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The Incorporation of Radioactive Inorganic Orthophosphate as Organic Phosphate by Collagen Fibrils *in vitro**

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The interaction of inorganic orthophosphate with reconstituted purified collagen fibrils obtained from a variety of tissues was studied *in vitro*. At initial solution concentrations of 1×10^{-2} M phosphate at 25°, collagen fibrils bound approximately 150–170 moles phosphate per mole collagen. Part of the bound phosphate was incorporated as organic phosphate. The acid and alkali stability and the electrophoretic behavior of the primary phosphate-32-labeled components make it unlikely that they are phosphorylated amino acids, but suggest that they are sugar phosphates. In addition to the readily dissociable and organic phosphate components, a fraction of the bound phosphate was very strongly complexed and difficult to separate from the collagen, but could be chemically extracted as inorganic orthophosphate. Therefore the interaction of collagen and inorganic orthophosphate is seen to encompass a wide spectrum of bond types.

An important consideration in understanding the mechanism underlying the nucleation of calcium and phosphate as apatite crystals by collagen fibrils *in vitro* (Neuman and Neuman, 1953; Sobel, 1955; Strates *et al.*, 1957; Glimcher *et al.*, 1957; Glimcher, 1959, 1960) are the interaction properties of collagen with calcium and phosphate ions.

Previous *in vitro* studies demonstrated that purified reconstituted collagen fibrils from fish swim bladder (ichthyocol) bound as much as 150 moles P/mole collagen, and that some of the bound phosphate had the characteristics of a covalent bond (Glimcher and Krane, 1962). The present study further characterizes the *in vitro* interaction of purified reconstituted collagen fibrils and inorganic orthophosphate.

EXPERIMENTAL

Preparation and Purification of Collagen.—Before the collagen was extracted, finely ground or hand-minced tissues were first washed for 24 hours in cold 1% NaCl, *p*H 7.4, extracted twice for 48 hours, and once for 24 hours at 2° with 3.9 M KCl, *p*H 8.3–8.5 (50–100 ml/g patted wet tissue), washed with 1% NaCl for 24 hours, and finally with cold distilled demineralized water until the fluid pressed from the tissue was chloride free. Acetic acid–soluble collagens from guinea pig skin and chicken leg tendon were obtained by extracting the washed tissues with approximately 15–20 volumes of 3% acetic acid at 2° for 48–72 hours. Rat tail tendon and mouse tail tendon were extracted in 20 volumes of 1% acetic acid, and carp swim bladder in 100 volumes of 0.05% acetic acid. The viscous collagen solutions were filtered either through Celite or through a 5-μ Millipore filter and prefilter using a specially designed pressure filtration device adapted to the standard large Millipore filter pressure unit.[1] The solutions were further clarified by centrifugation at 30,000 rpm for 2.5 hours in a Beckman-Spinco Model L ultracentrifuge.

The collagens, except for the carp swim-bladder collagen (ichthyocol), were purified as follows. Cold 25% NaCl, dissolved in the appropriate concentration of acetic acid, was added slowly with stirring at 2° to the clarified acetic acid solution of collagen until a final salt concentration of 5% was reached. The solution was stirred gently for approximately 1–2 hours, the stirring was discontinued, and the fluffy precipitate which settled out after 4–12 hours was collected by centrifugation at 6000–9000 rpm at 2°. The precipitate was washed three times by suspending and redispersing the collagen fibrils in cold 5% NaCl in the appropriate concentration of acetic acid, and was harvested at 6000–9000 rpm at 2°. The collagen fibrils were then redissolved in acetic acid, clarified by ultracentrifugation, and reprecipitated by the addition of NaCl. This procedure was repeated two more times. The collagen fibrils obtained from the last 5% NaCl precipitation were dissolved in 1% acetic acid and clarified by ultracentrifugation, cold concentrated NaCl in 1% acetic acid was added to a final concentration of 1% NaCl, and the *p*H was adjusted to 7.4 with NaOH. The temperature of the solution was then slowly raised to 25–30° and held for 0.5–1.0 hour (Gross and Kirk, 1958). The flocculent precipitate was separated from the supernatant, washed, redissolved in cold 1% acetic

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[1] Millipore Filter Corp., Bedford, Mass.

acid, and clarified by ultracentrifugation, and the procedure was repeated. The collagen fibrils were redissolved in 1% acetic acid, dialyzed exhaustively against 1% acetic acid, clarified, and either stored at 2° or lyophilized and kept in a vacuum dessicator at 2° until used.

Ichthyocol was precipitated five times by dialyzing 0.05% acetic acid solutions of collagen against 1% NaCl adjusted to *p*H 7.4 with 0.02 M Tris at 2° (Schmitt *et al.*, 1942).

Purification of $^{32}P_i$.—Carrier-free $^{32}P_i$ was obtained from the Oak Ridge National Laboratories and purified by column chromatography on Dowex 1-bicarbonate (Martonosi, 1960). Extraction of the purified $^{32}P_i$ by the Nielsen and Lehninger modification (1955) of the method described by Martin and Doty (1949), showed that all of the ^{32}P reacted as P_i.

Preparation of Reaction Tubes.—Preliminary studies showed that a variable and often significant amount of $^{32}P_i$ was bound by glass test tubes, particularly at low concentrations of phosphate. A number of procedures were investigated in order to eliminate this difficulty. The method finally adopted was as follows. Glass-stoppered Pyrex test tubes (10 ml) were heated at 90° for 24 hours with repeated changes of 15% trichloroacetic acid, and thoroughly washed with doubly distilled demineralized water at 100° and 20°. The test tubes and stoppers were then boiled in 0.5 M ethylenediaminetetraacetic acid, *p*H 9.0, for 4 days, and thoroughly washed with doubly distilled demineralized water. They were then placed in an ashing oven at 400° for 36 hours, and stored in a vacuum dessicator until used. Glassware treated in this fashion bound insignificant amounts of $^{32}P_i$ even at phosphate concentrations as low as 1×10^{-8} M.

Phosphate Binding by Collagen Fibrils: Experimental Technique.—Wet suspensions of collagen fibrils shown by electron microscopy to be of the typical native type (~700 A axial repeating structure) were thoroughly dispersed and suspended in 10 ml of 0.02 M Tris, Γ 2 0.165 NaCl or KCl, *p*H 7.4, purified $^{32}P_i$, and varying concentrations of nonradioactive P_i in previously prepared glass-stoppered test tubes. Toluene was added and the glass stoppers were fixed in place with wax and cellulose tape. Approximately 20 mg dry weight of collagen was used per 10 ml of solution. The experiments were done in triplicate. The dry weight of the samples was determined by drying down aliquots of the wet fibrils and by hydroxyproline determination (Stegemann, 1958). The suspensions of collagen fibrils were incubated with vigorous shaking at 25° for 2–120 hours. At the end of the incubation period the incubation test tubes were centrifuged, the supernatant fluid was filtered through a 100-A Millipore filter, and aliquots were plated on copper or aluminum planchets. Radioactivity of the samples was determined in a Nuclear-Chicago automatic gas-flow counter. The phosphorus bound per g or mole collagen was calculated from the difference between the initial and final net counts per minute (net cpm) ^{32}P ml of the supernatant fluid and the initial P_i concentration, taking into account the dilution which occurred because of the water associated with the collagen fibrils. The validity of this procedure was confirmed at the higher phosphate concentrations by chemical determination of phosphorus on the supernatant fluid. At low initial concentrations of phosphate these values were confirmed by blotting dry the collagen precipitate to remove solvent water, and either determining the total ^{32}P of the sample, or the ratio ^{32}P/hydroxyproline on samples hydrolyzed for 24 hours in 6 N HCl at 105°. In experiments where the aim was to characterize the bound phosphate, larger samples of collagen were used at approximately the same solid-to-solution ratio as above.

Characterization of the Collagen-bound Phosphate.—Collagen fibrils, which had been incubated with $^{32}P_i$, were blotted dry, redispersed, and dissolved in cold 1% acetic acid. The collagen solutions (~0.05%) were clarified by centrifugation, and an aliquot removed for hydrolysis in 6 N HCl, 105°, for 24 hours. The concentrations of ^{32}P and hydroxyproline were determined on this aliquot, and the total moles phosphate bound per mole collagen were computed.

Nondialyzable Collagen-bound ^{32}P.—Aliquots of the dissolved collagen as well as thermally denatured collagen solutions (heated to 70–90° for 10–30 minutes) were dialyzed against continuous changes of large volumes of 1% acetic acid in 0.1 M NaCl, followed by 1% acetic acid, or 1×10^{-3} M phosphate in 1% acetic acid for 7–14 days. After dialysis, aliquots of the collagen or gelatin solutions were hydrolyzed in 6 N HCl, 105°, for 24 hours and the concentrations of ^{32}P and hydroxyproline were determined.

Millipore Filtration.—Aliquots of the dissolved collagen or gelatin solutions both before and after dialysis were diluted and filtered through 100-A Millipore filters in specially constructed high-pressure filtration units. Under these conditions the solvent was forced through the filter, while the majority of the collagen remained above the filter. As the volume of the solution diminished, fresh acetic acid was added, and filtration continued until no further ^{32}P appeared in the filtrate. The filtrate was then analyzed for radioactivity and protein concentration (Lowry *et al.*, 1951).

Horizontal Paper Electrophoresis.—Collagen and gelatin solutions were applied to Whatman No. 1 and 3 MM paper and subjected to electrophoresis at field strengths varying from 40 to 240 v/cm over a wide range of *p*H. The location of the radioactivity was determined by an automatic recording strip counter and that of the collagen or gelatin by staining with Amido Black.

Deionization on Mixed-Bed Resin.—Samples of lyophilized collagen were converted to gelatin solutions of approximately 0.5–1.0% concentration by dissolving them in doubly distilled demineralized water at 60–70°. The hot gelatin solution was passed through a 2.1 × 45-cm jacketed column containing a mixed-bed resin (one part IR-120 and two parts Dowex 1)kept at 60°, and eluted at the rate of 1.0 ml/minute.[2] Fractions (10 ml) were collected, and both the radioactivity and the protein concentration determined on aliquots of each fraction.

Determination of P_i.—Collagen or gelatin solutions, collagenase digests, partial acid or alkali hydrolysates, and fractions eluted from paper electrophoresis or resin-column chromatography were extracted by the Nielson and Lehninger modification (1955) of the method of Martin and Doty (1949). The procedure described by Lipmann and Tuttle (1944) was also used.

Chromatography of Collagenase Digests, and Acid and Alkali Digests of Collagen.—Collagen or gelatin solutions were incubated with purified bacterial collagenase in 0.05 M Tris, *p*H 7.4, 1×10^{-3} M $CaCl_2$ from 2 to 24 hours at 37°. Approximately 1.5 mg of collagenase was used per 100 mg of collagen or gelatin. Toluene was added to prevent bacterial growth. The *p*H was checked at intervals and readjusted to *p*H 7.4,

[2] This procedure was developed and suggested to us by the Atlantic-Gelatin-General Foods Corp., Woburn, Mass.

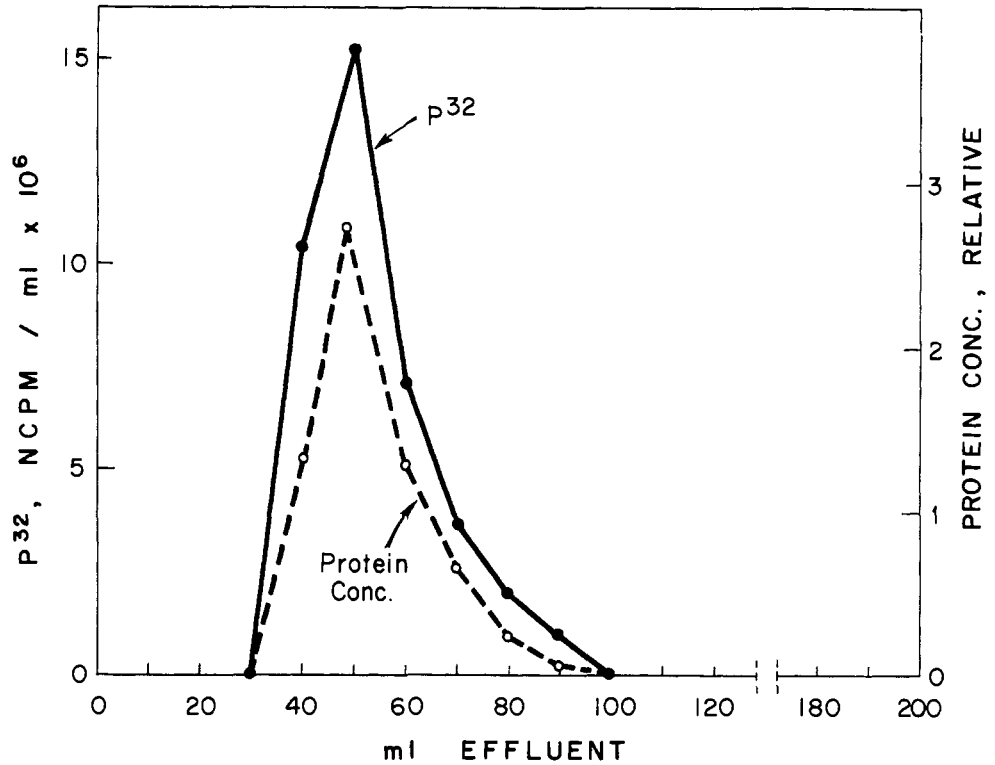

FIG. 1.—Deionization of approximately 200 mg of gelatin-^{32}P (thermally denatured guinea pig collagen-^{32}P) on mixed-bed resin at 60°. Conditions described in text.

and for the longer digestions additional collagenase was added. At the end of the incubation the solution was clarified either by centrifugation at 14,000 rpm for 30 minutes or by filtration through a fine sinter glass filter. The collagenase was precipitated by the addition of perchloric acid to a final concentration of 0.3 M at 2°, and most of the perchloric acid was removed by neutralizing with KOH. The clarified supernatant was reduced in volume on a rotary evaporator until precipitation of more $KClO_4$ occurred, the supernatant again was reduced in volume, and the procedure was repeated several times. The sample was then completely dried on the rotary evaporator and taken up in a small volume of water and filtered.

Samples of collagenase digests, alkali or acid hydrolysates, or collagenase digests further hydrolyzed by alkali or acid were acidified to a final concentration of 0.1 N HCl and chromatographed on Dowex 50-H^+, X-2 or X-8 resin. The resin had been previously equilibrated with 0.01 N HCl. The column was first eluted with either 0.01 N HCl or water, and the radioactivity of aliquots of the eluate was monitored. After elution of an early major asymmetrical peak, the eluting solution was changed to water in the case where 0.01 N HCl was used initially, or the water was continued until no further ^{32}P was eluted. The column was then stripped with 1 M NH_4OH. The columns were operated at approximately 6–10°.

High-Voltage Paper Electrophoresis, Paper Chromatography, and Radioautography.—Appropriate samples were subjected to electrophoresis on 58-cm strips of Whatman No. 3 MM paper at *p*H 1.9 (formic acid–acetic acid), or *p*H 6.5 (pyridine–acetic acid) at 3000–5000 v in a Servonuclear high-voltage paper-electrophoresis apparatus.[3] Descending paper chromatography was carried out on Whatman No. 3 MM paper in *n*-butanol–acetic acid–water (4:1:5). Radioautographs were prepared by exposing the dried paper strips on Ansco nonscreen X-ray film for periods varying from several hours to several days depending on the radioactivity of the samples.

Amino Acid Analysis.—Samples hydrolyzed in 6 N HCl at 105° for 24 hours were analyzed on a commercial model[4] of the automatic amino acid analyzer described by Piez and Morris (1960).

Chemicals, Reagents, and Standards.—*O*-phosphoserine, *O*-phosphothreonine, and phosphoethanolamine were obtained from Sigma Chemical Co. *O*-phosphohydroxyproline, *O*-phosphohydroxylysine, and *O*-phosphotyrosine were synthesized by the method of Plimmer (1941), and purified by column chromatography on Dowex-1 chloride. Glucose 1-phosphate, glucose 6-phosphate, galactose 1-phosphate, and ribose 5-phosphate were purchased from Sigma Chemical Co. Bacterial collagenase, purchased from Worthington Laboratories, was further purified by the method of Seifter *et al.* (1959). Purified prostatic phosphomonoesterase was a gift of Dr. Gerhard Schmidt. Wheat germ phosphatase was purchased from Mann Biochemical Co., and high-specific-activity calf-intestinal phosphatase was purchased from Worthington Laboratories. Phosvitin was purchased from Sigma Chemical Co. All other reagents used were analytical grade and were obtained commercially.

RESULTS

Not only did native type collagen fibrils reconstituted from acetic acid extracts of a variety of collagenous tissues bind inorganic phosphate from solution, but a variable proportion of the phosphate (dependent on the initial concentration) was nondialyzable and could not be removed by Millipore filtration. This fraction did not exchange with nonradioactive phosphorus, either by dialysis or after equilibration with high concentrations of nonradioactive phosphorus and subsequent high-voltage paper electrophoresis. The phosphate

[3] Servonuclear Corporation, Long Island City, N. Y.

[4] Phoenix Precision Instrument Co., Philadelphia, Pa.

TABLE I THE BINDING OF PHOSPHATE BY RECONSTITUTED GUINEA PIG SKIN COLLAGEN FIBRILS[a]

Phosphate Concentration in Solution M × 10^{-7}		Collagen (mg)	Total Phosphorus Bound			P Lost after Dissolving in 0.05% Acetic Acid and:			Extracted as P_i (Martin-Doty) after Dialysis vs. 1×10^{-3} M P in Acetic Acid (%)	Non-dialyzable P[c] (mole P/mole collagen)	Organic P[d] (mole P/mole collagen)
Initial[b]	Final[b]		(%)	(moles × 10^{-9})	(moles P/mole collagen)	Filtered 100 A Millipore (%)	Dialysis vs. 0.05% Acetic Acid (%)	Dialysis vs. 1×10^{-3} P in 0.05% Acetic Acid (%)			
0.94	0.093	17.7	90.3	0.089	0.018						
0.935	0.087	19.3	90.7	0.090	0.016	5.1	9.1	6.7	6.4	0.015	0.014
0.946	0.118	16.0	87.5	0.087	0.019						
4.70	0.338	19.6	92.8	4.64	0.083						
4.70	0.444	18.4	90.6	4.53	0.086	5.1	9.3	11.8	6.7	0.076	0.072
4.70	0.393	19.3	91.6	4.59	0.083						
9.35	0.804	19.4	95.5	9.56	0.173	1.9	8.8	8.1	4.6	0.159	0.151
47.0	25.9	17.8	45.5	22.7	0.447						
47.0	25.8	17.6	45.1	22.5	0.448	21.2	50.3	52.2	9.4	0.203	0.184
47.0	25.6	17.4	42.2	21.1	0.425						
470	389	20.7	17.2	86.0	1.46						
470	367	21.1	22.0	110.0	1.82	15.5	76.7	83.1	13.5	0.309	
4700	4160	21.1	11.4	570	9.45						
4660	4050	21.5	13.2	661	10.80	37.3	96.2	96.0	18.0	0.432	0.354

[a] Collagen fibrils incubated with $^{32}P_i$ at concentrations shown in 10 ml total volume of 0.02 M Tris, *p*H 7.4 Γ/2 0.165 KCl, for 24 hours at 25° with vigorous shaking. [b] Corrected for collagen water. [c] Nondialyzable vs. 1×10^{-3} M P in 0.05% acetic acid. [d] Computed by subtracting the P_i from the nondialyzable P.

TABLE II

LIBERATION OF $^{32}P_i$ FROM COLLAGEN-^{32}P AFTER INCUBATION WITH COLLAGENASE[a]

Sample	$^{32}P_i$ Liberated (Martin-Doty) (%)
Collagen	7.2
Collagen and Collagenase	8.1

[a] Reconstituted collagen fibrils (20 mg) prepared from rat tail tendon incubated in 10 ml total volume of 0.02 M Tris, *p*H 7.4, Γ/2 0.165 KCl, with $^{32}P_i$ at final concentration of 1×10^{-6} M for 24 hours at 25°. Collagen fibrils were washed, redissolved in 1.0% acetic acid, and dialyzed for 48 hours against 1% KCl in 1% acetic acid and for 72 hours against 1% acetic acid, all at 2°. The *p*H of the solutions was adjusted to 7.4 with Tris at final concentration of 0.05 M, $CaCl_2$ added to final concentration of 1×10^{-3} M, and collagenase was added. The mixture was then incubated at 37° for 24 hours. An additional sample was incubated under the same conditions but without collagenase.

TABLE III

THE EFFECT OF TIME OF INCUBATION AND INITIAL P_i CONCENTRATION ON P_i BINDING BY COLLAGEN FIBRILS[a]

Initial P_i Concentration (molar)	P_i Bound in Moles P × 10^{-8}		
	(4 hours)	(20 hours)	(44 hours)
1×10^{-7}	6	17	18
1×10^{-6}	19	102	152
1×10^{-5}	—	330	435

[a] Reconstituted collagen fibrils (ichthyocol, 20 mg/tube) incubated as in Table I for times shown at 25°. Binding determined by difference between initial and final concentrations of ^{32}P.

was not separated from the collagen by deionizing gelatin solutions on a mixed-bed resin at 60°. The majority of this nondialyzable fraction behaved chemically as organic phosphate, and could not be extracted as P_i even after digestion of the collagen or gelatin by collagenase. The results of typical experiments are shown in Tables I and II and Figure 1. Control experiments in which $^{32}P_i$ was added to collagen and gelatin solutions and equilibrated at 2° showed that the $^{32}P_i$ was dialyzable, could be separated from the collagen by paper electrophoresis, and reacted chemically as P_i.

As might be expected in the case of a protein in an ordered, aggregated solid state, the amount of phosphate bound at any one initial concentration of phosphate and fixed solid to solution ratio was found to vary considerably. Using the same preparation of collagen, it was found that the extent of binding was related to the density of the fibril preparation. Thus samples of collagen fibrils which had been tightly packed by centrifugation at high speed and/or extensively compressed and blotted dry bound less phosphate than finely dispersed preparations. When the phosphate was added to cold neutral solutions of collagen adjusted to the proper ionic strength, mixed, and the collagen precipitated from solution as fibrils by warming to 25–30°, the highest values for binding were obtained immediately. However, using finely dispersed collagen fibrils which had not been previously packed by high-speed centrifugation and blotting, similar amounts of phosphate were bound if the time of exposure were increased to 24 hours. The maximum amount of total phosphate bound under these conditions amounted to approximately 150–170 moles of P/mole collagen at an

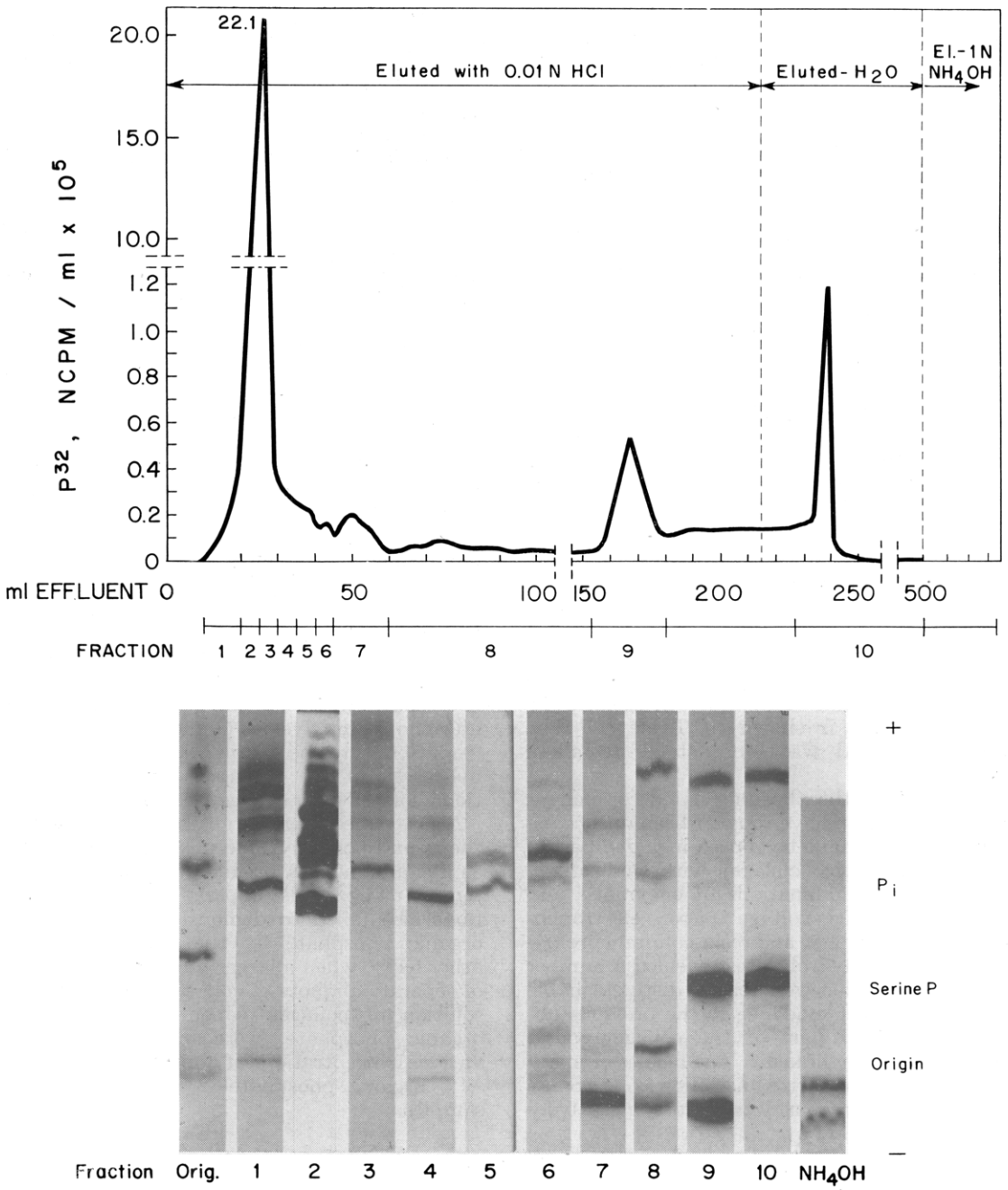

FIG. 2.—Chromatography and electrophoresis of alkali hydrolysate of collagenase digest of collagen-^{32}P. Collagen-^{32}P (guinea pig skin) prepared as in Table I and digested with collagenase for 3 hours. The collagenase digest was treated with 0.25 N NaOH at 100° for 24 hours prior to chromatography and electrophoresis. (A) Chromatography of sample on a 45 × 0.9-cm column of Dowex 50-H^+, X-8 resin, run at 15 ml/hour. (B) High-voltage paper electrophoresis of fractions eluted from Dowex 50-H^+ resin. Electrophoresis was carried out at *p*H 1.9 for 20 minutes on 58-cm strips of Whatman No. 3 MM paper at 5000 v. Orig. = aliquot of entire sample before fractionation on the Dowex 50-H^+ resin column; NH_4OH = entire fraction eluted with NH_4OH.

initial phosphate concentration of 1×10^{-2} M, similar to that previously reported for ichthyocol (Glimcher and Krane, 1962). Under these conditions of maximum binding the maximum amount of organic phosphate incorporated into the collagen, as determined by exhaustive dialysis followed by chemical extraction for P_i, varied from 1 to 3 moles P/mole collagen.

The effect of time of incubation and initial P_i concentration on the total amount of phosphate bound and the amount of organic phosphate formed is illustrated in Tables III and IV.

In addition to the phosphate which was easily removed from the collagen (by dialysis or Millipore filtration) and the organic phosphate, there was a variable amount of bound phosphate which was not organic as determined by chemical extraction, but which was exceedingly difficult to remove. This fraction remained with the collagen or gelatin after Millipore filtration and after all but the most extensive dialysis against nonradioactive phosphorus (Table I). Even after the latter procedure, a small fraction still remained which reacted chemically as P_i (Table I). Furthermore, a fraction of the ^{32}P which was eluted from the mixed bed resin with the gelatin could be extracted chemically as P_i, the amount depending on how well dialyzed the sample was prior to passing it through the

TABLE IV

EFFECT OF TIME OF INCUBATION ON THE BINDING OF $^{32}P_i$ BY COLLAGEN FIBRILS AND ITS INCORPORATION INTO COLLAGEN AS ORGANIC ^{32}P[a]

Time of Incubation (hours)	^{32}P Bound from Solution (%)	Collagen Solutions: ^{32}P Filterable thru 100 A Millipore Filter (%)	Collagen Solutions: $^{32}P_i$ Chemically Extracted (Martin-Doty) (%)
2	29.3	28.2	26.0
6	28.9	27.6	29.0
26	72.8	19.6	16.1
50	73.6	9.4	8.1
120	70.4	9.9	11.2

[a] Reconstituted collagen fibrils (ichthyocol) (20 mg/tube) incubated as in Table I for times shown with P_i at initial concentration of 1×10^{-6} M P. At the end of the incubation, fibrils were washed once, centrifuged, pressed dry on filter paper to remove solvent water, and redissolved in 1% acetic acid.

resin. Negligible amounts of $^{32}P_i$ could be eluted from these columns when $^{32}P_i$ or mixtures of $^{31}P_i$ and $^{32}P_i$ were chromatographed alone, or when $^{32}P_i$ was added to gelatin solutions just prior to chromatography.

The efficacy of various phosphomonoesterases in hydrolysis of the organic phosphate of gelatin-^{32}P compared to phosvitin is shown in Table V. The collagen-phosphate bond was more stable to alkali than to acid (Table VI).

Attempts to identify the phosphorylated component or components in collagen have thus far been unsuccessful. Procedures which have been used included partial digestion of the ^{32}P-labeled collagen with acid, alkali, and collagenase, and combinations of enzymatic and alkali degradation. High-voltage paper electrophoresis, paper chromatography, and resin-column chromatography of the partial hydrolysatesr evealed a number of ^{32}P-labeled fractions that were readily distinguishable from $^{32}P_i$, adding further evidence to the previous observations that at least part of the collagen-bound phosphate was covalently linked. The results of many experiments may be summarized as follows: (1) Some of the ^{32}P-labeled fractions were more negatively charged than P_i at *p*H 1.9, while others were either less negatively charged than P_i at this *p*H, moving in the region of phosphoserine, or had an overall net positive charge and moved slightly toward the cathode. (2) Further acid hydrolysis of many of these fractions produced the following: (a) The ^{32}P-labeled components which were more negatively charged than P_i were hydrolyzed to P_i only. (b) Those fractions which moved with phosphoserine at *p*H 1.9 released P_i and the more negatively charged components which moved more rapidly than P_i at *p*H 1.9. Furthermore, when the original fractions which moved with phosphoserine at *p*H 1.9 were electrophoresed at *p*H 6.5, they were shown to be distinct from phosphoserine. (c) The positively charged components also formed ^{32}P-labeled fractions which were more negatively charged than P_i at *p*H 1.9. It therefore appears that the primary ^{32}P-labeled compounds in collagen are represented by those negatively charged fractions moving more rapidly than P_i at *p*H 1.9. (d) The most prominent ^{32}P-labeled components which were more negatively charged than P_i at *p*H 1.9 were eluted from the paper electrophoretograms and on further electrophoresis were shown to move in the region of the sugar and glycerophosphates. A typical experiment in which many of these components can be seen is illustrated in Figure 2.

No ^{32}P-labeled component which could be identified as any of the *O*-phospho- amino acids possibly present in collagen (*O*-phosphoserine, *O*-phosphothreonine, *O*-phosphohydroxylysine, *O*-phosphohydroxyproline, and *O*-phosphotyrosine) was liberated after acid or alkali hydrolysis of the ^{32}P-labeled collagen or of the fractions isolated after collagenase degradation.

There were several preparations of collagen in which the bound nondialyzable phosphate reacted chemically as P_i, and not as organic phosphate. Repeated attempts using fibrils prepared from these collagen solutions also failed to demonstrate the incorporation of organic phosphate by the collagen fibrils. On the other hand, when a larger number (as many as twenty) of separate aliquots of collagen fibrils, prepared from a solution of collagen which did incorporate $^{32}P_i$ as organic phosphate, were incubated separately with various concentrations of nonradioactive phosphate and $^{32}P_i$, organic phosphate-32 could be detected in all samples.

TABLE V

LIBERATION OF P_i FROM GELATIN-^{32}P AND PHOSVITIN BY PHOSPHATASES[a]

Substrate	Enzyme	*p*H	P_i or $^{32}P_i$ Released: Time of Incubation: 1 Hour (%)	4 Hours (%)	24 Hours (%)
Gelatin-^{32}P	Prostatic	5.6	2.6	5.6	12.6
Phosvitin	Prostatic	5.6	0.8	0	1.5
Gelatin-^{32}P	Wheat Germ	5.6	24.7	29.5	48.5
Phosvitin	Wheat Germ	5.6	20.0	40.5	55.0
Gelatin-^{32}P	Intestinal	5.6	27.6	41.1	53.3
Phosvitin	Intestinal	5.6	3.4	8.0	24.3
Gelatin-^{32}P	Intestinal	8.8	46.3	58.4	70.6
Phosvitin	Intestinal	8.8	3.3	5.3	17.4

[a] Solutions of guinea pig skin gelatin-^{32}P, prepared by thermally denaturing dialyzed collagen-^{32}P (see Table I), and 10 mg phosvitin were adjusted to the appropriate *p*H and incubated at 37° for times shown with the various enzymes. Succinate buffer, 0.05 M, was used at *p*H 5.6, and glycine, 0.05 M was used at *p*H 8.8. $MgCl_2$ present at final concentration of 1.5×10^{-3} M. Release of $^{32}P_i$ from gelatin-^{32}P was analyzed directly on the solution by the modified Martin-Doty procedure. Release of P_i from phosvitin was measured by stopping the reaction with the addition of trichloroacetic acid, final concentration 10%, and determining the P_i in the supernatant by the method of Fiske and Subbarow. The amount of each enzyme used liberated the following amounts of P_i from *p*-nitrophenylphosphate at 1×10^{-3} M under the same conditions of incubation (μmoles P_i/hour): prostatic, 49.7; wheat germ, 27.0; intestinal at *p*H 5.6, 0.8; intestinal at *p*H 8.8, 50.0.

However, because of these results, and because of the unusual nature of the reaction described, the small amounts of phosphate incorporated as organic phosphorus, and the long incubations usually employed (20–24 hours), the possibility was considered that trace bacterial contamination might account for the results. The following observations make this possibility unlikely. (1) The reaction proceeded when the experiments were carried out under completely sterile conditions. Cultures of the collagen at the conclusion of the sterile experiments revealed no bacterial growth after 2 weeks. (2) In other experiments done with the usual precautions, but not with sterile technique, cultures of the collagen samples also revealed no bacterial growth. (3) The reaction proceeded and organic phosphate could be identified within 2 hours. (4) Dilute acetic acid solutions of ^{32}P-labeled collagen retained the ^{32}P after centrifugation for 3 hours at 4000 rpm or after filtration through bacterial filters. (5) Collagen fibrils prepared from collagen solutions which had been filtered through bacterial filters also incorporated $^{32}P_i$ as organic phosphate. (6) Ultraviolet-absorption spectra of concentrated ^{32}P-labeled collagen solutions revealed no additional shoulder or peak in the region 260–280 mμ. (7) Incubation of ^{32}P-labeled gelatin with purified ribonulease failed to release the ^{32}P from the gelatin. (8) None of the highly labeled fractions contained ultraviolet-absorbing material on scanning the paper strips used for electrophoresis. (9) RNA and various mononucleotides (labeled with ^{32}P and mixed with gelatin) could be separated from the gelatin on the mixed-bed resin column. Previous studies with ^{14}C-labeled ATP and ADP had shown essentially no binding of these substances by collagen fibrils (Krane and Glimcher, 1962). (10) The primary ^{32}P-labeled components in the collagen were not identified as ribose-5-phosphates.

Discussion

The incorporation of ^{32}P as protein-bound phosphoserine after the *in vitro* incubation of $^{32}P_i$ with intestinal and bacterial alkaline phosphatases has been reported by Ågren *et al.* (1959), Engström (1959, 1961, 1962), and Schwartz and Lipmann (1961).

There are many differences, however, between the *in vitro* reaction of P_i and collagen, in the present study, and that reported for the alkaline phosphatases. The phosphate incorporation into alkaline phosphatases occurs and reaches equilibrium within 10 seconds at 0° as well as 30° (Schwartz and Lipmann, 1961), and is reversible by dilution. With collagen, on the other hand, the reaction was first detected at approximately 2 hours, did not reach equilibrium until approximately 24 hours, and did not proceed at 0°, and the organic phosphate was not removed by dilution, dialysis, or exchange with $^{31}P_i$. Furthermore, the primary ^{32}P-labeled component in collagen does not appear to be *O*-phosphoserine or any other of the *O*-phospho- or *N*-phosphoamino acids. The evidence which supports this conclusion is as follows. In contrast to the reported alkali lability of *O*-phospho- bonds in protein-bound amino acids (Perlmann, 1955), and (except for imidazole *N*-phosphohistidine) (Boyer *et al.*, 1962) the extreme acid and alkaline lability of *N*-phospho- amino acid bonds in proteins (Perlmann, 1955), the collagen-phosphate bond was relatively stable in both alkali and acid, and more stable in alkali than in acid. Moreover, the primary ^{32}P-labeled component or components were more negatively charged and moved more rapidly than P_i on paper electrophoresis at *p*H 1.9, ruling out the possibility that these components are single *O*-phosphoamino acids or imidazole *N*-phosphohistidine.

Table VI

The Stability of the Collagen-^{32}P Bond to Acid and Alkali[a]

Experimental Conditions	$^{32}P_i$ Liberated (Martin-Doty) (%)			
	50°		70°	
	5 hr	24 hr	5 hr	24 hr
HCl, 0.25 N	18	18	27	41
NaOH, 0.25 N	3	4	5	7
HCl, 2.0 N	34	51	58	64
NaOH, 2.0 N	11	9	28	38

[a] Dialyzed collagen-^{32}P solutions prepared from rat tail tendon as described in text were adjusted to the condition shown and the $^{32}P_i$ liberated as determined by the modified Martin-Doty procedure.

Williams and Sanger (1959) have isolated polymers of *O*-phosphoserine from partial acid hydrolysates of phosvitin and casein. They also demonstrated that the polymers of *O*-phosphoserine phosphates moved faster than *O*-phosphoserine at *p*H 1.5. Polymers of *O*-phosphoserine phosphates, isolated from phosvitin under conditions described by Williams and Sanger (1959), were subjected to electrophoresis on paper under similar conditions used for collagen-^{32}P hydrolysates. The most negatively charged polymer of *O*-phosphoserine phosphate moved slightly more rapidly than P_i as compared to the very rapidly moving peaks isolated from collagenase and alkali digests of ^{32}P-labeled collagen. Furthermore, further acid hydrolysis of the polymer *O*-phosphoserine fractions resulted in the release of *O*-phosphoserine, whereas in the case of the fractions isolated from collagen only P_i was liberated under similar conditions of hydrolysis.

The liberation of P_i from collagen-phosphate by phosphatases roughly parallels that obtained with phosvitin as a substrate. In view of the relative specificity of the phosphatases towards phosphomonoesters, these data are consistent with the premise that the collagen-phosphate bond is a monoester.

The suggestion that N—P bonds in protein-bound amino acids could be distinguished from O—P bonds by the use of intestinal alkaline phosphatase at *p*H 5.6 and 8.6 (Perlmann, 1955) was not confirmed, since similar amounts of phosphate were removed at both *p*H values from phosvitin, in which only *O*-phosphoserine is known to be present. Moreover, the intestinal alkaline phosphatase had phosphomonoesterase activity at *p*H 5.6 using *p*-nitrophenylphosphate as substrate.

The relative alkali stability and acid lability of the phosphate bond in collagen, the enzymatic release of P_i by phosphomonoesterases, and the electrophoretic behavior of the primary collagen-^{32}P components suggest that the primary ^{32}P components are sugar phosphates. Both glucose and galactose (as well as other hexoses) have been identified in highly purified collagens (Beek, 1941; Grassman and Schleich, 1935; Gross *et al.*, 1958; Kuhn *et al.*, 1959).

Since the formation of a covalent phosphate bond requires energy, and is accomplished with collagen simply by incubating the protein with $^{32}P_i$ at 25°, some possible mechanisms should be offered. Although no definite answer can be given, there are at least three mechanisms which may be considered.

It is conceivable that the interaction of inorganic orthophosphate with collagen fibrils results in a local change in the configuration of the primary, secondary, or tertiary structure of the collagen, and that the

accompanying change in entropy due to the configurational change supplies the free energy required. In support of this possibility are the findings of Mandelkern and Meyer[5] who have recently studied the shrinkage temperature of collagen and elastoidin in the presence of varying concentrations of inorganic phosphate as compared to other ions at similar *p*H and ionic strength. They found striking increases in the shrinkage temperature with increasing concentrations of phosphate, suggesting some local rearrangement and stabilization of the collagen structure as the result of the collagen-phosphate interaction.

Secondly, the rearrangement of groups in the collagen as the result of its strong interaction with phosphate could also lead to the replacement of a preexisting ester of the type R—O—R′ by a phosphate ester. This possibility is supported by the findings of Gallop *et al.* (1959) and Blumenfeld and Gallop (1962) of the presence of ester or "esterlike" bonds in collagen.

The third possibility is that the $^{32}P_i$ is exchanging with organic phosphate already present in collagen. Recent studies on the protein-bound organic phosphorus content of various highly purified collagens (Fessler, 1960; Glimcher and co-workers[6]) have shown the presence of sufficient amounts of organic phosphorus to account for such an exchange. However, this seems unlikely in view of the findings that the $^{32}P_i$ once incorporated into the collagen as organic phosphate does not exchange with nonradioactive phosphorus.

In addition to the organic phosphate and to the rapidly reversible and presumably electrostatic collagen-phosphate interaction, there is also a very tightly bound phosphate moiety, which is difficult to remove either by dialysis, electrophoresis, or filtration, but which reacts as P_i on chemical extraction. There thus appears to be a whole spectrum of phosphate bonds formed with collagen after incubation with P_i, ranging from the simple reversible electrostatic bond on the one hand to an organic phosphate link at the opposite extreme.

These interaction properties of collagen and inorganic phosphate meet a number of the criteria described by Glimcher and Krane (1962), necessary in order that inorganic ions bound to organic matrices may participate in either crystal nucleation or crystal growth, and offer further evidence for the postulated role of phosphorus in the mineralization of collagenous matrices (Glimcher, 1960; Glimcher and Krane, 1962).

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[5] Personal communication.

[6] In preparation.

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