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LYSYL HYDROXYLASE

FURTHER PURIFICATION AND CHARACTERIZATION OF THE ENZYME FROM CHICK EMBRYOS AND CHICK EMBRYO CARTILAGE

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Summary

A purification of up to 4000-fold is reported for lysyl hydroxylase (EC 1.14.11.4) from extract of chick-embryo homogenate and one of about 300-fold from extract of chick-embryo cartilage. Multiple forms of the enzyme were observed during purification from whole chick embryos. In gel filtration the elution positions of the two main forms corresponded to average molecular weights of about 580 000 and 220 000. These two forms could also be clearly separated in hydroxyapatite chromatography. In addition, some enzyme activity was always eluted between the two main peaks both in gel filtration and in hydroxyapatite chromatography. The presence of the two main forms was also observed when purifying enzyme from chick embryo cartilage.

Both forms of the enzyme hydroxylated lysine in arginine-rich histone, which does not contain any -X-Lys-Gly- sequence. No difference was found between the enzyme from whole chick embryos and from chick embryo cartilage in this respect.

Lysyl hydroxylase was found to have affinity for concanavalin A, indicating the presence of some carbohydrate residues in the enzyme molecule.

Lysyl and prolyl hydroxylase activities increased when the chick embryo homogenate was assayed in the presence of lysolecithin. Preincubation of the homogenate either with lysolecithin or with Triton X-100 increased lysyl hydroxylase activity in the homogenate, and in the 15 000 × *g* and 150 000 × *g* supernatants, suggesting that the increase in the enzyme activity was due to liberation of the enzyme from the membranes.

Divalent cations were found to inhibit the activity of lysyl and prolyl hydroxylases in vitro. An inhibition of about 50% was achieved with 15 mM calcium, 60 μM copper and 3 μM zinc concentrations. The mode of inhibition was tested with Cu^{2+}, and was found to be competitive with Fe^{2+}.

Introduction

Hydroxylysine and hydroxyproline in collagen are synthetized by hydroxylation of certain lysyl and prolyl residues in peptide linkages (for review, see refs. 1—6). Both hydroxylations require specific enzymes, lysyl hydroxylase (EC 1.14.11.4) and prolyl hydroxylase, respectively, molecular oxygen, ferrous iron, α-ketoglutarate and a reducing agent, such as ascorbate [1—6]. The α-ketoglutarate is stoichiometrically decarboxylated during the hydroxylation reactions [7—9].

Prolyl hydroxylase has been purified to near homogeneity using conventional enzyme purification methods [10—12], and entirely pure preparations have been obtained by methods based on the affinity of the enzyme for a substrate [13] or a competitive inhibitor, polyproline [14]. Lysyl hydroxylase has been partially purified and characterized previously from chick embryo homogenate [9,15—19].

In the present work lysyl hydroxylase was purified further by a conventional enzyme purification procedure, and some unsuccessful attempts at affinity chromatography are described. Additional characterization of the enzyme was carried out, and some comparisons of the characteristics of lysyl hydroxylase and prolyl hydroxylase were made.

Materials and Methods

Materials

Fertilized eggs of white Leghorn chickens were purchased from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and incubated in humidified incubators at 37°C for 14 days, to be used as material for enzyme purification, or for 17 days when used for preparation of the substrate. Lysolecithin and arginine-rich histone were purchased from Sigma Chemicals Co. (St. Louis, Mo.), [^{14}C] lysine and [^{14}C] proline from New England Nuclear Corp., and α-[1-^{14}C] ketoglutarate from Calbiochem. The peptides L-I, having the amino acid sequence Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly [9] and (Pro-Pro-Gly)$_5$ · $4H_2O$ were synthetized at the Protein Research Foundation (Minoh, Osaka, Japan), and used as substrates for lysyl and prolyl hydroxylases.

[^{14}C] Lysine- and [^{14}C] proline protocollagen substrates

[^{14}C] Lysine- and [^{14}C] proline-labelled protocollagen substrates were prepared in isolated chick-embryo tendon cells as described previously [20], except that instead of precipitating protocollagen by $(NH_4)_2SO_4$ the dialyzate was heated for 10 min at 100°C after dialysing against 0.2 M NaCl and 50 mM Tris · HCl, adjusted to pH 7.9 at 4°C, and after heating, centrifuged at 20 000 × *g* for 30 min. Aliquots of 150 000 dpm were used when assaying lysyl hydroxylase and 60 000 dpm when assaying prolyl hydroxylase. The substrates were stored as aliquots at −20°C.

Defatted albumin

Fatty acids were removed from bovine serum albumin (Sigma Chemicals Co.) by the method published previously [21]. The treatment removes at least 99%

of the radioactively-labelled fatty acids bound to albumin and the protein is native after treatment, when judged by several criteria [21].

Purification of lysyl hydroxylase from homogenate of whole chick embryos

All procedures were carried out at 0—4°C and samples were stored at 0—4°C without freezing between steps. All centrifugations were carried out at 15 000 × *g* using a Sorval RC2-B refrigerated centrifuge.

Preparation of the initial extract. A total of 150—200 14-day-old chick embryos were homogenized in batches of 30 embryos in a solution of 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 0.1% Triton X-100 and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C (1 ml solution per g of embryo) in a Waring blender at full speed twice for 30 s. The homogenate was allowed to stand with occasional stirring for 30 min, and then centrifuged for 30 min.

$(NH_4)_2SO_4$ fractionation I. Solid $(NH_4)_2SO_4$ was slowly stirred into the supernatant fraction to a final concentration of 17% saturation. The pellet obtained by centrifugation for 20 min was discarded and solid $(NH_4)_2SO_4$ was slowly stirred into the supernatant fraction to a final concentration of 55% saturation. The pellet obtained by centrifugation for 20 min was dissolved in 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C. The sample, in a final volume of about 2300 ml, was dialyzed for 3 and 16 h against two separate 24-l volumes of this solution, and centrifuged for 10 min to remove a small amount of insoluble material.

Calcium phosphate gel fractionation. A suspension of calcium phosphate gel (Calbiochem) diluted with distilled water to a concentration of 30 mg/ml was added to the $(NH_4)_2SO_4$ enzyme preparation in a protein concentration of 30 mg/ml. The ratio was generally 85 mg solid/100 mg protein, but this was varied depending on the absorption capacity of the gel preparation. The mixture was stirred for 15 min and the gel removed by centrifugation for 10 min. The pellet was eluted successively with the following solutions, which were adjusted to pH 7.5 at 4°C: (a) 0.04 M KH_2PO_4, 0.1 M NaCl, 0.1 M glycine; (b) 0.06 M KH_2PO_4, 0.15 M NaCl, 0.1 M glycine; (c) 0.09 M KH_2PO_4, 0.15 M NaCl, 0.1 M glycine; (d) 0.15 M KH_2PO_4, 0.15 M NaCl, 0.1 M glycine; (e) 0.18 M KH_2PO_4, 0.15 M NaCl, 0.1 M glycine. At each step, 2300 ml were used and the pellet was homogenized with a Teflon and glass homogenizer. After stirring for 20 min, the sample was centrifuged for 10 min. Solid $(NH_4)_2SO_4$ was stirred into the eluate to a final concentration of 55% saturation, and the pellets obtained after centrifugation for 20 min were dissolved in 0.05 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C. They were then dialyzed for 3 and 12 h against two separate 24-l vol. of this solution; after dialysis, the samples were centrifuged for 10 min in order to remove a small amount of insoluble material. Two or three of the eluates having the highest specific activity were combined and purified further.

DEAE-cellulose chromatography I. The calcium phosphate gel enzyme was applied to a DEAE-cellulose column (DE 23, Whatman), 7 × 40 cm, equilibrated with 0.07 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C. The column was eluted at a flow rate of 400 ml/h, first with 500 ml of equilibrating buffer, and then with the

same solution, with the NaCl concentration increased step-wise as follows: 0.09 M, 250 ml; 0.12 M, 500 ml; 0.16 M, 500 ml; 0.18 M, 500 ml; and 0.20 M, 2000 ml. Fractions having at least 60% of the specific activity of the fraction with the highest specific activity were pooled.

$(NH_4)_2SO_4$ fractionation II. The DEAE-cellulose enzyme pool was precipitated by stirring solid $(NH_4)_2SO_4$ into the solution to a final concentration of 60% saturation. The pellet was eluted successively with solution containing 0.15 M NaCl, 10 μM dithiothreitol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C, and decreasing concentrations of $(NH_4)_2SO_4$ as follows: 45, 42, 39, 30, 25, 20, 15, and 10%. In the elution with the 45% saturated solution, 20 ml was used, elsewhere 10 ml. $(NH_4)_2SO_4$ solution having a 90% saturation, pH 7.5, was slowly stirred into the eluates to a final concentration of 50% saturation, and the pellets obtained by centrifugation were dissolved in 2.5—5 ml of 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C. The samples were dialyzed for 3 and 12 h against two separate 8-l volumes of this solution, and the eluates with the highest specific activities, usually those with 39, 35 and 30% of $(NH_4)_2SO_4$ saturation, were then pooled.

Bio-Gel A-1.5 m gel filtration. The $(NH_4)_2SO_4$ II enzyme pool was applied to a Bio-Gel A-1.5 m column (200—400 mesh, Bio-Rad), 2.5 × 85 cm in size, equilibrated and eluted with 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C. Fractions of 5 ml were collected, and the fractions containing most of the enzyme activity were pooled and concentrated by ultrafiltration with a Diaflo PM-30 membrane (Amicon) to a final volume of 10 ml.

DEAE-cellulose chromatography II. The Bio-Gel enzyme pool was diluted with 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4° C, to final concentration 0.06 M NaCl. The sample was then immediately applied to a DEAE-cellulose (DE 52, Whatman) column, 2.5 × 10 cm in size. The column was equilibrated with 0.06 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C, and eluted first with 20 ml of the same buffer followed by 100 ml linear gradient from 0.06 to 0.18 M NaCl containing 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl buffer, pH 7.5, at 4°C. The gradient was followed by 50 ml of 0.18 M NaCl, 0.1 M glycine, 10 μM dithiothreitol and 20 mM Tris · HCl buffer, pH 7.5, at 4°C. 3-ml fractions were collected, and those with most of the enzymic activity were pooled.

Hydroxyapatite chromatography. The DEAE-cellulose II enzyme pool was applied to a hydroxyapatite column (Bio-Gel HTP, Bio-Rad), 0.9 × 5 cm in size, which was equilibrated with 0.01 M KH_2PO_4, 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol and 1% glycerol, pH 7.5. The column was eluted with the same buffer, except that the KH_2PO_4 concentration was increased step-wise as follows: 0.06 M, 4 ml; 0.09 M, 4 ml; 0.12 M, 4 ml; 0.15 M, 4 ml; 0.18 M, 4 ml; 0.21 M, 4 ml and 0.30 M, 10 ml. Fractions of 1 ml were collected. The fractions with best specific activities were pooled and ultrafiltrated with a Diaflo PM-30 membrane. The product constituted the purified enzyme.

Purification of lysyl hydroxylase from homogenates of chick embryo cartilage

Cartilage and cartilagenous bone from about 400 15-day-old chick embryos

was carefully minced in a solution of 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol and 20 mM Tris · HCl buffer adjusted to pH 7.5 at 4°C (4 ml solution per g of cartilage). The mixture was homogenized with an Ultra-Turrax homogenizer, three times for 5 s and the homogenate further blended in a Waring blender at full speed twice for 30 s. The homogenate was then centrifuged, and the supernatant fractionated with $(NH_4)_2SO_4$, as described above for whole chick embryos, except that a fraction with 17—45% $(NH_4)_2SO_4$ saturation was purified further. Calcium-phosphate gel fractionation and DEAE-cellulose chromatography were carried out as described above.

The DEAE-cellulose enzyme pool was concentrated by ultrafiltration in an Amicon ultrafiltration cell with a PM-30 membrane, and the enzyme was applied to a second DEAE-cellulose column (DE 52, Whatman). The column was equilibrated with a solution of 0.06 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, and 0.01 M Tris · HCl buffer, pH 7.5, at 4°C, and eluted first with 80 ml of the same solution followed by 400 ml linear gradient from 0.06 to 0.2 M NaCl. The fractions which contained most of the total enzyme activity were pooled and concentrated by ultrafiltration, and the concentrate was gel filtrated by a Bio-Gel A-1.5 m as described above. The fractions with most of the enzymic activity were again concentrated, and this concentrate constituted the purified enzyme.

Affinity chromatography of lysyl hydroxylase on concanavalin A-Sepharose column

In these experiments, 0.5—1 g of $(NH_4)_2SO_4$ I or calcium-phosphate gel enzyme was applied to a column of concanavalin A-Sepharose (Pharmacia Fine Chemicals), 5 ml in volume, which was equilibrated with 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol and 20 mM Tris · HCl adjusted to pH 7.5 at 4°C. The column was washed with 15—20 ml of the equilibrating buffer, and elution was carried out with the same solution with the addition of 0.5 M α-methyl-D-glucoside (Sigma Chemicals Co.), or 0.5 M α-methyl-D-mannoside (Sigma Chemicals Co.), or both 0.5 M α-methyl-D-glucoside and -mannoside, or 0.1 M of glycosides and 1.5 or 20 or 150 mM EDTA, or with a buffer of 0.1 M tetraborate, 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol and 1% glycerol, pH 7.5, at 4°C.

Assay procedures

Lysyl and prolyl hydroxylase activities were assayed with [^{14}C]lysine- or [^{14}C]proline-labelled protocollagen substrate [10,15,22], respectively, or by the method involving the determination of the $^{14}CO_2$ released from α-[^{14}C]-ketoglutarate during hydroxylation of lysyl residues in peptide L-I and prolyl residues in peptide (Pro-Pro-Gly)$_5$ · $4H_2O$ [7,9]. The incubations were carried out in a final volume of 1 ml when assaying lysyl hydroxylase, and in 2 ml for prolyl hydroxylase.

In attempts to purify lysyl hydroxylase by affinity chromatography on a Concanavalin A-Sepharose column, chelating agents were used in the elution of the enzyme from the column. An attempt was made to compensate for the chelation of iron by adding extra ferrous iron to the incubation mixture when assaying the enzyme. Also, in some experiments the fractions eluted were

dialyzed against the equilibrating buffer of the affinity column, and assay of the lysyl-hydroxylase activity was carried out after the dialysis.

When testing the effect of lysolecithin on the activities of lysyl and prolyl hydroxylases, the incubations were carried out as "in standard conditions" [10,15], except that albumin was omitted and lysolecithin was added to the incubation mixtures. In these experiments, the activities of lysyl and prolyl hydroxylases were assayed in chick embryo homogenate and in enzyme preparations with somewhat higher purity, as described in Results.

In experiments in which the effect of metals on the activity of lysyl and prolyl hydroxylases was tested, the additions to the incubation mixture were carried out as described in Results.

All ^{14}C counts were performed in a Wallac liquid scintillation spectrometer with an efficiency of 85% and a background of 25 cpm.

The protein content of the enzyme preparations was measured by peptide absorbance at 225 nm using serum albumin as a standard.

Disc electrophoresis of native lysyl hydroxylase was carried out using 5.3% polyacrylamide gels at 4°C with 42.5 mM Tris · HCl and 46.3 mM glycine buffer adjusted to pH 9.6 at 4°C as the upper buffer, and 120 mM Tris · HCl buffer adjusted to pH 8.8, at 4°C as the lower buffer. The gels were stained with Coomassie brilliant blue. Disc electrophoresis of the denatured enzyme polypeptide chains was carried out in the presence of sodium dodecyl sulphate as described by Weber and Osborn [23].

Results

Partial purification of lysyl hydroxylase from whole chick embryo homogenate

Seven separate steps were used to purify the enzyme in the final purification

TABLE I

PURIFICATION OF LYSYL HYDROXYLASE FROM EXTRACT OF WHOLE CHICK EMBRYOS

The starting material was the 15 000 × *g* supernatant of the homogenate of 2250 g wet weight of 14-day-old chick embryos. One unit of enzyme activity is defined as the amount of enzyme present in 1 mg of the $(NH_4)_2SO_4$ fraction (17—55% saturation) obtained from the 15 000 × *g* supernatant of the homogenate.

Enzyme fraction	Total protein (mg)	Total units of enzyme [b]	Recovery (%)	Specific activity	Purification
15 000 × *g* supernatant	111 500	55 700	100	0.5	1
$(NH_4)_2SO_4$ I	40 200	40 200	72	1.0	2
Calcium phosphate gel	4 090	14 300	26	3.5	7
DEAE-cellulose I	245	5 980	11	24	48
$(NH_4)_2SO_4$ II	50.6	3 410	6	67	134
Bio-Gel A-1.5 m	10.0	1 580	2.8	158	316
DEAE-cellulose II	2.9	905	1.6	312	624
Hydroxyapatite pool I + II [a]	0.48	350	0.6	729	1460
Hydroxyapatite pool II [a]	0.18	220	0.4	1220	2440

[a] The hydroxyapatite enzyme was collected in two separate pools, termed I and II, the former containing the enzyme eluted with 0.12 M phosphate buffer, and the latter containing that eluted with 0.18 and 0.21 M phosphate buffer.

[b] One unit of enzyme activity is defined as amount of enzyme present in 1 mg of the $(NH_4)_2SO_4$ fraction (17—55% saturation) obtained from the 15 000 mg supernatant of the chick embryo homogenate.

procedure (Table I). A 2440-fold purification was obtained with the best enzyme preparation, and the recovery of the enzyme activity was about 0.4%.

In gel filtration on Bio-Gel A-1.5 m column, the enzyme was eluted in two separate peaks. The first was broad and had an elution position corresponding to a molecular weight of 490 000—670 000 compared with globular protein standards. The elution position of the second peak corresponded to a molecular weight of 220 000. The distribution of the major part of the enzyme activity between the two peaks varied greatly in different enzyme preparations, and some enzyme was always eluted between the two peaks.

In hydroxyapatite chromatography the enzyme was recovered in two peaks, the first with 0.12 M phosphate buffer and the second with 0.18 M phosphate buffer (Fig. 1). In this purification step, too, the distribution of the major part of the enzyme activity between the two peaks varied in different enzyme preparations, and some enzyme always eluted between the peaks. Also, in almost all the enzyme preparations, there was some elution of enzyme activity with 0.21 M phosphate buffer.

When purifying one enzyme preparation, the DEAE-cellulose II chromatography was carried out before the gel filtration, and the two peaks of enzyme activity recovered in gel filtration on a Bio-Gel A-1.5 m column were pooled separately. Both pools were then chromatographed separately on the hydroxyapatite column. With the enzyme pool having the higher molecular weight, the major part of the enzyme activity was recovered with 0.12 M phosphate buffer, and with the pool having the lower molecular weight, most of enzyme was recovered by elution with 0.18 M phosphate buffer. However, in both cases, the presence of the other activity peak was also observed even though it was considerably smaller than the major peak. Most of the total enzyme activity in

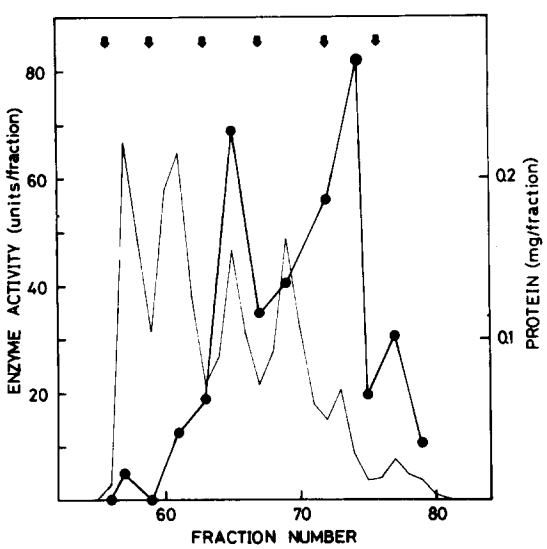

Fig. 1. Elution of lysyl hydroxylase activity from the hydroxyapatite column. Conditions as described in Materials and Methods. Fractions Nos. 64—68 (enzyme pool I) and 72—78 (enzyme pool II) were pooled and constituted the purified enzyme preparations shown in Table I. The arrows indicate changes to buffers with the following concentrations: 0.06, 0.09, 0.12, 0.15, 0.18 and 0.21 M. Enzyme activity (●); protein (———).

this preparation was recovered in the form having the lower molecular weight and the property of being eluted with 0.18 M phosphate. The highest specific activity in a single fraction of the eluate obtained in this experiment with 0.18 M buffer corresponded to approx. a 4000-fold purification.

Polyacrylamide gel electrophoresis of the native enzyme or enzyme dissociated by sodium dodecyl sulphate was carried out with the fraction having the highest specific activity. In the electrophoresis of native lysyl hydroxylase, the protein did not enter to the separating gel, but was precipitated as a single band just below the stacking gel. In experiments with less pure enzyme preparations (0.12 M phosphate peak of larger form of the enzyme) an additional band was seen in the separating gel. In sodium dodecyl sulphate disc electrophoresis three major bands and one minor band were seen with the purest enzyme fraction (Fig. 2A). The positions of the three major bands corresponded to molecular weights of 74 000, 53 000 and 45 000, and that of the minor band to a molecular weight of 96 000. The sodium dodecyl sulphate disc electrophoresis was

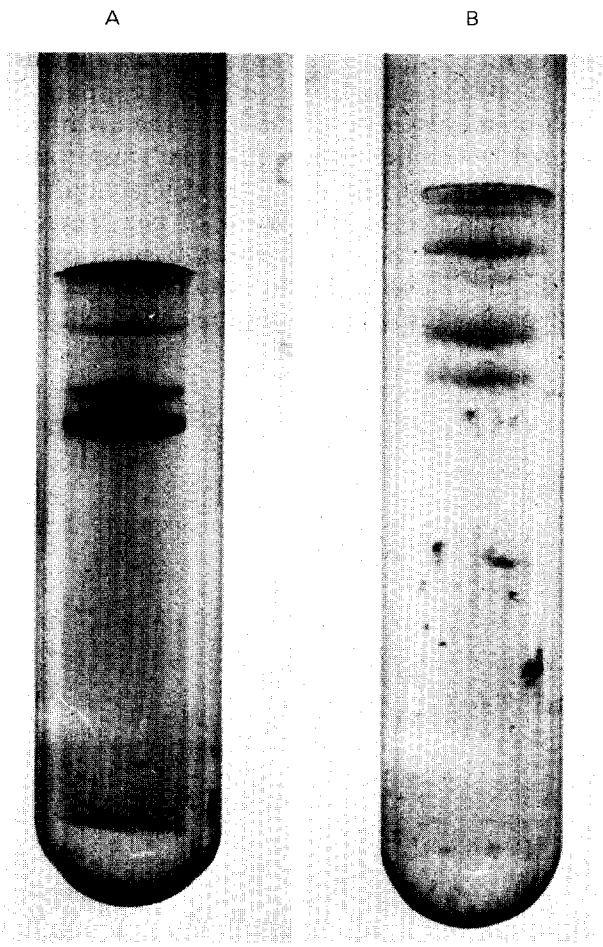

Fig. 2. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis from partially purified lysyl hydroxylase preparations. (A) The purest enzyme preparation, with purification of about 4000-fold (see text). (B) Hydroxyapatite pool II (see Table I). Conditions as described in Materials and Methods.

also carried out with fractions of the 0.12 M phosphate eluate in the chromatography of the smaller form, and 0.12 and 0.18 M phosphate eluate in the chromatography of the larger form in the experiment reported in the preceding paragraph. The bands with molecular weights of 96 000, 53 000 and 45 000 were present in all fractions, whereas that with a molecular weight of 74 000 was not found consistently. The three bands mentioned above and two additional bands were also present in the hydroxyapatite enzyme poole II of a different enzyme preparation (Fig. 2B), shown in Table I. Unfortunately, there was no way of demonstrating whether only one of these bands was derived from the enzyme protein or whether two or three were so derived.

Lysyl hydroxylase has previously been shown to hydroxylate lysyl residues in arginine-rich histone [19], which does not contain any -X-Lys-Gly- sequences but does contain some -X-Lys-Z- sequences. To study whether a separate enzyme is involved in the hydroxylation of the lysyl residue in the triplet not containing a glycyl residue, and whether only one of the two forms of lysyl hydroxylase observed here is able to hydroxylate a lysyl residue in such a triplet, arginine-rich histone was studied as a substrate to both forms of the enzyme. In this experiment hydroxylation of lysyl residues in both arginine-rich histone, and peptide L-I was observed with both forms of the enzyme, and the ratio of the hydroxylation of arginine-rich to that peptide L-I proved to be about the same with both forms of the enzyme.

Attempts to purify lysyl hydroxylase by affinity chromatography on concanavalin A-Sepharose and other columns

Enzyme preparations purified through either $(NH_4)_2SO_4$ I step or through the calcium phosphate gel step were applied to a concanavalin A-Sepharose column as described in Materials and Methods, and the column washed with the equilibrating buffer. About 75—80% of the protein applied to the column was recovered in the effluent, whereas only about 20% of the lysyl hydroxylase activity was recovered. The relative specific activity of the enzyme in the effluent was about 0.2 compared with that of the preparation applied to the column (Table II). The protein concentration in the fractions of the column effluent increased to the same value as in the original enzyme preparation after about 1000 g of protein had passed through the 5 ml column. During chromatography on plain agarose, about 90% of the activity of lysyl hydroxylase was recovered. Thus, the low recovery of enzyme activity in the effluent is obviously due to the binding of the enzyme to the column made of agarose-bound concanavalin A rather than inactivation during chromatography on this column.

The elution of the concanavalin A-Sepharose column was first carried out with a buffer containing a 0.5 M concentration of both α-methyl-D-glucoside and α-methyl-D-mannoside. About 20% of the protein bound to the column could be eluted with these glycosides, but only about 3% of the bound enzyme activity was recovered. An additional 5% of the bound protein and 4% of the bound enzyme activity were eluted with a buffer containing 1.5 mM EDTA and 0.1 M glycosides. The total enzyme activity that could be eluted with both of these buffers was thus only about 7% of that bound to the column. The relative specific activity in the best fraction in this experiment was 4.2 (Table II).

Elution of the concanavalin A-Sepharose column was also tested with several

TABLE II

AFFINITY CHROMATOGRAPHY OF LYSYL HYDROXYLASE ON A CONCANAVALIN A-SEPHAROSE COLUMN

An $(NH_4)_2SO_4$ I enzyme preparation was applied to a concanavalin A-Sepharose column, 5 ml in size, as described in Materials and Methods, and the column eluted first with 0.5 M α-methyl-D-glucoside and 0.5 M α-methyl-D-mannoside in 0.05 M NaCl, 0.1 M glycine, 10 μM dithiothreitol and Tris · HCl buffer, pH 7.5, at 4°C, and then with the same buffer except that the glycoside concentrations were 0.1 M and EDTA was present in a concentration of 1.5 mM. The flow rate was about 30—40 ml/h.

	Total protein		Enzyme units		Specific activity	Specific activity in the best fraction
	mg	%	Units	%		
Enzyme preparation applied to column	1110	100	1110	100	1.0	
Effluent from sample and washing	860	77	210	19	0.2	
Elution with glycosides	51	5	26	0.02	0.5	1.3
Elution with EDTA plus glycosides	13	1	35	0.03	2.7	4.2

different modifications. When elution was carried out with 0.5 M α-methyl-D-glucoside or with α-methyl-D-mannoside alone, both glycosides recovered some enzyme activity, with the relative specific activities in the best fractions being about twice that in the enzyme preparation applied to the column. The recovery of the enzyme activity was very poor. The EDTA concentration in the buffer was also increased from 1.5 to 20 mM and to 150 mM, but the results were no better than in the elution with buffer containing 1.5 mM EDTA. In fact that recovered in elution with 150 mM EDTA was scarcely detectable. In addition, attempts were carried out to elute the concanavalin A-Sepharose column with a solution containing 0.1 M sodium tetraborate, 0.2 M NaCl, 0.2 M glycine, 10 μM dithiothreitol and 1% glycerol, pH 7.5, at 4°C, but this elution was found to inactivate the enzyme. Attempts to restore enzyme activity, as described in Materials and Methods, also failed.

Affinity chromatography based on the affinity of the enzyme for its substrate was also tried. Gelatin or collagen was bound to agarose with CNBr activation (ref. 25), or lysine-rich histone (ref. 19) to AH-Sepharose 4B carbodiimide activation. Lysyl hydroxylase had no affinity for gelatin or collagen coupled to agarose under the conditions used (0.05—0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 20 mM Tris · HCl buffer, pH 7.5, at 4°C), and the lysine-rich histone coupled to the AH-Sepharose 4B, behaved as an ion exchanger rather than as an affinity adsorbent. The latter observation is probably explained by the basicity of lysine-rich histone.

Purification of lysyl hydroxylase from chick-embryo cartilage

Experiments to purify lysyl hydroxylase from homogenate of chick embryo cartilage were carried out because the enzyme activity had been shown to be higher in this tissue than in any other tissue except tendons at the 15th day of embryo development [24]. In addition, the question of whether the enzyme from cartilagenous bones was different from that obtained from chick embryos was studied. A final purification of about 200—300-fold compared with that in

TABLE III

PURIFICATION OF LYSYL HYDROXYLASE FROM EXTRACT OF CARTILAGENOUS BONES OF CHICK EMBRYOS

The starting material was the 15 000 × *g* supernatant of the homogenate of 400 g wet weight of cartilagenous bones of 15-day-old chick embryos. The specific activities are expressed with $(NH_4)_2SO_4$ Fraction I from the whole chick embryos (Table I) as reference, corresponding to a specific activity of 1.00. Purification is calculated with the 15 000 × *g* supernatant from whole chick embryos as reference.

Enzyme fraction	Total protein (mg)	Total units of enzyme	Recovery (%)	Specific activity	Purification
15 000 × *g* supernatant	18 160	43 580	100	2.4	4
$(NH_4)_2SO_4$	9 950	39 800	90	4.0	8
Calcium phosphate gel	1 320	13 700	30	10.4	16
DEAE-cellulose I	134.5	5 600	13	41.6	68
DEAE-cellulose II	45.0	3 850	9	85.6	144
Bio-Gel A-1.5 m	11.1	1 500	3	135.2	224 *

* Purification in the best fraction was about 300-fold.

the 15 000 × *g* supernatant of the homogenate from whole chick embryos was achieved with a 3% recovery of the enzyme activity (Table III).

In gel filtration on Bio-Gel A-1.5 m, two peaks of enzyme activity were recovered. As with the enzyme from whole embryos, the larger form dominated in some preparations, whereas in others most of the activity was recovered in the smaller form. The molecular weights of the two forms were the same as observed with the enzyme from whole chick embryos.

In addition, enzyme purified from chick embryo cartilage was tested with arginine-rich histone as substrate, and hydroxylation of lysine residues was found to take place in this substrate.

TABLE IV

EFFECT OF TRITON X-100 AND LYSOLECITHIN ON LYSYL HYDROXYLASE ACTIVITY IN THE CHICK EMBRYO HOMOGENATE AND HOMOGENATE FRACTIONS

One 10- or 11-day-old chick embryo was homogenized in 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, and 0.02 M Tris · HCl buffer adjusted to pH 7.5 at 4°C. 15 ml of solution per g of embryo was used, and the homogenate was diluted 1 : 3 with 50 mM Tris · HCl buffer adjusted to pH 7.8 at 25°C before addition to the incubation system. Part of the homogenate was treated with Triton X-100 (in 0.1% concentration) and another part with lysolecithin (in 0.9 mM concentration) for 1 h in 4°C before incubation.

	Homogenate	15 000 × *g* supernatant	150 000 × *g* supernatant
(1) No pretreatment			
dpm	490	430	360
Percent of value in homogenate	100	88	73
(2) Pretreatment with Triton X-100			
dpm	1210	1100	880
Percent of value in homogenate	100	91	73
Ratio (2) : (1) × 100	246	256	244
(3) Pre-treatment with lysolecithin			
dpm	1550	852	950
Percent of value in homogenate	100	55	61
Ratio (3) : (1) × 100	316	198	264

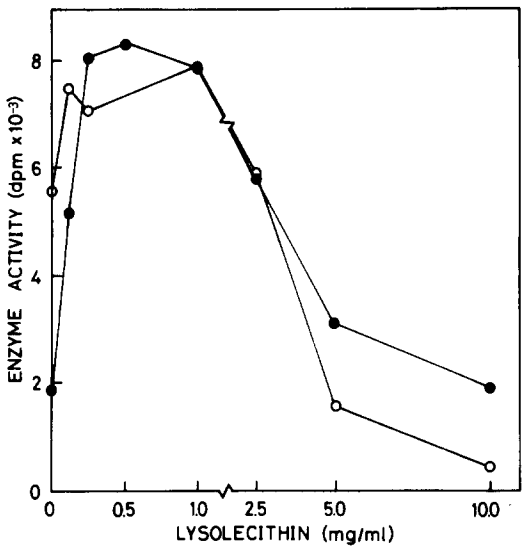

Fig. 3. Effect of lysolecithin on lysyl hydroxylase activity in vitro. The activity of lysyl hydroxylase was assayed in chick embryo homogenate, prepared as described in Table IV with [^{14}C]lysine-labelled protocollagen as substrate with lysolecithin concentrations of 0.00, 0.125, 0.25, 0.5, 1.0, 2.5, 5.0 and 10 mg/ml in the incubation mixture. The assay was carried out with (○), or without (●) pre-treatment with 0.1% Triton X-100, and albumin was omitted from the incubation mixture, as described in Materials and Methods.

Effect of Triton X-100 on lysyl-hydroxylase activity in chick-embryo homogenate

To study the effect of Triton X-100 on the extraction of lysyl hydroxylase, one 10- or 11-day-old chick embryo was homogenized in 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol and 20 mM Tris · HCl buffer, pH 7.5, as described in Table IV, and an aliquot of the homogenate treated with Triton X-100. The homogenates with and without Triton X-100 treatment were centrifuged first at 15 000 × *g* and then at 150 000 × *g*. The assay of lysyl hydroxylase with ^{14}C-labelled protocollagen was carried out in the homogenate, 15 000 × *g* supernatant and 150 000 × *g* supernatant (Table IV).

Treatment of the chick embryo homogenate with Triton X-100 increased the enzyme activity in the homogenate and supernatants by about 200%, but no change was observed in the distribution of the enzyme activity between the soluble fractions. Addition of Triton X-100 into the incubation mixture also increased enzyme activity by about 200% when assaying the untreated homogenate, but the same addition did not have any effect when assaying homogenate pretreated with Triton X-100. With 15 000 × *g* and 150 000 × *g* supernatants the addition of Triton X-100 to the incubation did not have any significant effect.

Lysyl hydroxylase activity has been previously reported to increase by 130—140% in the 15 000 × *g* supernatant after treatment with Triton X-100 [24]. The difference between these earlier results and the present ones is probably explained by the more gentle homogenization used here.

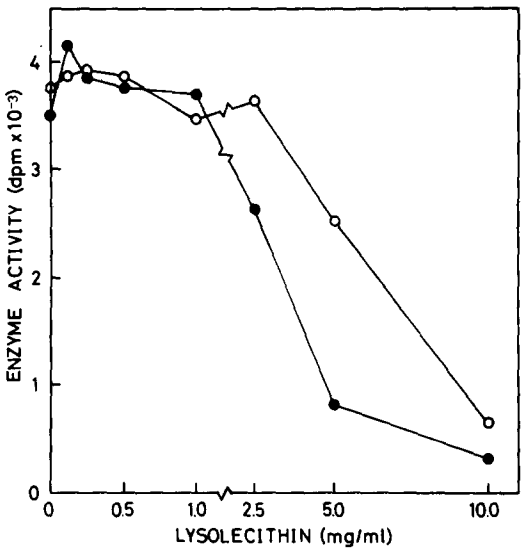

Fig. 4. Effect of lysolecithin on prolyl-hydroxylase activity in vitro. The activity of prolyl hydroxylase was assayed in chick-embryo homogenate with [^{14}C] proline-labelled protocollagen as substrate. The conditions were the same as in Fig. 4, except that 5 ml homogenating buffer per g of embryo were used and no dilution was carried out before incubation. ○, assay with pre-treatment of 0.1% Triton X-100; ●, assay without pretreatment of Triton X-100.

Effect of lysolecithin on lysyl and prolyl hydroxylase activities

The addition of lysolecithin was found to increase lysyl hydroxylase activity in chick embryo homogenate (Fig. 3). A maximal increase in the enzyme activity of about 4.8-fold was observed with a lysolecithin concentration of 0.5 mg/ml, which corresponds to about 0.9 mM lysolecithin, with a molecular weight of 536 (calculated as oleolyl lysolecithin). There was a decline in the activity with higher concentrations of lysolecithin, and with about 20 mM lysolecithin it reached the control value (Fig. 3).

Pretreatment of the chick embryo homogenate with Triton X-100 also resulted in an increase in lysyl hydroxylase activity, this being about 3-fold (Fig. 3). After this pretreatment, lysolecithin still increased lysyl hydroxylase activity slightly, and the maximal activity was about 1.3 times that in the Triton X-100-treated control. With higher lysolecithin concentrations a decline in activity was again observed (Fig. 3).

Prolyl hydroxylase could be activated by lysolecithin in the homogenate in the same manner as lysyl hydroxylase, though lysolecithin increased its activity by only about 1.2-fold with a 0.23-mM concentration (Fig. 4). Also preincubation with Triton X-100 had less effect on the activity of prolyl hydroxylase than on that of lysyl hydroxylase, this treatment giving only slight or no increase in the prolyl hydroxylase activity. No additional increase in prolyl hydroxylase activity was found with lysolecithin after Triton X-100 treatment. With higher lysolecithin concentrations a clear inhibitory effect on the enzyme activity was seen (Fig. 4).

The effect of lysolecithin on lysyl hydroxylase activity was also studied with purer enzyme preparations. No activatory effect on calcium phosphate gel en-

zyme or DEAE-cellulose I enzyme was found with 2 mM lysolecithin in the incubation mixture, although an activation of about 4.3-fold was found wich chick embryo homogenate with the same lysolecithin concentration. With a concentration of 4.6 mM or greater, inhibition of lysyl hydroxylase activity was found in these enzyme preparations.

In order to study whether the effect of lysolecithin on the activity of lysyl and prolyl hydroxylases is a true activatory effect or whether it is due to solubilization of the enzyme by detergency, an experiment was carried out in which chick embryo was homogenized and the homogenate then centrifuged at 15 000 × *g* and at 150 000 × *g*. Part of the homogenate was preincubated at 4°C with 0.9 mM lysolecithin before the centrifugations. The assay of lysyl-hydroxylase activity was carried out in the homogenate, 15 000 × *g* supernatant, and 150 000 × *g* supernatant. The results suggest that the effect of the lysolecithin was not a true activatory one (Table IV), since pretreatment with lysolecithin seemed to increase the soluble enzyme found in the 15 000 × *g* and 150 000 × *g* supernatants. Addition of extra lysolecithin (0.9 mM concentration) directly to the incubation mixture did not increase lysyl-hydroxylase activity in lysolecithin pretreated homogenate, 15 000 × *g* supernatant or 150 000 × *g* supernatant.

Effect of albumin-bound lipids on the activity of lysyl and prolyl hydroxylase

Albumin has been found to stimulate the activity of lysyl and prolyl hydroxylase [12,15,16]. In order to examine the possible role of albumin-bound lipids in the stimulatory effect of albumin on these enzyme activities, experiments were carried out with pure prolyl hydroxylase purified on a poly(L-proline)-agarose column [14]. When defatted albumin, prepared as described in Materials and Methods, was added to the incubation mixture (concentration 2 mg albumin/ml of incubation mixture), inhibition of enzyme activity was observed, though the enzyme could be entirely reactivated by adding extra Fe^{2+} to the incubating mixture. These results indicate that the stimulatory effect of albumin on the activity of prolyl hydroxylase in vitro is not due to the albumin-bound fatty acids, and that the iron-binding capacity of albumin increases markedly once the fatty acids are removed from it. Results consistent with those above were obtained in experiments with lysyl hydroxylase.

Inhibitory effect of certain cations on the activity of lysyl and prolyl hydroxylases

Several divalent cations were found to have an inhibitory effect on lysyl hydroxylase activity (Table V). The most potent inhibitors were Zn^{2+}, Cd^{2+} and Co^{2+} (Table V), and similar results were obtained when prolyl hydroxylase was assayed in the presence of these divalent cations.

More detailed studies of the inhibitory effect of divalent cations on the activity of lysyl hydroxylase were carried out with Ca^{2+}, Cu^{2+} and Zn^{2+}. These experiments were carried out by a method involving the measurement of the radioactive $^{14}CO_2$ released during the hydroxylation reaction. Albumin was omitted from the incubation mixture.* An inhibition of the hydroxylation of lysyl residues amounting to about 50% was achieved with concentrations of about 15 mM Ca^{2+}, 60 μM Cu^{2+} or 3 μM Zn^{2+} (Fig. 5). To eliminate the possible

TABLE V

EFFECT OF METALS ON THE ACTIVITY OF LYSYL HYDROXYLASE IN VITRO

The effect of metals on the activity of lysyl hydroxylase was tested by assaying lysyl hydroxylase with [^{14}C]lysine-labelled protocollagen as substrate. Albumin was omitted from the incubation mixture, and cations were added to the incubation system in the form of chlorides except for $CdSO_4$. Calcium phosphate gel enzyme (about 8-fold purification) was used in these experiments.

Cation	Concentration (mM)	dpm	Percent of control
Control		3000	100
Mg^{2+}	25	780	25
	2.5	2880	95
Mn^{2+}	25	1020	35
	2.5	2900	95
Ni^{2+}	0.03	1500	50
	0.003	2760	90
Co^{2+}	0.03	1200	40
	0.003	3060	100
Zn^{2+}	0.03	60	2
	0.003	1320	45
Cd^{2+}	0.03	1260	40
	0.003	2940	100
Na^{+}	25	2460	80
	2.5	3000	100
K^{+}	25	2340	80
	2.5	3120	105

effect of divalent cations bound to other proteins present in the enzyme preparations used in these experiments, the enzyme preparation was dialyzed against 10 mM EDTA, and the enzyme activity assayed in the presence of different concentrations of Ca^{2+}. Partial inactivation of lysyl hydroxylase during EDTA treatment was observed, but the concentration of Ca^{2+} required for 50% inhibition of the enzymic activity remained essentially the same as without the EDTA treatment (Fig. 5).

The K_i of Cu^{2+} for lysyl hydroxylase was determined by the method of Dixon and Webb [26], and was found to be about 30 μM. From the same plot it can be deduced that the inhibition is a competitive one, Cu^{2+} competing with Fe^{2+} for binding to the enzyme molecules (Fig. 6).

Discussion

An up to 4000-fold purification of lysyl hydroxylase from extract of whole chick embryos and a lower degree of purification from extract of embryo cartilagenous bones were obtained in the present study by conventional enzyme purification methods. The procedures are partly based on a previous one [15] with several modifications, but the degree of purification was more than 10-fold compared with that reported previously [15]. Completely pure enzyme could not be obtained, however, and recovery of the enzyme activity was poor. The difficulty experienced in purifying lysyl hydroxylase can be explained

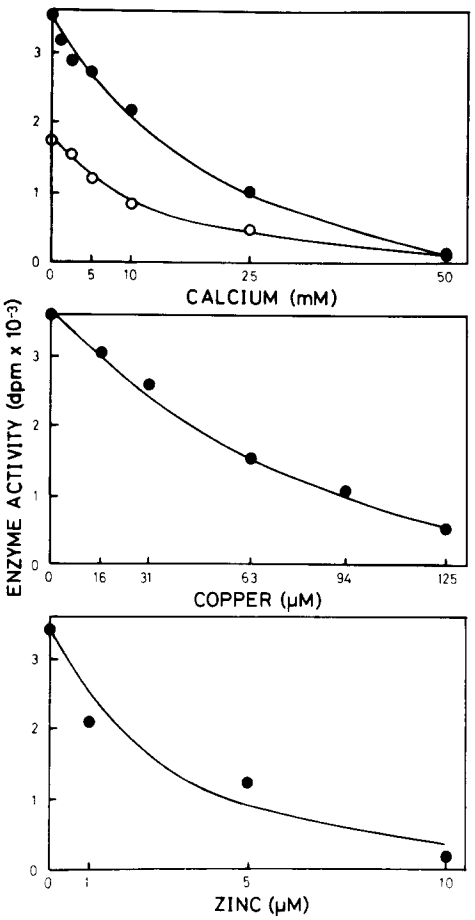

Fig. 5. Effect of divalent cations on the hydroxylation of lysyl residues in vitro. Lysyl-hydroxylase activity was assayed in the presence of different Ca^{2+}, Cu^{2+} and Zu^{2+} concentrations by a method involving measurement of $^{14}CO_2$, as described in Materials and Methods, except that albumin was omitted from the incubation mixture. Upper panel: effect of calcium on the activity of lysyl hydroxylase (●), and EDTA-treated lysyl hydroxylase (○). Middle panel: effect of copper on the activity of lysyl hydroxylase. Lower panel: effect of zinc on the activity of lysyl hydroxylase.

partly by the lability of the enzyme and partly by its existence in multiple forms.

The latter could be demonstrated in gel filtration and in hydroxyapatite chromatography. The larger form of the enzyme recovered during gel filtration showed a very broad peak, whereas the smaller form had a sharper elution profile. It is not known whether the larger form represented aggregates of the smaller form, or whether it was due to membrane particles still bound to the enzyme molecule, or whether two forms are separate enzymes with entirely different physical properties. However, the broadness of the peak of the larger form is suggestive of aggregation or the presence of membrane particles. In addition, a tendency was observed during the purification procedure for the enzyme to aggregate. The same phenomenon was observed when purifying lysyl hydroxylase from cartilagenous bones of chick embryo.

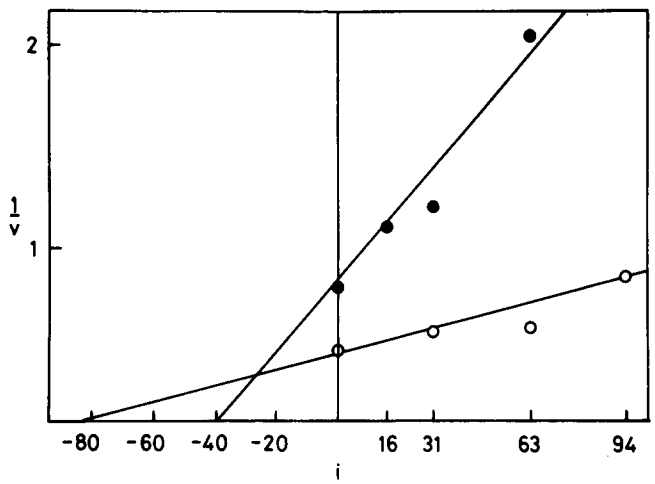

Fig. 6. Determination of K_i of Cu^{2+} for lysyl hydroxylase. Calcium phosphate gel enzyme was assayed in the presence of different concentrations of Cu^{2+} with [^{14}C]lysine-labelled protocollagen as substrate. The incubation was carried out as described in Materials and Methods, except that albumin was omitted from the incubation mixture. The assay was carried out with two different iron concentrations, 1 μM (•) and 5 μM (○). Abscissa: concentration of the inhibitor in μM. Ordinate: reciprocal values of the initial velocity in 10^{-3} × dpm.

Lysyl hydroxylase was also eluted in two separate peaks in hydroxyapatite chromatography. The peak eluting with 0.12 M phosphate buffer was found to correspond to the larger form, and that eluting with 0.18 M phosphate buffer to the smaller form, but the presence of both forms was always observed in the chromatograms of single forms of the enzyme. This can be explained either by contamination with or by transition of the enzyme from one form to the other. In addition, some enzyme activity was always recovered between the two main peaks both in gel filtration and in hydroxyapatite chromatography. This may represent either transition between the two main forms of the enzyme, or still another independent form of lysyl hydroxylase.

It has been suggested that there may be two lysyl hydroxylases [19,27], one of them hydroxylating lysyl residues in the -X-Lys-Gly- sequences, and the other hydroxylating lysyl residues in the -X-Lys-Ser- sequences, the latter being located mainly in bone cells. Both forms of the enzyme were therefore studied with arginine-rich histone as substrate [19], as this substrate contains no -X-Lys-Gly- sequences. However, no differences were found between the two forms in their ability to hydroxylate this substrate, nor was any difference found between the enzyme from whole chick embryos and that from cartilagenous bones in this respect.

Attempts to purify lysyl hydroxylase by methods based on the affinity of the enzyme for the substrate were not successful. Lysyl hydroxylase was found to have affinity for a concanavalin A-Sepharose column, indicating some residue or residues of carbohydrates present in the molecule [28].

There is agreement at present about the location of lysine and proline hydroxylation in the cisternae of the rough endoplasmic reticulum, but some controversy exists about whether the enzymes are bound to membranes [6]. Triton

X-100 has been shown to solubilize lysyl and prolyl hydroxylases from tissues [24,29,30], and the present results support to this finding. Such results suggest binding of the hydroxylases to the membranes, but the solubilizing effect could also be explained by the detergent rupturing the cisternae of the rough endoplasmic reticulum, thus liberating lysyl and prolyl hydroxylases from inside the cisternae. In the present study, Triton X-100 caused a greater increase in lysyl hydroxylase activity than in prolyl-hydroxylase activity in chick-embryo homogenate, this quantitative difference suggesting that at least lysyl hydroxylase is membrane bound, or if both enzymes are membrane-bound, lysyl hydroxylase seems to be more tightly so.

The stimulatory effect of lysolecithin and some other lipids on certain hydroxylases and glycosyltransferases has been reported earlier [31—33]. Some lysolecithin is always present in the tissues [34], and an increase of 5% in the lysolecithin content of phospholipids has been found in the intima and media of aterosclerotic aortas [35,36]. Consequently, lysolecithin might have a physiological role in regulating the activity of certain hydroxylases and glycosyltransferases. Lysolecithin has been presumed to cause a conformational change in the molecule of some enzymes leading to enzyme activation [31,32]. In the present study, activation of lysyl hydroxylase in the chick-embryo homogenate was observed with lysolecithin. Preincubation of the homogenate with lysolecithin released more lysyl hydroxylase into the 15 000 × *g* and 150 000 × *g* supernatants, thus suggesting a detergent-like solubilizing effect rather than a true activatory one. In addition, the effect of lysolecithin was very similar to that of Triton X-100, and again its effect on lysyl-hydroxylase activity was much more pronounced than on prolyl-hydroxylase activity. A detergent-like effect on the membranes was further suggested by the finding that no increase in enzyme activity occurred when the 15 000 × *g* or 150 000 × *g* supernatant of the homogenate, or partially purified enzyme preparations were assayed in the presence of lysolecithin in the incubation mixture.

Hormones affecting mineral metabolism and vitamin D are known to affect collagen metabolism (see ref. 37) and an increased hydroxylation of lysyl residues in bone collagen has been found in vitamin-D deficiency [38—40]. The present results are indicative of the inhibitory effect of several divalent cations on lysyl and prolyl hydroxylase activity. Thus it seems possible that the changes in collagen metabolism caused by the agents mentioned above are at least partly mediated by changes in the levels of divalent cations, principally calcium. It has also been found that calcium and some other divalent cations inhibit these hydroxylations in isolated chick-embryo leg-tendon fibroblasts (Oikarinen, A. and Ryhänen, L., unpublished results). However, it is not known whether the concentration of divalent cations in the cell, and especially in the cisternae of the endoplasmic reticulum, is high enough to cause inhibition, though it is known that the mitochondria and endoplasmic reticulum of cells have a powerful calcium-concentrating property [41—44]. Thus it does not seem impossible for at least the sum of calcium and other divalent cations to reach a concentration high enough to affect lysyl and prolyl hydroxylase activities in the cisternae of fibroblasts. The mode of inhibition by divalent cations, tested with Cu^{2+}, was found to be competitive, the inhibiting ions competing with Fe^{2+} for the binding site in the enzyme molecule.

Acknowledgements

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