Studies in Procollagen. I. Solubility.* (20668)

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In the speculations regarding the mechanism of formation of collagen fibers, the existence of a soluble collagen precursor has frequently been suggested. Such a possibility was amplified by the demonstration by Nageotte(1) that collagen fibrils could be precipitated by sodium chloride from dilute acetic acid solutions obtained from rat tail tendon. More recently Orekhovitch *et al.*(2) have demonstrated that treatment of mammalian skin with citrate buffers (pH 3.0-4.0) extracts a protein which readily forms fibrils resembling collagen both morphologically and in amino acid composition (3-6). These workers have named this substance "procollagen" because it appears to be a metabolic precursor of collagen.§ Although interactions of collagen with hyaluronic acid (HA), chondroitinsulfuric acid (CSA), and with heparin have been observed, it has not yet been demonstrated that these acid mucopolysaccharides are combined with collagen in ground substance or participate in fibrogenesis (4,5,7-9) The possible existence of a soluble collagen precursor in connective tissue is of great importance with respect to the metabolism of collagen as well as the behavior of ground substance. In view of the demonstrated existence of the above named polysaccharides their possible interaction with soluble collagen precursors is obvious. Most investigations have been concerned with various means of producing collagen fibrils from solutions of procollagen. It seemed of importance, however, to determine the factors which may be responsible for increasing the solubility of this protein and its possible interactions with other components of ground substance.

This report presents experiments concerned mainly with the effect of certain small ions on solubility of procollagen.

Experimental. Preparation of procollagen. After sacrifice by air embolism, adult albino rabbits were immediately skinned. After removal of the subcutaneous layer, the skin was ground in an electrical meat grinder and either stored in the deep freeze (for periods up to 2 months) or immediately extracted by the procedure of Orekhovitch, *et al.*(2). This involved extraction by stirring at 1° with a 5-fold volume of 0.3 M disodium phosphate for 24 hours. The supernatants obtained after centrifugation were combined and dialyzed against distilled water at 1°. The "globulins" were precipitated by 50% saturation with ammonium sulfate and removed by centrifugation; the "albumins" were then precipitated by saturation of the supernatant with ammonium sulfate. The residue was washed with small amounts of citrate buffer at pH 4.0 and then extracted 6 times with 5-fold portions of McIlvaine (phosphate-citrate) buffer of pH 4.0 by stirring at 1° for 24 hours.‖ Each

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§ Metabolic studies of procollagen of the rat have been carried out with the use of carboxyl-labeled glycine by Orekhovitch, *et al.*(2). They concluded therefrom that animal skin contains a series of procollagens which are metabolic precursors of collagen. The same conclusion was reached by Harkness and Neuberger(6) and was based upon similar metabolic experiments with the rabbit.

‖ Electron micrographs of the skin residues remaining after each extraction showed that extraction with disodium phosphate solutions caused little, if any, change in the structure of the collagen fibril. However, extraction with citrate buffer had a progressive effect resulting in very extensive swelling and disorganization of the collagen fibrils. A similar disintegration of collagen fibrils is obtained by treatment of rat tails tendon with dilute acetic acid(10). It may be that procollagen is a structural component of the collagen fibril. We are grateful to Dr. Friedrick Wasserman of the Argonne National Laboratories for aid with electron microscope studies.

extract was dialyzed against a large excess of 0.01 M disodium phosphate at 1°. The voluminous, fibrous precipitate was washed several times with distilled water and stored moist at 4°. Solutions of the above preparations were obtained by extraction with citrate buffers at pH 3.0 or 4.0 followed by centrifugation for 1 hour at about 20,000 times gravity. The protein content was determined by nitrogen analysis and calculated, based upon a nitrogen content of 16%. Even after prolonged centrifugation at 20,000 times gravity the solutions were frequently faintly hazy. However, it was observed that procollagen which yielded clear solutions could be obtained if dialysis of the extract was allowed to proceed against the same buffer in 2 stages, the first stage yielding a small precipitate of material which was discarded, the second yielding the bulk of the precipitable protein.

Uniformity of procollagen preparations. Procollagen, unlike most animal proteins, yields highly viscous solutions at low concentrations. It appeared that viscosity measurements would furnish an index to uniformity and relative freedom from contamination. Procollagen preparations obtained in successive extracts from skin were dissolved in McIlvaine buffer pH 4.0 and adjusted to a concentration of 0.060%. The viscosity relative to buffer (ratio of respective flow times) was determined for each solution in an Ostwald viscometer at 30°. It was found that the viscosity of a solution declined with time at 30° but appeared to become constant after about one hour. For the 1st, 2nd, 5th and 6th extracts the relative viscosities obtained were respectively 2.00, 2.19, 2.15 and 2.24 indicating that, with the exception of the first extract, the preparations were of uniform viscosity (within experimental error). The ultraviolet absorption spectrum of preparations from six successive extractions of skin all closely resembled those reported for gelatin(11), and were similar to those previously reported for procollagen preparations(2). In no case was there any indication of a tyrosine peak in the range of 278-282 mμ.

Solubility of procollagen. Procollagen is soluble in dilute acid, insoluble at neutral or alkaline pH and readily precipitated by low concentrations of neutral salts(2,12). The effect of pH and ion concentration on the solubility of procollagen was further investigated by two separate technics. In a dilution technic, a concentrated solution of procollagen in citrate buffer was mixed with solutions of various ions, whereas in another more direct method, precipitated collagen was shaken with the solution to be tested. In either case the solutions were centrifuged and the amount of dissolved protein calculated from the nitrogen content. A 0.7% solution of procollagen (2nd extract) in McIlvaine buffer, pH 4.0, was dialyzed in a cellophane casing against 100 volumes of 0.1 M citrate or acetate buffer at 4° for 24 hours. Precipitation of protein was noted only in the solution dialyzed against the pH 5.42 acetate buffer. The contents of each casing were diluted 10-fold with distilled water and allowed to stand at room temperature for 3 hours. Precipitates were removed by centrifugation and supernatants were analyzed for nitrogen. Table I shows the amounts of procollagen remaining in the supernatants in the 0.01 M buffers at several pH's. The data suggest that at 0.01 M buffer concentration procollagen is equally soluble in the presence of citrate and acetate ions. However, at higher buffer concentration citrate is more effective than is acetate at pH's above 5.4. A separate dialysis experiment with 0.1 M acetate buffer at pH 5.4 showed that less than 0.15% procollagen was in solution as compared to 0.7% in 0.1 M citrate buffers of pH 6.0 and 7.1. In other experiments, a 0.5% solution of procollagen in 0.1 M citrate buffer pH 6.8, was diluted 10-fold with various solutions to yield a final pH of 5.8 to 6.2. All of the protein remained in solution when the diluent was 0.1 M citrate buffer or 0.1 M $CaCl_2$. Less than 10% of the protein remained dissolved when the diluting solution was 0.1 M $NaHCO_3$,

TABLE I. Effect of pH on Solubility of Procollagen in 0.01 M Citrate and 0.01 M Acetate Buffers.

Citrate		Acetate	
pH	Protein in sol., %	pH	Protein in sol., %
4.24	.065	3.92	.057
4.98	.003	4.86	.030
6.02	.002	5.42	.002
7.08	.002		

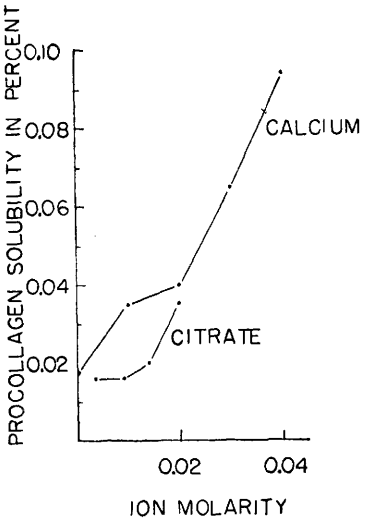

FIG. 1. Effects of citrate and calcium concentration on solubility of procollagen in Krebs-Ringer-carbonate, pH 7.4-7.5 by the dilution method. The solutions containing various amounts of added calcium also contain 0.003 M citrate.

0.1 M NaCl solutions containing 0.3% skin albumin, 0.3% skin globulin, or 0.1% mixed acid mucopolysaccharides from rabbit skin.

Effect of calcium and citrate on solubility. Procollagen in 0.03 M citrate, pH 6.8, was diluted with a solution of Krebs-Ringer-carbonate buffer of pH 7.4-7.5 containing various amounts of citrate and calcium. From the results shown in Fig. 1, it appears that calcium and citrate exert a strong solubilizing effect on procollagen. It was also found that phosphate inhibited the solubilizing action of calcium on procollagen. This action of phosphate may be interpreted as due to association with calcium in competition with procollagen. The increased solubility of procollagen in the presence of calcium can be reversed by dialysis of the solution against the same buffer without calcium. Preliminary experiments had indicated that the rate of solution of protein from the first extract was slightly greater than that of material from succeeding extracts. This finding suggested the possible presence of a small amount of contaminating protein in procollagen from the first extract. When different amounts of protein (pooled fractions) were shaken for 8 hours at 0° with a fixed volume (5 ml) of Krebs-Ringer-carbonate buffer of pH 8.5 containing 0.02 M citrate but no calcium, the amount of protein in the supernatant increased with increase in the amount of solid protein taken in the test (Fig. 2). No difference was found between solubility values obtained in 8 hours or in 16 hours. It appears possible that the contaminating protein is a minor component (estimated as about 3%) with a solubility greater than that of procollagen. Thus, in the following solubility experiments it would be expected that a small but fixed amount of impurity would go into solution, but that changes in protein content of the supernatant found would be due only to the changed solubility of procollagen. Moist procollagen (combined extracts) was shaken with Krebs-Ringer-carbonate solutions at 0° for 8-24 hours. Between 60 and 70 mg of total protein was taken for each 5 ml of solution. After 8 hours, the samples were centrifuged at 0°, and a small amount of supernatant removed for analysis. The remainder of the material was shaken for an additional 8 or 16 hours and the supernatants analyzed once more. No change in solubility was noted after 8 hours.

The results are shown in Fig. 3 and 4. In the presence of citrate, calcium addition at first reduces the amount of procollagen dissolved, but when present in molar excess over citrate, increases the protein solubility. This mutual antagonism of calcium and citrate suggests that the free ionic concentrations of these

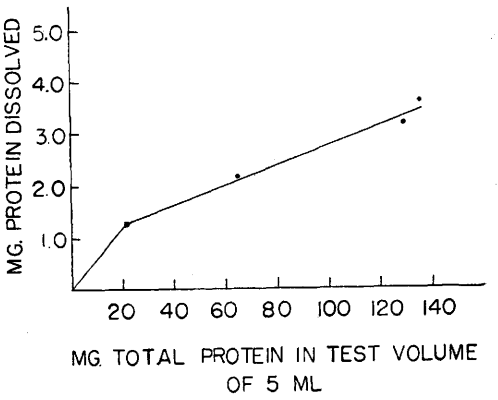

FIG. 2. Effect of total protein present on solubility. Protein present in supernatant after 16 hr at 0° when various amounts of procollagen pooled from first 3 extracts are shaken with 5 ml of Krebs-Ringer-carbonate solution, pH 8.5, containing 0.02 M citrate and no calcium.

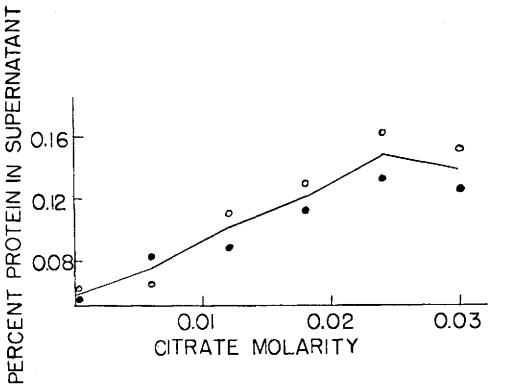

FIG. 3. Solubility of procollagen in Krebs-Ringer-carbonate solution with varying citrate concentrations. No calcium was present and pH was 7.8 to 8.0. Solubility after 8 hr indicated by ○; after 16 hr by ●.

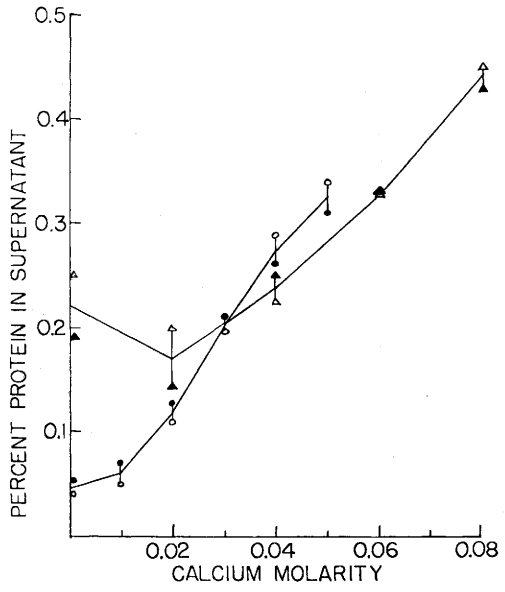

FIG. 4. Solubility of procollagen in Krebs-Ringer-carbonate solution with varying calcium concentrations. The pH was 6.7 to 6.8. Solubility in absence of citrate after 8 hr indicated by ○; after 24 hr by ●. Solubility in presence of 0.03 M citrate after 8 hr indicated by △; after 16 hr by ▲.

substances have been reduced by complex formation with each other.

It is of interest that glucuronic acid had a definite but much weaker solubilizing action on procollagen than does citrate. Under comparable conditions, 0.1 M glucuronic acid is equivalent in effect to 0.012 M citrate (Fig. 3). In these experiments, however, an unknown amount of the uronic acid is present as lactone. Chondroitinsulfuric acid from bovine cartilage in concentrations up to 1% or mixed acid mucopolysaccharides from rabbit skin (0.3%) had little or no effect on the solubility of procollagen in neutral Ringer-carbonate buffers.

Discussion. The solubilizing action of citrate may be interpreted as due to binding of citrate by procollagen, which reduces the electrostatic attractive forces between procollagen molecules in the fibril. A similar interpretation may be given for calcium ion. However, an alternative explanation may also be offered based upon the assumption that part of the strong molecular interaction in procollagen is due to the presence of an acid mucopolysaccharide which interacts with calcium(13). Some evidence in support of this assumption is given by the experiments of Jackson(8) who showed that prior treatment of collagen (calf tendon) with hyaluronidase (presumably to remove chondroitinsulfuric acid) very greatly increased the amount of collagen brought into solution by dilute acetic acid. Thus, the absence of demonstrable effect of added acid polysaccharides on the solubility of procollagen should not be taken to exclude the possibility of interaction between procollagen and acid mucopolysaccharides already present in the protein preparation.

Summary. 1. Procollagen has been prepared from rabbit skin and its solubility characteristics studied. 2. Both citrate and calcium ions exert a strong solubilizing action on procollagen. Added acid mucopolysaccharides from skin and chondroitinsulfuric acid from cartilage show little, if any, influence on the solubility of procollagen.

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Influence of Dietary Bile Salts on Blood Cholesterol Levels. (20669)

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Mueller(1) carried out the first critical experiments on the role of bile in absorption of cholesterol. When a cholesterol or cholesterol oleate emulsion was fed to dogs in which the bile was diverted from the intestinal tract there was no significant rise in the total cholesterol of the lymph as compared to normal dogs. Schoenheimer(2) also observed that the serum cholesterol of rabbits was increased more after feeding cholesterol with desoxycholic acid than after cholesterol alone. Kim and Ivy(3) found that the serum cholesterol of rats was significantly raised when cholic acid was added to a diet containing cholesterol and oleic acid. When bile acid was added to a cholesterol-free diet no significant increase in blood cholesterol was observed. Recently Siperstein *et al.*,(4) reported that when labeled cholesterol was fed to bile fistula rats none of the labeled compound could be recovered in the lymph. It was suggested that bile was obligatory for the absorption of cholesterol. In addition to bile, pancreatic secretions and fat have been shown to be intimately related to cholesterol absorption(5,6). There is good evidence that esterification of cholesterol is an important step in its absorption and that this process takes place in the intestinal wall(7,8) under the influence of pancreatic cholesterol esterase. In connection with studies (9-11) on this enzyme, it has been demonstrated that it will synthesize and hydrolyze the long-chain fatty acid esters of cholesterol only in the presence of bile salts. In the preceding paper(11) the effect of different bile salts on cholesterol esterase activity *in vitro* was demonstrated. The type and amount of bile salt present in substrate mixture is important in the activity of the enzyme. Bile salts having 3, 2, and 1 hydroxyl groups respectively showed a decreasing order of activity while dehydrocholate with no hydroxyl groups was found to be inactive in promoting either synthesis or hydrolysis. Also the conjugated bile salts, taurocholate and glycocholate showed differences in activity when compared to cholate.

The purpose of the present investigation was to determine, using the blood cholesterol level as an indicator of cholesterol absorption, if different bile salts had different effects on cholesterol absorption comparable to the relations between bile salt structure and cholesterol esterase activity *in vitro* demonstrated in the preceding paper(11). If comparable effects could be demonstrated this would be added evidence that the requirement of bile salts for the absorption of cholesterol is due to its being necessary for cholesterol esterase activity and that cholesterol esterase is directly involved in cholesterol absorption.

Methods and materials. Forty-five rats from our stock colony and raised on Purina pellet chow were used. The animals were housed in separate cages and fed the experimental diets and water *ad libitum.* Following a control period on pellet chow during which the blood cholesterol fractions were determined, the rats were divided into two series.