimated in the supernatant. ■, 'Light' mitochondrial fraction; ●, 'heavy' mitochondrial fraction; ▲, whole mitochondrial fraction.

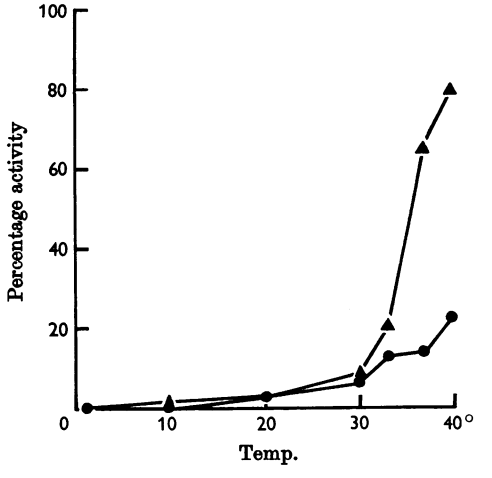

Fig. 3. Effect of temperature upon the release of cathepsin by vitamin A. Rat-liver mitochondria, suspended in 0·25 M-sucrose, were incubated for 45 min. under nitrogen with vitamin A (1 μg./mg. of tissue). At the end of the experimental period the suspensions were spun at 15 000*g* for 20 min. and proteolytic activity was measured in the supernatant. ▲, Vitamin A (1 μg./mg. of tissue); ●, control.

Release of the protease from various 'mitochondrial' fractions. The 'light' mitochondrial fraction that was obtained in this work appears to be more sensitive to the action of the vitamin than the 'heavy' or the whole fractions. 50 % of the total activity of the 'light' fraction is released by 0·25 μg. of vitamin A/mg. of original tissue after 45 min. at 37°, whereas three times this concentration is required to liberate 50 % of the activity of the whole fraction (Fig. 2). This difference may be due to higher ratios in the light fraction of lysosomal to non-lysosomal protein, or to lipoprotein, both of which may compete for the available vitamin.

Though all three preparations show an increasing release of protease with rising concentrations of vitamin A, the 'light' fraction exhibits a considerable release of enzyme over a narrow range of concentrations.

This rise in proteolytic activity under the influence of increasing amounts of the vitamin must be due to an increased liberation of the enzyme, since the vitamin has no effect upon the enzyme after it has been liberated under hypo-osmotic conditions.

Effect of temperature and pH *upon enzyme release.* The action of the vitamin upon the enzyme-containing particles is influenced by temperature (Fig. 3). Between 1° and 30° the vitamin has little or no effect, but above 30° there is an extremely rapid increase in the percentage activity released after incubation for 45 min. with 1 μg. of vitamin A/mg. of original tissue. At 40° there is also an increase in the thermal activation but this is small compared with the effect of the vitamin.

All the experiments so far reported have been performed by incubating the intracellular particles in 0·25 M-sucrose dissolved in glass-redistilled water of approximately neutral pH. Appelmans & de Duve (1955) reported that increase in acidity favoured the thermal activation of rat-liver lysosomes; it was therefore decided to compare the effects of different pH values on the thermal activation. The release of protease by the lysosomes was measured after incubation, with and without vitamin A, for 45 min. at 37° in 0·25 M-sucrose containing 0·02 M-sodium acetate buffers of differing pH values.

Below pH 5 (Fig. 4) there is a considerable increase in thermal activation and this is not appreciably altered by the presence of the vitamin. Above pH 5 the particles are stable in the absence of vitamin A, but the addition of the vitamin (1·0 μg./mg. of original tissue) is much more effective at neutral than at acid pH.

Effect of pH *upon the released enzyme.* Gianetto & de Duve (1955) reported that cathepsin released from rat-liver lysosomes by hypo-osmotic treat-

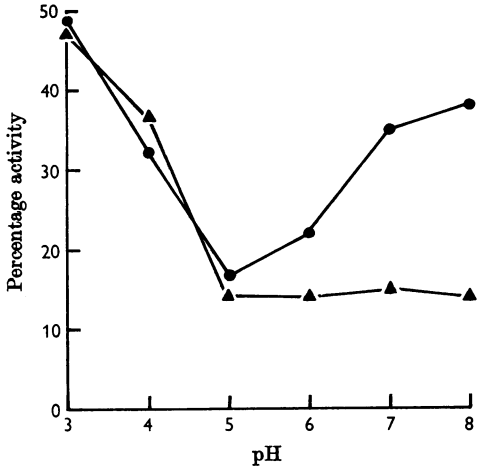

Fig. 4. Effect of pH upon the release of proteolytic activity from rat-liver lysosomes by vitamin A. 'Mitochondria' were suspended in 0·25M-sucrose containing 0·02M-sodium acetate buffer. The suspensions were incubated at 37° for 45 min. under nitrogen with 1 μg. of vitamin A/mg. of tissue. At the end of the incubation period the suspensions were spun at 15 000g for 20 min. and proteolytic activity was estimated in the supernatant. ●, Vitamin A (1 μg./mg. of tissue); ▲, control.

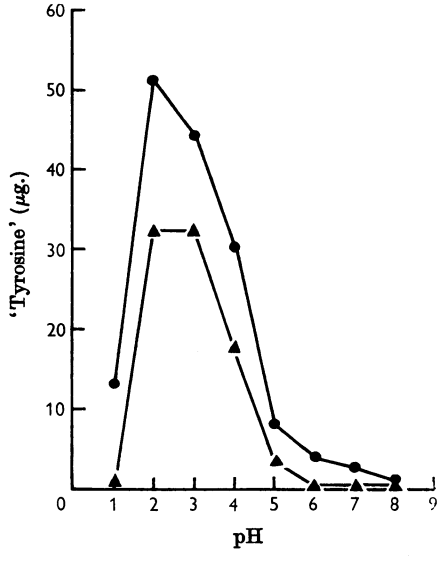

Fig. 5. Effect of pH upon the released enzyme. The cathepsin was released from rat-liver lysosomes by treatment with vitamin A (1 μg./mg. of tissue) and also by hypo-osmotic treatment. The enzyme solutions were incubated for 20 min. at 37° with 2% haemoglobin in 0·1M-sodium acetate buffers of different pH values. The acid-soluble tyrosine released was estimated as indicated in the text. ●, Enzyme released by vitamin A; ▲, enzyme released by hypo-osmotic treatment.

ment was more active at pH 3·6 than at pH 5·0; this is confirmed by the results of the present experiments (Fig. 5), which also show that the enzyme released by vitamin A has the same pH optimum against haemoglobin as that liberated by hypo-osmotic treatment.

DISCUSSION

In the preceding paper (Dingle *et al.* 1961) it was shown that the effects of vitamin A on cartilaginous limb-bone rudiments could be simulated by hypo-osmotic treatment, and also that the hypo-osmotic effects were probably due to the action of an intracellular protease, which was able to break some part of the protein–polysaccharide complex of the matrix. In order to relate this model system, in which the enzyme is released by hypo-osmotic treatment, to the action of the vitamin on living tissues an important step was to find if the vitamin was able to liberate a protease by the direct addition of the chemically pure compound to the enzyme-containing particulate components of the cells.

The results presented in this paper show that a proteolytic enzyme, with an acid pH optimum, similar to the enzyme liberated from the cartilaginous rudiments by hypo-osmotic treatment, is rapidly released by treatment of the 'mitochondrial' fraction of rat liver with vitamin A. Rat liver was used as a source of material as Gianetto & de Duve (1955) have shown that stable cathepsin-containing particles, the lysosomes, can easily be obtained from this tissue. Preliminary observations upon homogenates of embryonic cartilage indicate that the vitamin probably has a similar effect on the particulate components of this tissue also. Since no evidence could be found that the activity of the protease liberated from rat-liver lysosomes by hypo-osmotic treatment is affected by the presence of the vitamin, it is probable that the increased proteolytic activity of the supernatant is due to release of the bound enzyme under the influence of vitamin A. There is no direct evidence at present to indicate the way in which the vitamin alters the permeability of the particles, though the dependence of the vitamin A effect upon temperature and pH might perhaps suggest an enzymic mechanism.

The above results may indicate the mechanism by which the vitamin, acting at a subcellular level, is able to alter so drastically the composition of the extracellular material. Mainly because the breakdown of matrix was initially of greatest interest and also because of the ease of assay, this paper has dealt only with the release of a proteolytic enzyme from the particulate preparations. de Duve (1959) has demonstrated the presence of a number of

hydrolases in the particles he terms lysosomes. Besides a cathepsin, these include a ribonuclease, a deoxyribonuclease, a phosphatase, a glucuronidase and a sulphatase. As described in the first paper of this series, the action of the vitamin upon the cartilaginous rudiments grown in culture causes a complex series of changes, including loss of ribonucleic acid, amino sugars and dry weight. It seems very likely that the vitamin may release some, or all, of these bound hydrolases and that this complex of enzymes may cause many of the changes seen in the tissue treated with vitamin A.

SUMMARY

1. Particulate preparations from rat liver suspended in 0·25M-sucrose and treated with vitamin A release a proteolytic enzyme with an acid pH optimum.

2. The action of the vitamin on the particles is dependent upon temperature and pH.

3. The enzyme, after release by hypo-osmotic treatment, is not affected by the vitamin.

4. The relationship of this effect to the action of vitamin A on intact tissue is discussed.

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Biochem. J. (1961) **79**, 512

A Method for the Chromatographic Detection of Penicillins and Related Compounds and of Penicillinase

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The intact penicillin molecule is not oxidized by molecular iodine, but after the β-lactam ring is opened—for example, when penicillin is inactivated by the enzyme penicillinase—the resulting penicilloic acid rapidly reacts with 8–9 equiv. of iodine (Alcino, 1946).

Iodine also reacts with many sulphur compounds related to penicillin and can be used to detect these on paper chromatograms. In combination with penicillinase it will detect those penicillins whose β-lactam ring is opened by the enzyme. Alternatively, the method can be used to detect penicillinase. The method has been developed in this Institute over the past few years.

EXPERIMENTAL

The reagents are made up as follows:

Starch–iodine solution: 0·01M-iodine in 3 mM-potassium iodide, 10 ml.; M-phosphate buffer, pH 7·0 (54 g. of KH_2PO_4 and 84·6 g. of Na_2HPO_4 in 1 l. of water), 1 ml.; 2% (w/v) sodium starch glycollate (British Drug Houses Ltd.) in water (a crystal of thymol is added as preservative), 9 ml. The three stock solutions are stable, and the mixture is stable for several days at least.

Penicillin solution: sodium benzylpenicillin, 60 mg./ml. (100 000 i.u./ml.), freshly prepared, 1 ml.; M-phosphate buffer, pH 7·0, 1 ml.; water, 8 ml. This solution must be freshly made each day.

Penicillinase solution: Penicillinase solution of at least 1000 enzyme units/ml. A convenient method for preparing high-titre penicillinase from *Bacillus cereus* (strain 569/H or 5/B) has been given by Pollock (1957). This preparation usually contains 2000–10 000 enzyme units/ml., and is stable for some months at 4°: the enzymes from these two strains have given identical results when used in the methods described below.

Two methods are suggested for general use:

(1) Method A, for the detection of penicillin and related compounds. The paper is sprayed with starch–iodine solution and observed for 2 min. Most compounds, related to penicillin, containing potential sulphydryl groups immediately give pale spots on a deep-blue background, except for those with a closed β-lactam ring. The paper is now sprayed with a freshly made mixture of equal amounts of the starch–iodine and the penicillinase solutions. Additional pale spots will appear at the site of penicillins whose β-lactam ring is opened by the penicillinase. The