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Biosynthesis of Collagen Performed *in vitro* with the Aid of a Matrix Occurring in Reticulin

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Experiments have been carried out showing that it is possible to perform the last step in the biosynthesis of collagen peptide chains *in vitro*. An enzyme from liver has been used together with the performed matrix occurring in reticulin. Ascorbic acid is necessary for the reaction.

In the previous paper [1] a matrix was described containing all the elements necessary for the biosynthesis of collagen except the coupling enzyme. In this paper some experiments will be described in which the synthesis was performed using an enzyme obtained from homogenates of mouse liver.

EXPERIMENTAL

Preparation of the enzyme. It was considered that the enzyme would be the same as that transferring activated amino acids from the soluble RNA to their places in the peptide chain. Therefore a modification of the methods given by Nathans and Lipmann [2] and by Takanami [3] was used to prepare it.

One gram of fresh liver from mouse was homogenized in 9 ml 0.25 M cold sucrose solution and then centrifuged at low speed to free it from cell debris, nuclei and mitochondria. Sodium deoxycholate to a concentration of 0.5 % was added and the solution was centrifuged at 100 000 *g* for 2 h so that the microsomes were spun down. The solution was dialysed against water for 3 h and the pH was lowered to 5.0 with 1 M acetic acid. The precipitate was immediately spun down and the pH adjusted to pH 7.6. This solution was put on a DEAE-cellulose column and eluted with increasing concentrations of potassium chloride. The enzyme appeared between 0.18 – 0.25 M KCl. It was passed once more through the column. This enzyme solution has been used in the experiments for coupling the peptides in the reticulin. The absorption at 280 mμ in the enzyme solution has generally been 0.040 or if higher has been adjusted to this value.

Investigations on the phosphate release of reticulin. A buffer solution was used containing 0.1 M Tris-HCl buffer pH 7.6, 0.05 M KCl and 0.006 M $MgCl_2$. 50 mg reticulin, milled to a powder in a mortar were suspended in 2 ml of the buffer, and 10 μl of the enzyme solution was added. The suspension was incubated at 37°C for 30 min. The solution was acidified to pH 6 and the powder was centrifuged. Labile phosphate was determined in the supernatant according to Fiske and Subbarow [4]. There was no increase in the phos-

phate content compared with a solution where no enzyme had been added. The experiment was repeated but this time 2 mg ascorbic acid were added to the suspension before the incubation. Now it was found that the reticulin had released to the supernatant 6.4 μM labile phosphate. The original reticulin contained 6.7 μM labile phosphate, so practically all the phosphate was released.

If cysteine hydrochloride was used instead of ascorbic acid there was no increase of phosphate in the solution. If *p*-hydroxymercuribenzoate was added to a solution as well as ascorbic acid there was no effect.

The nucleotide released. The nature of the nucleotide in the supernatant has been investigated. One ml supernatant was diluted with 2 ml water and put on a column of DEAE-cellulose. The material was eluted with ammonium carbonate solutions of different molarities according to Staehelin [5]. All the nucleotide material came out as guanosine monophosphate at 0.03 M ammonium carbonate. Phosphate could also be detected at 0.05 M ammonium carbonate, where pyrophosphate is to be expected. No peptide could be detected in the solution. Thus the nucleotide part is released and broken to give GMP and pyrophosphate.

Investigation on the reticulin powder. In order to control if dialysable peptides occurred in the incubated reticulin it was dissolved in the urea buffer. About 80 % went up into the solution. No dialysable peptides could be detected. A chromatographic analysis was done on the urea extract using the same technique as before. As in the case of collagen from rat tail tendon one component came out soon after the hold up volume. It comprised 98 % of the protein content in the supernatant assayed with the method of Lowry *et al.*[6]

DISCUSSION

The investigation indicates that a synthesis of collagen chains or collagen molecules went on in the reticulin powder when a coupling enzyme from liver was used. For the reaction the presence of magnesium ions and ascorbic acid was indispensable. During the peptide bond synthesis guanosine monophosphate and pyrophosphate were given off from the reticulin powder and were found in the surrounding solution. The investigations confirm the hypothesis previously given that in reticulin there exists a system able to synthesise collagen.

A confirmation of considerable interest that a synthesis has taken place is the dependence of the reaction on ascorbic acid. Wolbach and Howe [7] have already shown that the synthesis of collagen is dependent on ascorbic acid, while investigating the healing of wounds in ascorbic acid deficient guinea pigs. Bradfield and Kodicek,[8] continuing the work, showed that in scorbutic guinea pigs the wounds contained swollen anomalous fibres imbedded in a periodic acid—Schiff positive mass. Gould [9] implanted polyvinyl sponges in both flanks of guinea pigs and made the animals ascorbic acid deficient. When he injected a small amount of ascorbic acid into one of the sponges collagen was synthesised only at this side. It seems, thus, that even *in vivo* the synthesis of collagen chains is dependent on ascorbic acid.

From investigations which will be published later it seems that the enzyme acts even on the nucleotide peptides of collagen in solution. During this reaction the amount of free amino groups is decreased and at the same time the amount of peptide groups is increased. The presence of ascorbic acid is also necessary here. As the enzyme is taken from another animal and organ it cannot be said that the enzyme is specific for collagen biosynthesis, but takes part in the general biosynthesis of proteins. It is an enzyme which mediates the transfer of activated amino acids from s-RNA to their place in the sequence

of a peptide chain growing on the ribosome. Guanosine triphosphate is also necessary for this process [10].

It might therefore be that ascorbic acid is necessary in the general process for good protein biosynthesis. This does not, however, seem to have been investigated. In the special case of collagen where the last step of the synthesis occurs outside the cell the ascorbic acid is necessary. If it was acting only as an activator of the enzyme analogous effects should also be able to activate the enzyme, but they are often inactive in scurvy. It seems therefore most probable that the ascorbic acid takes part directly in the process.

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