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THE SIZE OF THE POLYPEPTIDE PRECURSOR OF COLLAGEN HYDROXYPROLINE*

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Studies with both cell-free¹ and intact cell systems² from chick embryos indicated that the hydroxyproline present in collagen is synthesized by hydroxylation of proline residues already incorporated into polypeptide linkage. This finding raised the question of the nature of this polypeptide material. In this paper we report studies on (1) the size of this substrate, (2) the possibility that it terminates in an sRNA molecule, and (3) some of its properties, in particular its stability.

Materials.—Uniformly labeled C¹⁴-proline, approximately 186 $\mu\text{c}/\mu\text{mole}$, was obtained from New England Nuclear Corporation in 0.01 *N* HCl and was added as such to incubations. Puromycin dihydrochloride was purchased from Sigma Chemical Co., and α,α' -dipyridyl from the Matheson Co., as was the prepurified nitrogen used in anaerobic experiments. The crystalline RNase and the purified collagenase (no. CLSP-A) were products of the Worthington Biochemical Corp. White Leghorn 7-day embryonated eggs were purchased from Hall Bros. Hatchery, Wallingford, Conn. Ichthyocol was generously provided by Drs. Blumenfeld and Gallop. The conditions of gelatinization of ichthyocol and of treatment with NH₂OH (for 2.5 hr) were as described by Blumenfeld, Rojkind, and Gallop.³ The fragments obtained after this treatment are referred to in the present paper as subunits, in accordance with the usage of these authors.

Methods.—*Preparation of acetic acid extracts of minces of chick embryos:* The embryos were minced in Krebs-Ringer phosphate,⁴ in ratios of embryos to medium indicated below, with 5–6 complete strokes of a loosely fitting Potter-Elvehjem homogenizer, turning at low speed. After addition of C¹⁴-proline and other materials where indicated, the minces were incubated at 37° with shaking. At the end of the incubation, the entire incubation was chilled to near 0° and centrifuged at 3° for 2 min at about 10,000 $\times g$ in a Servall SS-1 centrifuge. The sediment was dispersed in ice-cold 0.5 *N* acetic acid, 6 ml per 10 ml of the original incubation mixture, with a Dounce homogenizer and was shaken for 1 hr at 3° on a Burrell wrist-action shaker, prior to centrifugation at about 15,000 $\times g$ for 5 min. The sediment was discarded and the supernatant was dialyzed overnight against at least two batches of distilled H₂O.

Gel filtration on Sephadex G-200: One column of Sephadex G-200, bead form (3 \times 74 cm), was used without repacking for all experiments. The dialyzed acetic acid extracts were concentrated before gel filtration either by means of a flash evaporator or by treatment with dry Sephadex G-200. The sample in 0.25 *N* acetic acid was applied in a final volume of 4.7 ml. The column was developed at room temperature with 0.25 *N* acetic acid, and 5-ml fractions were collected. The extracts from minces contained material absorbing at 280 $m\mu$, and the fraction containing the maximum concentration of this material (32–35 in different runs) was used as an internal marker to bring all elution profiles into register with that of the ichthyocol subunits shown in Figure 1.

Disc electrophoresis: The conditions of Nagai *et al.*⁵ were used.

Conditions of the incubation to prepare the substrate for the hydroxylation reaction: The incubation mixtures contained, per ml, 0.36 embryos (7-day, 1.1 gm wet weight), 0.6 ml of Krebs-Ringer phosphate, 0.7 μc of C¹⁴-proline (186 $\mu\text{c}/\mu\text{mole}$), and 1 μmole of α,α' -dipyridyl. After incubation

at 37° for 1 hr, the radioactive substrate was extracted with 0.5 *N* acetic acid as described above, and after dialysis, could be stored in the frozen state.

Conditions of the enzymic hydroxylation reaction: The composition of this incubation mixture (usually 4.8 ml) was patterned directly after that of Prockop and Juva⁶ except for the addition of ascorbic acid (not proved necessary), and contained, unless otherwise noted, per ml, the amount of acetic acid-extracted substrate derived from 0.42 ml of the substrate-synthesizing incubation and 0.4 ml of the extract prepared by blending 8-day embryos (1.5 gm wet weight) in one ninth their weight of 2.5 *M* sucrose for 10 sec in a Servall Omni-Mixer and centrifuging the homogenate for 1 hr at about 28,000 rpm in the 30-head of the Spinco model L centrifuge. The other components were, per ml, 60 μ moles of Tris·HCl, pH 7.6, 5 μ moles of MgCl₂, 25 μ moles of KCl, 0.6 μ mole of ascorbic acid, 0.06 μ mole of FeSO₄, and 0.012 μ mole of EDTA. The vessels were incubated for 1 hr at 37°. In cases where the size of the hydroxylated substrate was to be examined by gel filtration (see Fig. 2), glacial acetic acid was added to the chilled incubation mixture to give a final concentration of 0.5 *N* acetic acid, and the subsequent preparation of the acetic acid extract followed the procedure given above. For the determination of the extent of hydroxylation the samples were prepared in the manner described previously.²

Measurement of the total radioactivity in hydroxyproline: The method consisted of multiplying the total μ moles of hydroxyproline originally present in a sample, or added as carrier, by the specific activity of the hydroxyproline. The latter was determined after acid hydrolysis followed by fractionation on columns of Dowex 50-H⁺, with, where indicated, further purification by paper chromatography, as described previously.² The concentration of hydroxyproline was determined colorimetrically by procedure A of Bergman and Loxley,⁷ scaled down to one fifth the published volumes, and with the final dilution step omitted. When the concentration of protein-bound hydroxyproline was determined, the samples were hydrolyzed² and taken to dryness in a vacuum desiccator, prior to the colorimetric assay.

Assay for the substrate for the hydroxylation reaction: The quantity of substrate in a sample was determined by analysis of the total radioactivity in hydroxyproline before and after incubation under the conditions of the enzymic hydroxylation reaction. The samples were added to identical reaction mixtures except that in the former case the embryo extract was added after the addition of the trichloroacetic acid. The increase in radioactivity in hydroxyproline as a result of the enzymic hydroxylation reaction was found to be directly proportional to the amount of substrate added.

Results.—The size of the material containing newly synthesized C¹⁴-hydroxyproline: Preliminary experiments showed (1) that 0.5 *N* acetic acid at 3° extracted up to 70 per cent of the total radioactive hydroxyproline newly synthesized by chick embryo minces, and (2) that the usual conditions of extraction with hot trichloroacetic acid² caused the breakdown of essentially all of the radioactive material extractable by acetic acid, as judged by gel filtration experiments with Sephadex G-100. Therefore, minces were extracted with acetic acid after incubation with C¹⁴-proline, and such extracts were examined after gel filtration through a column of Sephadex G-200. Analysis of the effluent fractions showed that most of the radioactive hydroxyproline was in material of smaller size than ichthyocol gelatin, which was added as a marker. Therefore, a comparison was made of the distribution of hydroxyproline and of the subunits derived from ichthyocol gelatin upon gel filtration through this column (Fig. 1). In this experiment, the extract from a 15-min incubation was mixed with a sample of NH₂OH-treated ichthyocol gelatin prior to gel filtration. The elution of the subunits (dashed line) was followed by colorimetric assay of the hydroxyproline released by acid hydrolysis (the 0.5 μ mole of hydroxyproline contributed by the mince extract were considered negligible in comparison with the 20 μ moles of hydroxyproline present in the subunit sample). The two peaks in the elution profile of untreated ichthyocol gelatin (solid line) are believed to correspond to, respectively, the β and α components of ichthyocol gelatin, which has been reported to

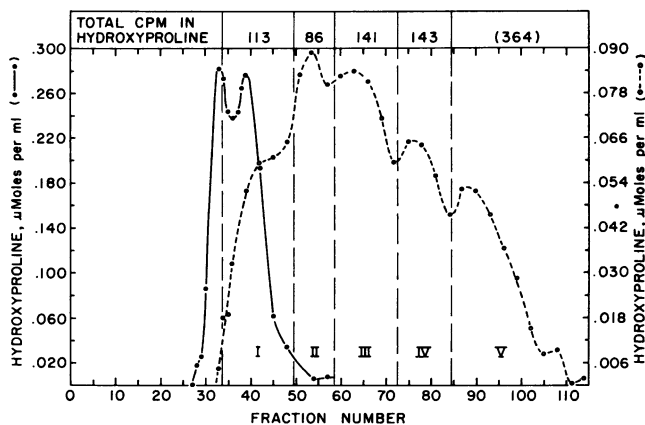


FIG. 1.—Comparison of distribution of radioactive hydroxyproline and of ichthyocol subunits on Sephadex G-200. The acetic acid extract was prepared in the usual way from a 15-min incubation containing, per ml, 0.24 embryos (7-day), 0.72 ml Krebs-Ringer phosphate, and 0.34 μC of C^{14} -proline (186 μC per μmole) in a final volume of 104 ml. The extract, after dialysis, was lyophilized and the collagenous material in the residue was solubilized by adding 3 ml of H_2O and placing the suspension in a 60° bath for 10 min. After centrifuging the suspension for 5 min at about $10,000 \times g$, the clear supernatant (2.3 ml) was mixed with 2.3 ml containing the subunits derived from 36 mg of ichthyocol and this mixture was then fractionated on a column of Sephadex G-200. Aliquots (0.1 and 0.2 ml) were taken for colorimetric determination of hydroxyproline after acid hydrolysis. The total hydroxyproline and its specific activity were also determined in the pooled fractions. The specific activities in the five peaks after the Dowex-50 step were, respectively, 16.3, 17.6, 22.6, 38.3, and 259 cpm per μmole and, after chromatography in the phenol-ammonia solvent, 30.1, 24.1, 28.2, 45.6, and 103.5 cpm per μmole . The increase in the specific activities of the hydroxyproline of the first four peaks is believed to be due to removal of impurities causing self-absorption, whereas the decrease in specific activity of the peak V hydroxyproline indicates the presence of radioactive contaminants after the Dowex 50 step; in the absence of further purification, the radiochemical purity of this sample is still uncertain after the chromatographic step. The elution pattern of ichthyocol gelatin (solid line), derived from 28 mg of ichthyocol, was determined in a separate experiment.

contain 30 per cent β -component,⁸ on the basis of their behavior on disc electrophoresis.

It may be concluded that the first of the five peaks in the NH_2OH -treated sample represents material of the same size as undegraded α chains. The fractions corresponding to the ichthyocol-derived peaks, referred to throughout this paper as peaks I–V, respectively, were pooled, as indicated by the vertical lines, and analyzed for radioactive hydroxyproline with the results shown at the top of the figure. The radioactivity present in the hydroxyproline of peak V is subject to uncertainty since this sample, unlike the other four samples, showed a decrease in specific activity on further purification. It is clear, however, that most of the radioactive hydroxyproline is present in material that is smaller in size than α chains, but is comparable in size to the subunits of these chains. A similar elution pattern of the ichthyocol subunits and of the radioactive hydroxyproline has been found in a total of seven other gel-filtration experiments with the same column, including an experiment in which the extract was not heated at 60° for 10 min prior to gel filtration. The reason for the appearance of radioactive hydroxyproline in material smaller than α chains remains uncertain. It is pertinent to note, however, that reincubation of isolated peak I material with such minces causes a partial breakdown of the material containing the radioactive hydroxyproline into fragments of smaller size.

Studies with the cell-free hydroxylation system: While the above studies were in progress, Prockop and Juva⁹ reported that a cell-free synthesis of hydroxyproline could be observed if they used, as a substrate for the cell-free hydroxylation, material that had been synthesized in a previous anaerobic incubation of tibia from chick embryo with C¹⁴-proline. Subsequently, the same workers⁶ reported that the radioactive substrate could be extracted from a particulate, presumably microsomal, fraction with 1 M NaCl. We have confirmed these findings in a system in which the substrate was labeled by anaerobic incubation with minces of entire 7-day embryos, and have found, further, that 0.5 N acetic acid extracted approximately as much of the substrate after 1 hr at 3° as did 1 M NaCl. The low yields of radioactive substrate, however, based on the added C¹⁴-proline, prevented the immediate application of this system to a determination of the size of the substrate. Subsequently, Juva and Prockop¹⁰ reported that α, α' -dipyridyl could be used instead of anaerobic conditions to cause accumulation of the substrate. Upon testing this compound as a possibly more convenient and reproducible way of preparing the radioactive substrate, the unexpected finding was obtained that, in our system, α, α' -dipyridyl was some 30 times more effective than anaerobic conditions in causing accumulation of the radioactive substrate (Table 1). From 11 such incubations run at different times, the yield of substrate, expressed as the increased radioactivity present in hydroxyproline after the enzymic hydroxylation reaction, has varied from a low of 66 cpm per ml of the substrate-synthesizing incubation mixture, to a high of 917 cpm per ml, with an average of 439 cpm per ml. Some of this variability may be due to the cell-free enzymic hydroxylation reaction since, although the increase in hydroxyproline has been found to be directly proportional to the quantity of added substrate within a single experiment, there is no assurance that, with different batches of embryo extract, the maximum extent of hydroxylation is always obtained.

The size of the substrate for the cell-free hydroxylation reaction: The increased yields of radioactive substrate obtained in the presence of α, α' -dipyridyl made it feasible to determine the size of this material by examining its behavior on columns of Sephadex G-200. The results of an experiment of this type are shown in Figure 2A. The fractions corresponding to the five peaks obtained after treatment of ichthyocol gelatin with NH₂OH (see Fig. 1) were combined and assayed for their content of substrate. It may be seen that the bulk of the substrate is in peak I and therefore of the same size as the α or β chains of ichthyocol gelatin, an unexpected result in view of the finding described above, that considerable radioactive hydroxyproline is found in material smaller than these chains after incubation of minces with

TABLE 1
COMPARISON OF EFFECTIVENESS OF ANAEROBIC CONDITIONS AND α, α' -DIPYRIDYL IN CAUSING ACCUMULATION OF SUBSTRATE

Conditions of incubation to form substrate	Cpm in Hydroxyproline		
	Before enzymic hydroxylation	After enzymic hydroxylation	Increase
Under N ₂	35	192	157
With 1 μ mole per ml of α, α' -dipyridyl	280	5375	5095

The final volume of the incubation mixture in the substrate-synthesizing step was 20 ml in each case. The extract from each incubation was then divided equally between two vessels of 12.5 ml final volume which contained the usual components of the enzymic hydroxylation reaction. The difference in the radioactivity present in hydroxyproline before and after the enzymic hydroxylation served as a measure of the amount of substrate present (see *Methods*). The total radioactivity in the substrate-containing extract added to each vessel was 14,100 cpm in the extract prepared from the anaerobic incubation and 184,000 cpm in the extract prepared from the incubation containing α, α' -dipyridyl.

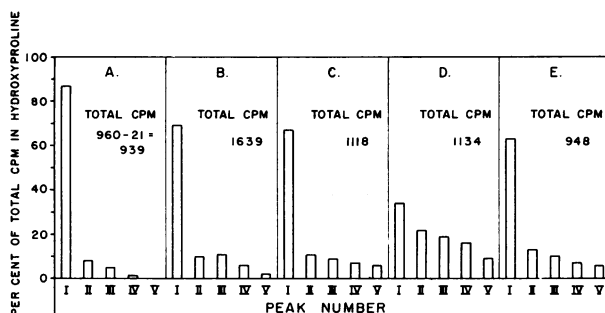


Fig. 2.—The size of the substrate and of the hydroxylated substrate before and after treatment with heat or hydroxylamine. In (A), the acetic acid extract derived from 12.3 ml of the usual incubation to form the substrate was subjected to the usual conditions of gel filtration on Sephadex G-200. The fractions corresponding to the five peaks obtained from NH_2OH -treated ichthyocol gelatin (Fig. 1) were combined and assayed for substrate. Before the enzymic hydroxylation reaction, peaks I through V contained, respectively, 17, 4, 3, 0, and 0 cpm, in hydroxyproline, compared with 830, 77, 47, 6, and 0 cpm, respectively, after the hydroxylation reaction. For (B) through (E), another aliquot of the same extract used in (A), derived from 24.6 ml of the substrate-synthesizing incubation, was subjected to the usual conditions of the enzymic hydroxylation reaction in a final volume of 18.6 ml. After an hour at 37° , the hydroxylated substrate was re-extracted (see *Methods*) and the extract reduced in volume to 18 ml. This extract was divided into 4 aliquots, one of which was fractionated on Sephadex G-200 without further treatment (B). A second aliquot was fractionated after placing in a 60° bath for 10 min and centrifuging at $105,000 \times g$ for 20 min to remove denatured protein (C). A third aliquot was treated with NH_2OH under the same conditions used to prepare the ichthyocol subunits (D), and the fourth aliquot was treated identically except that the NH_2OH was omitted (E).

C^{14} -proline in the absence of α, α' -dipyridyl. Since the elution of ichthyocol gelatin under these conditions has been found to be incomplete until fraction 53 or 54, the presence of some substrate in peak II would not be surprising even if it was identical in size with ichthyocol α chains.

The size of the substrate after hydroxylation: In addition to determining the size of the material that could be hydroxylated, the size of the material containing the newly synthesized hydroxyproline was examined. For this purpose another aliquot of the same preparation of substrate analyzed in Figure 2A was subjected to the usual conditions of the enzymic hydroxylation reaction and was then re-extracted and fractionated on Sephadex G-200. The fractions corresponding to the peaks given by NH_2OH -treated ichthyocol gelatin were pooled and analyzed for the radioactivity in hydroxyproline with the results shown in Figure 2B. It may be seen that most of the hydroxylated material is still of the same size as the substrate, that is, comparable to the α and β components of ichthyocol, although a small loss of material from peak I has occurred during incubation under the conditions of the hydroxylation reaction, since this peak contains only 69 per cent of the total radioactivity in hydroxyproline compared with 86 per cent of the substrate in Figure 2A.

Effect of denaturation and of hydroxylamine on the substrate after hydroxylation: To test whether the conditions used to convert ichthyocol to its gelatin had any effect on the size of the hydroxylated substrate, another aliquot of the hydroxylated substrate examined in Figure 2B was heated for 10 min in a 60° bath, prior to gel filtration on Sephadex G-200. It can be seen (Fig. 2C) that these conditions cause no detectable change in the size of the hydroxylated substrate.

The effect of treating the hydroxylated substrate with hydroxylamine under the same conditions used to prepare the ichthyocol subunits was also examined (Fig.

2D), as well as the effect of an identical treatment, but with the hydroxylamine omitted (Fig. 2E). For these tests, two other aliquots of the same hydroxylated substrate examined in Figures 2B and C were used. It may be seen (Fig. 2D) that the hydroxylamine treatment has caused a drastic decrease in the size of the hydroxylated substrate, of which only 33.5 per cent is present in peak I, compared to 69 per cent in peak I of the untreated control (Fig. 2B). Only a small breakdown of the hydroxylated substrate occurs under the same conditions when the hydroxylamine is omitted since 63 per cent of the radioactive hydroxyproline is still in peak I of Figure 2E. This result suggests that the substrate, consistent with its size, has a subunit type of structure such as is present in ichthyocol, and may similarly be broken down by treatment with NH_2OH .

This conclusion has been strengthened by two experiments in which the peak I fraction of the hydroxylated substrate was isolated by the usual fractionation on Sephadex G-200 and was then treated with NH_2OH . This material was again fractionated on Sephadex G-200 and single fractions were analyzed for the total radioactivity present in hydroxyproline. In each case radioactive hydroxyproline was found to occur in several peaks of smaller size than the peak I material, a finding that further confirmed that a similar breakdown of hydroxylated substrate and of ichthyocol gelatin occurs on treatment with NH_2OH .

Effect of heat or hydroxylamine treatment on ability of substrate to be hydroxylated: Treatment of the substrate with hydroxylamine under the usual conditions destroyed all but 30 per cent of its activity (Table 2, expt. 1), a figure in agreement with the per cent of hydroxylated substrate that remains intact (in peak I) under these conditions, as judged by gel filtration on Sephadex G-200 (Fig. 2D). It appears, however, that it is possible to destroy the ability of the substrate to be hydroxylated without significantly reducing its size, since treatment under identical conditions (pH 10 at 40° for 2.5 hr) in the absence of hydroxylamine allowed retention of only 46 per cent of the active substrate, although these conditions cause little change in size of the hydroxylated substrate (Fig. 2E). The conditions used to convert ichthyocol collagen to gelatin also cause little change in this size (Fig. 2C) and, in this case, little loss of active substrate occurred (Table 2, expt. 2). Exposure to strongly basic conditions for 1 hr at 30° destroyed the ability of the substrate to be

TABLE 2
EFFECT OF HYDROXYLAMINE, HEATING, AND ALKALI ON THE ABILITY OF THE
SUBSTRATE TO BE HYDROXYLATED

	Treatment of substrate	Total Cpm in Hydroxyproline		
		Before enzymic hydroxylation	After enzymic hydroxylation	Increase
Expt. 1	Untreated	69	579	510
	NH_2OH -treated	50	203	153
	NH_2OH conditions, minus NH_2OH	36	269	233
Expt. 2	Untreated	44	736	692
	Heated 10 min at 60°		654	610
	Incubated 60 min at 30° in 0.16 N KOH		260	216

The same preparation of substrate was used in experiments 1 and 2. The concentration of KOH was chosen so that on neutralization (with HCl), the concentration of KCl during the second incubation would be the same in these vessels as the others (0.025 M), so that no additional KCl was added to this vessel. The radioactivity reported for the hydroxyproline samples in experiment 2 was measured after their further purification by paper chromatography in the phenol-ammonia solvent since the sample from the heat-treated vessel was clearly contaminated after the Dowex 50 step. The specific activities of the hydroxyprolines from the other vessels of experiment 2 did not decrease after this paper chromatographic step; as usually found (compare Fig. 1), an increase of 1.1- to 1.4-fold was observed.

hydroxylated (Table 2, expt. 2); the effect of these conditions on the size of the hydroxylated substrate has not been investigated.

Evidence that the substrate does not require attachment to sRNA in order to be hydroxylated: Because of the possibility that hydroxylation *in vivo* occurs on nascent polypeptide chains which are still attached by their terminal carboxyl groups to sRNA molecules, an attempt was made to decide whether the substrate for the cell-free hydroxylation reaction is bound to sRNA. Samples of substrate derived from 2 ml of the substrate-synthesizing incubation were incubated for 1 hr at 30° with 40 μ g of RNase and 0.05 M Tris·HCl, pH 7.6, in a final volume of 2 ml, and were then assayed in the usual enzymic hydroxylation reaction. The presence of RNase had no effect on the ability of the substrate to be hydroxylated, in agreement with the findings of Prockop and Juva⁶ with the substrate accumulated under anaerobic conditions. In the absence of a suitable control to show that sRNA attached to nascent polypeptides would be broken down under these conditions, the above experiment, while suggestive, cannot be considered conclusive.

More convincing evidence that the substrate does not require attachment to an sRNA molecule was provided from experiments in which puromycin was added to the substrate-synthesizing system. After the usual 1-hr incubation, puromycin was added to give a concentration of 0.2 μ mole per ml (a level found in other experiments to inhibit protein synthesis completely in 5–10 min), and the incubation was continued for another 10 min. Since puromycin has been shown to exert its inhibition by replacing the carboxyl-terminal sRNA molecule of nascent polypeptides, which are then released from ribosomes,^{11,12} one would expect a drastic decrease in active substrate under these conditions, if the substrate required attachment to an sRNA molecule for activity. On the contrary, 1216 cpm were found in the substrate after 1 hr, and 1270 cpm after the additional 10 min in the presence of puromycin. The lack of effect of puromycin when incubated with the mince containing the newly formed substrate strongly suggests that the substrate does not require attachment to an sRNA molecule to be hydroxylated by the cell-free system. It might be mentioned in this connection that the substrate appears to be reasonably stable *in vivo*, since it showed essentially no loss (434 compared to 452 cpm) when the mince at the end of the usual 1-hr substrate-synthesizing incubation was thoroughly washed and was then reincubated for 25 min in fresh medium of the same composition as before except containing 5 μ moles per ml of C¹²-proline.

Sensitivity of the substrate to digestion by collagenase: Incubation of the substrate for the hydroxylation reaction with collagenase completely destroyed its ability to be hydroxylated. The substrate derived from 2.5 ml of the substrate-synthesizing incubation was digested for 1 hr at 37° in 0.025 M Tris, pH 7.2, and 0.005 M CaCl₂ with 5 or 10 μ g per ml of Worthington purified collagenase in a final volume of 2 ml, and was then heated in a boiling water bath for 10 min, prior to performing the usual enzymic hydroxylation reaction. The digested substrates showed no increase in radioactivity in hydroxyproline after the enzymic hydroxylation reaction, compared with an undigested aliquot, similarly heated, which showed an increase of 1583 cpm. The presence of nonspecific proteinases in this collagenase preparation was not tested; it is stated by Worthington, however, to be inactive toward casein, and similarly purified enzyme preparations have been reported¹³ to be free from activity toward proteins other than collagens or their derived gelatins. The

destruction of the substrate for the hydroxylation reaction by this collagenase preparation is therefore consistent with the assumption that the substrate contains the amino acid sequence of collagen, but with the hydroxyproline residues replaced by proline residues.

Discussion.—The finding that the substrate for the cell-free hydroxylation reaction is comparable in size to the α or β components of ichthyocol does not prove that, *in vivo*, hydroxylation occurs on material of this size. Udenfriend has recently suggested¹⁴ that, *in vivo*, hydroxylation keeps pace with polypeptide synthesis with the result that as proline residues satisfying the substrate specificity of the hydroxylase are added to nascent polypeptides they become hydroxylated. The presence of substrate of the size described here might then be a result of inhibiting the hydroxylation reaction, in this case with α, α' -dipyridyl. Although very low levels of substrate are present in smaller size species, this result might be due to a very low steady-state concentration of these smaller species. The appearance in uninhibited minces of considerable radioactive hydroxyproline in species of smaller size than the α or β components of ichthyocol (Fig. 1) would appear to support Udenfriend's suggestion, although the possibility remains that this finding is due to enzymic breakdown of the newly formed hydroxylated material. It is hoped that future work will provide the answer to this question as well as to the problem of whether the substrate is entirely of the size of α chains or of β chains.

Summary.—The bulk of the radioactive hydroxyproline synthesized from C¹⁴-proline by minces of chick embryo and extractable with cold 0.5 N acetic acid is present in material that is smaller in size than the α chains of ichthyocol and comparable in size to the materials obtained on treating ichthyocol gelatin with hydroxylamine. In contrast, the substrate for the cell-free hydroxylation reaction that converts protein-bound proline residues to hydroxyproline residues is of a size comparable to the α or β components of ichthyocol. This substrate is apparently similar in structure, also, to ichthyocol gelatin since, after hydroxylation, it can be broken down to a number of smaller species by treatment with hydroxylamine. The substrate appears not to require attachment to an sRNA molecule in order to be hydroxylated by the cell-free system.

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INTRAMITOCHONDRIAL PH CHANGES IN CATION ACCUMULATION

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One aspect of mitochondrial function of considerable current interest is the reaction of the mitochondria with monovalent and divalent cations.^{1, 2} The addition of small concentrations of Ca^{++} ($100 \mu\text{M}$) causes an intense stimulation of respiration, large shifts in the steady state of the respiratory carriers,¹ and eventual accumulation of nearly all of the Ca^{++} .^{3, 4} A number of other reactions accompany cation accumulation, for example, ejection of protons,³ the uptake of anions,^{5, 6} and particularly the establishment of an inhibited state of electron transport when none of the appropriate species of permeant anion is present.⁷

Electron transport is identified as a membrane-bound phenomenon by the isolation of submitochondrial particles in which the electron transport occurs as effectively as it does in the intact mitochondria,⁸⁻¹⁰ leading generally to the idea of a membrane-bound respiratory assembly or oxysome which embraces the functions of forward and reversed electron transport, oxidative phosphorylation, and more recently, ion accumulation.

Demonstration that ion accumulation takes place directly in the cytochrome-containing membrane is a problem of considerable importance in the development of ideas on reaction mechanisms. However, the only evidence available so far on this point is the rapid response of the respiratory carriers to Ca^{++} addition¹ and suggestions from electron microscopy that Ca^{++} is accumulated not only in the matrix space of the mitochondria but also in the cristal membrane (see ref. 1). The description in this communication of a cation responsive indicator bound within the cristal membrane opens new possibilities for the investigation of reaction mechanisms of cation uptake.^{11, 12}

Experimental Methods.—Preparations: The results presented here are obtained with rat liver mitochondria,¹³ but pigeon heart mitochondria have been used as well (Chance and Mela, in preparation). The suspension medium for the mitochondria has been reduced to its simplest ingredients and contains 0.225 *M* mannitol, 0.075 *M* sucrose for osmotic balance, and sodium succinate to serve as substrate and a "minimal" buffer; all other cations and anions that would permeate the mitochondria and interfere with the observation of intramitochondrial events are omitted. It is important to note that our preparation method for rat liver mitochondria involves the use of 10^{-4} *M* versene in the first two centrifugations. The mitochondria are subsequently washed twice to free them of the versene. Mitochondria prepared in this way contain a minimal amount of cations (5 $\mu\text{moles Ca}^{++}/\text{mg protein}$).

The technology of simultaneous measurements of pH of the external medium, oxygen concentration, absorbance changes of the indicator, and light scattering is described elsewhere^{1, 14} (Chance and Mela, in preparation), and the diagrams presented here are principally summaries of these data emphasizing the absorbance changes of the indicator.