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Calcification XXVII. Collagen Transformation under Influence of Calcium and Phosphate Ions.*† (25231)

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Earlier studies have shown that fibers of acid-soluble collagen reconstituted with chondroitin sulfate mineralize *in vitro* with formation of apatite crystals(1-4). Such fibers have the non-striated form(5,6). The collagens occurring in animal tissues generally have a periodicity of 640 Å, although in preosseous cartilage which mineralizes, no periodicity is discernible under electron microscopic examination. Glimcher, *et al.*(7), have reported that, of the various types of reconstituted collagen fibers, only those with 640 Å spacings are able to nucleate hydroxyapatite. The question arose whether non-striated collagen fibers undergo any structural changes during incubation in calcifying solution.

Methods. Solutions of acid soluble collagen in 0.04% acetic acid were prepared from rat tail tendon as previously described(4). Collagen fibers were reconstituted by mixing samples of 5 ml of the collagen solution with 0.25 ml of a 1% solution of whale tracheal chondroitin sulfate‡ in 0.04% acetic acid. Fibers were further reconstituted from the collagen solution by adding a 30% NaCl solution up to a final concentration of 1% NaCl. The precipitates obtained with chondroitin sulfate were washed with distilled water, and those obtained with NaCl were washed with a solution of 1% NaCl. The washed precipitates were employed for further studies.

The basal salt solution used in the experiments was a carbonate buffer containing in mE/L, sodium: 95.0; chloride: 80.0; bicarbonate: 22.0; and potassium: 5.0. This solution was diluted 10 times with doubly-distilled water. If needed, Na_2HPO_4 and/or $CaCl_2$ stock solutions were added to the diluted basal salt solution, resulting in concentrations of 5 mg % P and 15 mg % Ca. The pH of the solutions was adjusted to 7.3 ± 0.05, as described earlier(8). The ionic strength of the solutions employed was about 0.10.

Collagen fibers reconstituted with chondroitin sulfate were incubated in diluted basal salt solution containing calcium or phosphate or both calcium and phosphate (calcifying solution) at pH 7.3 ± 0.05 and 37°C. Time of incubation in the various solutions was 1 and 2 days. Incubation in calcifying solution was also carried out for 6 hours. At the end of incubation the fibers were washed with distilled water until, after adding a few drops of 3% $AgNO_3$ solution, no precipitation of AgCl occurred in the washing fluid. The fibers were then stored in distilled water in the refrigerator for use in electronmicroscope studies. Collagen fibers reconstituted with NaCl were used without further pretreatment.

Samples used for the electronmicroscope studies§ were cut in fine segments with surgi-

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‡ We wish to thank Prof. Erik Jorpes for this preparation of chondroitin sulfate, prepared according to method of Strandberg (*Acta Physiol. Scand.*, 1950, v21, 222) and probably consists of a mixture of chondroitin sulfate A and C.

§ Electronmicroscopy done by Ladd Research Industries, Roslyn Heights, L. I., N. Y.

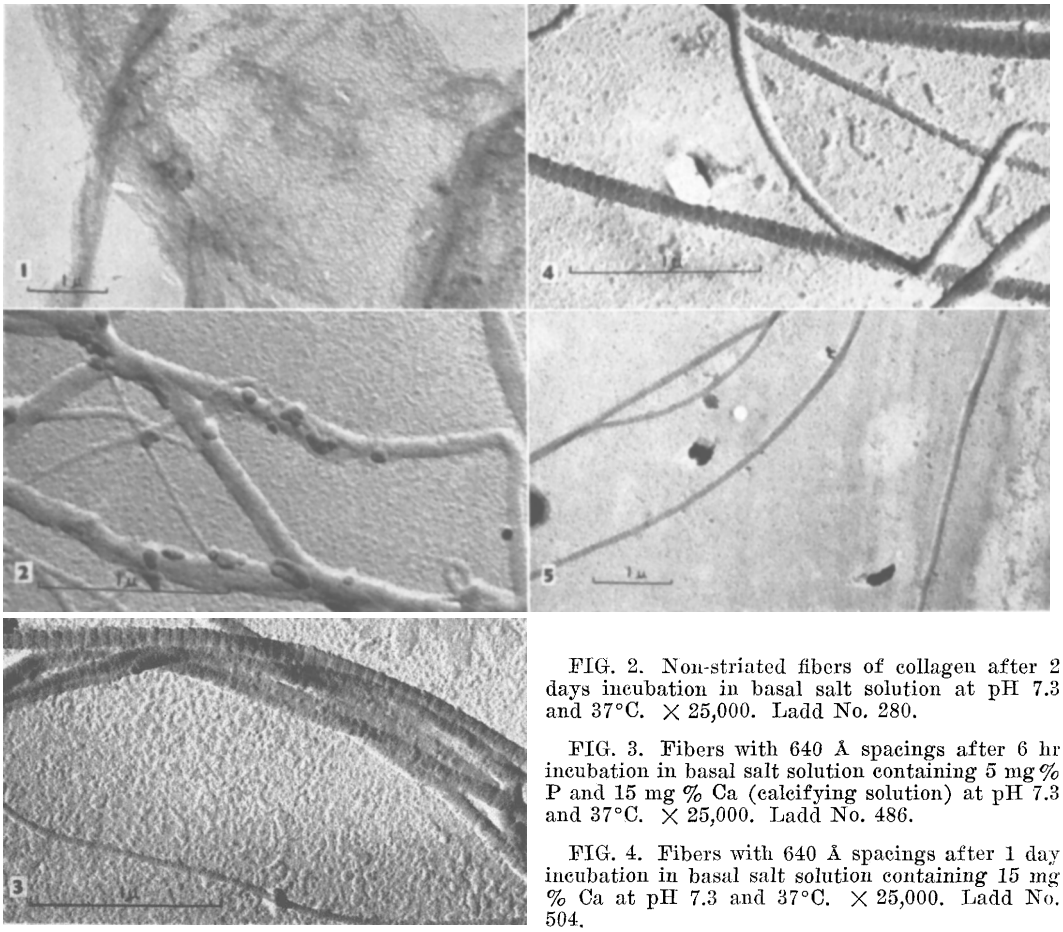

FIG. 1. Non-striated sheets of collagen fibers reconstituted with chondroitin sulfate. × 10,000. Ladd No. 264.

FIG. 2. Non-striated fibers of collagen after 2 days incubation in basal salt solution at pH 7.3 and 37°C. × 25,000. Ladd No. 280.

FIG. 3. Fibers with 640 Å spacings after 6 hr incubation in basal salt solution containing 5 mg % P and 15 mg % Ca (calcifying solution) at pH 7.3 and 37°C. × 25,000. Ladd No. 486.

FIG. 4. Fibers with 640 Å spacings after 1 day incubation in basal salt solution containing 15 mg % Ca at pH 7.3 and 37°C. × 25,000. Ladd No. 504.

FIG. 5. Fibers with 640 Å spacings after 1 day incubation in basal salt solution containing 5 mg % P at pH 7.3 and 37°C. × 10,000. Ladd No. 439.

cal scissors and dispersed in distilled water. No fixation was used. A drop of the suspension was put down on a Formvar film supported by a microscope grid. Before the specimen was completely dry, it was repeatedly washed with distilled water using a fine bore pipette. When dry the fibers were shadowed with platinum and lightly coated with evaporated carbon for stabilization under the electron beam. Unshadowed specimens were also lightly coated with carbon.

The complete experiments were repeated 3 times and the results were confirmed each time. The prepared specimens were examined in a Philips E.M.—100 B electronmicroscope and micrographs taken of representative fields. In all, some 1,100 micrographs were taken in the study.

Results. Fibers reconstituted with 1% NaCl showed very clearly the 640 Å spacings under the electronmicroscope. Collagen reconstituted with chondroitin sulfate, however, usually showed sheet-like structures without indication of spacings (Fig. 1). After treatment with basal salt solution at pH 7.3 and 37°C for 2 days some rearrangement of the sheet-like structures had occurred, giving rise to fibers of 300-1500 Å thickness, in which no indication of spacings was discernible (Fig. 2). After incubation for only 6 hours in calcifying solution (basal salt solution + 5 mg % P + 15 mg % Ca) at pH 7.3 and 37°C, virtually all sheet-like structures had transformed to the fibrous form with 640 Å spacings (Fig. 3). Treatment with basal salt solution containing either 5 mg % P or 15 mg % Ca also caused

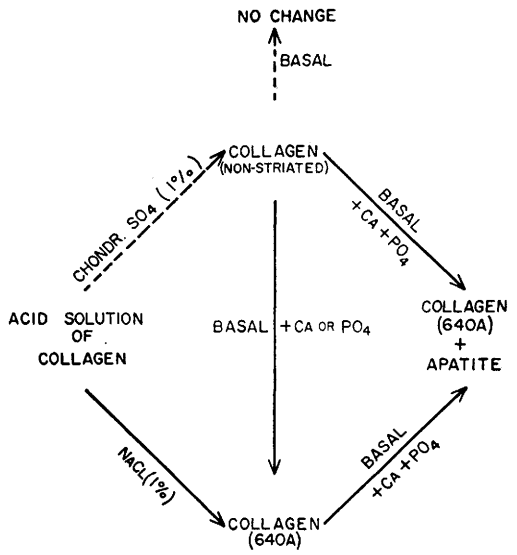

FIG. 6. Influence of calcium and phosphate on transformation of reconstituted acid-soluble collagen fibers (non-striated) to 640 Å spaced striated form.

transformation to this form (Fig. 4, 5), but in several experiments this transformation was only partial, even after 2 days. Transformation to fibers with 640 Å spacings in basal salt solution containing calcium only, occurred to a larger extent than in this solution containing phosphate only.‖

Discussion. These studies show that either calcium or phosphate ions, when present in a basal salt solution at 37°C and pH 7.3, will cause rapid transformation of non-striated collagen fibers placed in this solution to the form with spacings of 640 Å. If both ions are present the transformation is more rapid and more complete. In contrast to this, basal salt solution containing neither calcium nor phosphate ions does not bring about this transformation (Fig. 6). Our finding that fibers reconstituted with 1% NaCl show predominantly 640 Å spacings confirms results obtained by other investigators(9).

That the various types of collagen fibers are interconvertible after reextraction with acid buffers has been reported(10,11). To our knowledge this is the first report on direct transformation of one type of fiber into another by specific agents.

From the results obtained in the transformation studies, one may cautiously suggest that either calcium or phosphate ions interact with a critical site in the non-striated collagen, where an electrostatic bond between positively and negatively charged groups is split, allowing the rearrangement of the patterns of aggregation of tropocollagen units to the 640 Å spaced fibers. Alternately, but less likely, 2 independent sites, one binding calcium and the other binding phosphate, might produce such rearrangement. To illustrate, electrostatic bonds within the collagen fibril which might be ruptured could be bonds between terminal amino and carboxyl groups on the tropocollagen units, or more likely, side chain bonds between the ϵ-amino groups of lysine or hydroxylysine and δ-carboxylic group of glutamic acid or the σ-carboxylic group of aspartic acid(12,13).

A further study to determine degree of specificity of calcium and phosphate ions in this transformation may shed light on the intriguing question whether the groups whose interaction with calcium and phosphate bring about transformation are the same as those responsible for formation of nuclei of hydroxyapatite crystal growth(1-4,7).

Summary. 1) Fibers of acid-soluble collagen reconstituted with chondroitin sulfate showed sheet-like structures without indication of spacings. After treatment for 2 days with basal salt solution at pH 7.3 and 37°C, containing neither calcium nor phosphate ions, non-striated fibers were formed of 300-1500 Å thickness. If either calcium or phosphate ions were present partial transformation to the form with 640 Å spacings took place. If both of these ions were present this transformation occurred more rapidly and more completely. 2) These findings suggest that calcium and phosphate ions interact with sites on the collagen fiber where the breaking of electrostatic bonds catalyzes transformation to the 640 Å spaced fiber.

‖ Crystals seen in photographs originate from salts of basal salt solution, which were not completely washed out during preparation of samples for electronmicroscopy.

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Influence of Bile Acids on Cholesterol Metabolism in the Mouse.* (25232)

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Cholic and dehydrocholic acids alter rates of cholesterol synthesis and mobilization in rat liver and adrenal(1,2,3). Since the results of these and other investigations(4,5) have implicated the bile acids in accumulation of dietary cholesterol and in homeostatic control of cholesterol metabolism; cholic, hyodeoxycholic, deoxycholic and lithocholic acids, were selected for this study of cholesterol metabolism in mouse tissues.

Procedure. Seventy Webster strain female albino mice, weighing 18-20 g, were placed on synthetic basal cholesterol free diet (CFD) (6) for 2 weeks, then divided into 7 equal groups. Groups A and F were continued on CFD; Group B received CFD plus 0.5% cholic acid; Groups C and G, CFD plus 0.5% hyodeoxycholic acid; Group D, CFD plus 0.5% deoxycholic acid; and Group E, CFD plus 0.5% lithocholic acid. After receiving these diets *ad lib.* for 3 weeks, all animals were given an intraperitoneal injection of 5 μc sodium acetate-1-C^{14} in saline. Mice in Groups A, B, C, D and E were sacrificed one hour after injection; those in groups F and G, 15 minutes after injection. Liver, small intestine and kidneys were removed for determination of total cholesterol, as previously described (7), and cholesterol-x-C^{14}. The latter was isolated as the digitonide and counted in a Windowless Flow Counter(1).

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Results. The effects of bile acids on cholesterol levels and synthesis rates in mouse liver, small intestine, and kidney are presented in Tables I and II.

Cholic acid produced a 51% increase in total cholesterol level in the liver, a small but significant increase in intestinal cholesterol, and no change of this level in kidney. At the same time there was a striking decrease in the rate of hepatic cholesterol synthesis. Despite

TABLE I. Effect of Bile Acids on Cholesterol Levels in Mouse Tissues.

Group	Total cholesterol, mg/g* tissue		
	Liver	Small intestine	Kidney
A. Control	5.14 ±1.10	2.21 ±.20	4.42 ±.39
B. Cholic acid, .5%	7.76 ±1.74	2.80 ±.18	4.58 ±.42
C. Hyodeoxycholic acid, .5%	2.82 ± .38	2.23 ±.16	4.43 ±.31
D. Deoxycholic acid, .5%	3.88 ±1.16	1.49 ±.54	3.96 ±.28
E. Lithocholic acid, .5%	3.28 ± .75		

Statistical comparison of values:

Groups	Liver	Intestine	Kidney
A and B	P <.01	P <.01	P = .38
A " C	"	P = .80	P = 1.00
A " D	P = .03	P <.01	P <.01
A " E	P <.01		

* Wet wt.