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*EXTRACTION OF COLLAGEN FROM CONNECTIVE TISSUE BY NEUTRAL SALT SOLUTIONS**

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One of the most challenging biological problems concerning the connective tissue relates to the mechanisms by which collagen is synthesized. Presumably, precursors are formed within the connective-tissue cells and transformed into the organized fibrils in extracellular space. Some information concerning this problem has been obtained in electron microscope studies of collagen-forming connective-tissue cells in cultures and in sections.[1] However, in such preparations structures identified as collagen must have axial periodicity characteristic of this protein. Extremely thin collagen particles whose structure may be beyond the resolution obtainable in such preparations would be either missed or unrecognized as collagen. It has thus far been impossible to demonstrate morphologically the step between the intracellular synthesis of the collagenous protein and its appearance extracellularly in the form of characteristically striated collagen fibrils. The missing link is believed to be the biological "monomer" or collagen particle which presumably is synthesized in the cell and transformed into fibrils in the environment of the extracellular space.

Previous in vitro studies of the precipitation of collagen fibrils have been carried out in acid solutions of collagen.[2, 3] However, physical-chemical studies of such solutions, particularly in the presence of citrate buffer,[4] indicate that the collagen exists as long chains of protofibrils or thin bundles thereof, thousands of angstrom units long and having particle weights of the order of 10^6–10^7.[5] Previous studies by the authors[6, 7] suggested the existence of a type of collagen particle having a length of the order of 2,000 A and being very thin, possibly one or a few polypeptide chains in cross-section. This particle was given the name "tropocollagen."[7] Whether or not tropocollagen is present in the tissues in the dispersed state as a biological precursor of the fibrils is yet to be determined.

In 1951 Highberger, Gross, and Schmitt[3] demonstrated that mildly alkaline phosphate buffers could extract from fresh whole connective tissues soluble collagenous material which, after further manipulation, could be transformed to

characteristic collagen fibrils. The possible relationship of this material to precursors of the fibrils was evident.

Studies, described below, were begun on the properties of collagen extracted in its isoelectric range and on mechanisms involved in its precipitation from solution. During the course of this work several papers appeared by Harkness *et al.*,[8, 9] who, using procedures previously described,[3] demonstrated that the collagen thus extracted had a higher glycine turnover rate than that of any of the proteins of the skin and, indeed, higher than that of the blood proteins. They also showed that, although the glycine turnover of acid-extracted collagen ("procollagen") was somewhat higher than that of the residual collagen fibrils, it was not high enough to indicate an early precursor stage. Possibly the acid-dissolved material derived from freshly formed collagen fibrils.

It seems probable that the collagen extractable from connective tissue by neutral or slightly alkaline solutions of phosphate and other salts is present in the ground substance rather than in the organized fibrils, because (1) purified collagen swells little and dissolves hardly at all in the pH range 5–10.5 (Highberger,[10] Bowes and Kenten[11]); and (2) electron microscope examination of fibrils after salt extraction shows that most of the fibrils are relatively normal in appearance, whereas after extraction by acid or acid buffers (such as in the extraction of the so-called "procollagen" of Orekhovitch) the structural organization of the fibrils is badly disrupted.

MATERIALS AND METHODS

The corium of fresh[12] 3–7-day-old calf skin was cut into strips and coarsely ground in a meat grinder with chips of dry ice to avoid excessive heating. Portions were taken for dry weight and collagen estimation. Except where noted, all steps were performed at 0°–5° C. Aliquots of wet tissue, each weighing 50 gm., were extracted for 18 hours on a shaking machine with 50 or 100 ml. of salt solutions (see Table 1). The extraction media included serum ultrafiltrate. In most cases only one or two extractions of the same tissue sample were carried out. In some experiments the tissue was extracted four times, and each extract was treated separately. In one instance the extraction was carried out 24 times with 0.15 *M* phosphate, pH 8.0 ($\Gamma/2 = 0.45$). The experiment eventually had to be discontinued because of excessive bacterial growth.

The extracts were separated from the tissue by centrifugation in the Model L Spinco preparative centrifuge at 80,000 g for 1 hour and then passed through fine sintered glass filters. The filtrates were usually amber- or red-colored, water-clear, and somewhat viscous.

Aliquots of each extract were dialyzed against repeated changes of cold distilled water. The fluffy precipitates which formed were separated by centrifugation, washed in water in the centrifuge, and lyophilized. The supernates were also lyophilized. All transfers and lyophilizations were performed as carefully as possible to avoid loss of material. Precipitates were examined in the electron microscope. Glycine was determined by the method of Christensen, Riggs, and Ray[13] and hydroxyproline according to Martin and Axelrod's modification[14] of the method of Neuman and Logan[15] on lyophilized samples dried *in vacuo* at 108° C.

TABLE 1

COLLAGEN CONTENT OF SALT EXTRACTS OF CALF CORIUM*

EXTRACTANT	Γ/2	pH	PRECIPITATE		SUPERNATE		COLLAGEN (MG/ML IN EXTRACT)
			Mg. Lyophilized Material/Ml	Gly/Hypro	Mg. Lyophilized Material/Ml	Gly/Hypro	
[3]K_2HPO_4-KH_2PO_4	1.5	7.6	0.84	7.2/3.3	4.9	3.5/0.5	0.2
[1]Na_2HPO_4-NaH_2PO_4	0.45	8.0	2.17	16.1/7.2	4.2	3.2/0.2	1.1
[2]Na_2HPO_4-NaH_2PO_4	0.45	7.6	1.42	15.2/7.35	5.5	3.4/0.3	0.74
[1]Na_2HPO_4-NaH_2PO_4	0.45	7.0	3.82	19.0/10.0	4.4	3.3/0.3	2.7
[4]Na_2HPO_4-NaH_2PO_4	0.19	5.9	2.74	9.2/3.9	3.76	3.2/0.4	0.76
[4]$NaC_2H_3O_2$-$HC_2H_3O_2$	0.45	4.0	2.4	15.3/7.0	6.9	17.8/8.4	5.3
[4]$NaC_2H_3O_2$-$HC_2H_3O_2$	0.19	4.0	4.22	20.4/10.9	2.78	7.1/2.7	3.8
[3]NaCl	2.57	Unbuffered	1.71	8.5/2.8	6.71	3.5/1.3	0.34
[3]NaCl	1.7	Unbuffered	2.2	12.5/5.1	7.23	3.5/0.3	0.8
[2]NaCl	1.5	Unbuffered	1.69	14.0/5.6	7.27	2.9/0.3	0.68
[2]NaCl	1.0	Unbuffered	2.73	15.6/7.7	8.1	3.6/0.3	1.5
[2]NaCl	0.7	Unbuffered	1.87	13.2/6.7	7.0	3.3/0.3	0.89
[2]NaCl	0.45	Unbuffered	1.27	14.0/6.1	7.3	3.3/0.3	0.55
[2]NaCl	0.2	Unbuffered	0.48	14.9/6.7	7.0	3.4/0.3	0.23
[1]Ox serum ultrafiltrate	..	7.4	1.05	10.7/5.8	5.1	3.5/10.2	0.42

* Extracts were dialyzed against water until salt-free. Precipitates and supernates were lyophilized, weighed, and analyzed for glycine and hydroxyproline. Collagen content of extracts was estimated from hydroxyproline values (based on 14 per cent for pure bovine corium collagen), and the amounts of material precipitated from the extract. Superscripts in first column indicate groups of extractions performed on the same sample of tissue.

In order to obtain information concerning the effect of temperature on the dissolved collagen, an aliquot of each extract was heated at 37° C. under toluene or filtered cyclohexane until a gelled opalescent precipitate formed. This was separated by centrifugation, resuspended and washed in fresh buffer in the centrifuge, dialyzed free of salt, and lyophilized. The degree of gelation and precipitation was determined every 15 minutes in all extracts by measuring turbidity in 2-ml. samples (held at 37° C.) in a photoelectric colorimeter.

RESULTS

Preliminary ultracentrifuge and electrophoretic studies were made on the phosphate and NaCl extracts. Extracts made with serum ultrafiltrate were examined by ultracentrifuge only. In all cases two or three slow-moving boundaries were present, together with at least one hypersharp peak. Four or five distinct components were observed electrophoretically. Attempts at separation and characterization of the components are in progress.

Ultraviolet absorption maxima occurred at 2600 A in a number of phosphate extracts, as reported earlier,[6] although this was by no means the rule, and, indeed, in some similar extracts maxima occurred at 2750–2800 A.

Collagen Content of Extracts.—White flocculent precipitates formed on dialysis against water in all cases except in the 1.0 and 1.5 *M* NaCl extracts, which set to stiff, slightly opalescent gels. The data concerning the lyophilized weights of the several salt-free fractions and their collagen content in terms of hydroxyproline and

glycine percentages are given in Table 1. Hydroxyproline content in the precipitates ranged from 4 to 11 per cent for all the salt extracts except at the highest ionic strength. Glycine was estimated relative to hydroxyproline in order to give some indication as to whether or not the collagen was abnormal with respect to hydroxyproline content. In all instances in which appreciable amounts of hydroxyproline were present, the ratio of glycine to hydroxyproline was about the same as that for purified bovine collagen. Thus the amount of hydroxyproline provides a good measure of the proportion of collagen in the various fractions. The hydroxyproline content of the lyophilized supernatant fluids were uniformly below 1 per cent except in the acetate extracts at pH 4, where they ranged as high as 8 per cent. Thus practically all the collagen in the neutral or near-neutral extracts was precipitated by dialysis against water. There was considerable variation in the amount of material extracted from different calf skins by the same extractant. For this reason the quantitative data in Table 1 are of comparative value only where they relate to simultaneous extractions of the same tissue samples by the different media. These groups are identified by superscripts.

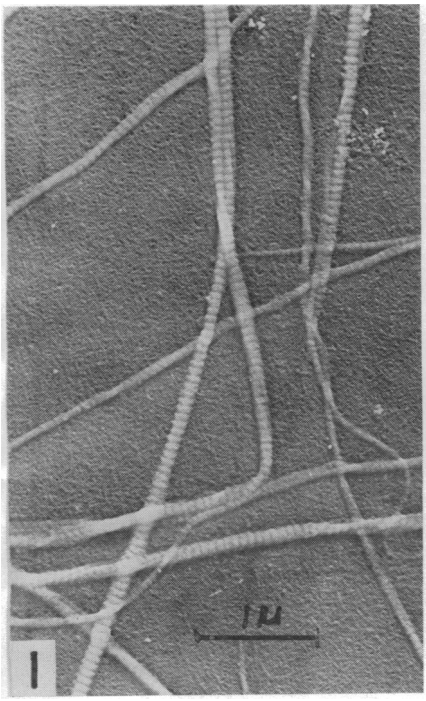
The fact that collagen may be extracted from fresh native connective tissue by physiological media such as 0.2 *M* NaCl and bovine serum ultrafiltrate is in itself evidence that such soluble collagen could hardly have been derived from the formed fibrils of the corium.

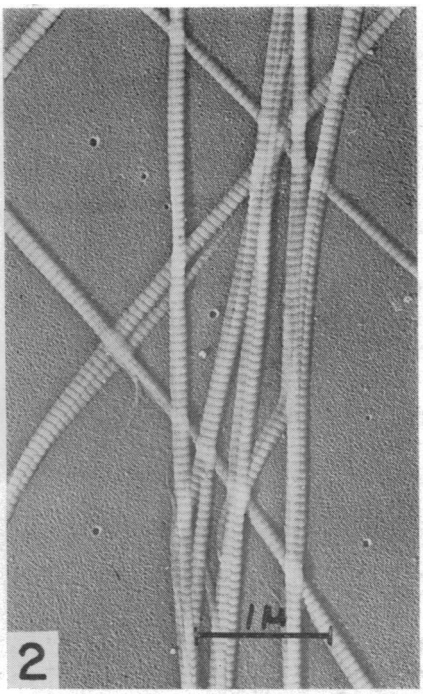
Electron microscope examination of the precipitates revealed amorphous and thin filamentous material in some instances and in other cases large tangled masses of a long-spacing type of fibril displaying strongly accentuated double bands about 2000 A apart. This variability is as yet unexplained; it may relate to undetermined differences in composition between tissue samples. The precipitated collagen is completely soluble in the cold extraction media. However, much of the precipitated noncollagenous material remains insoluble.

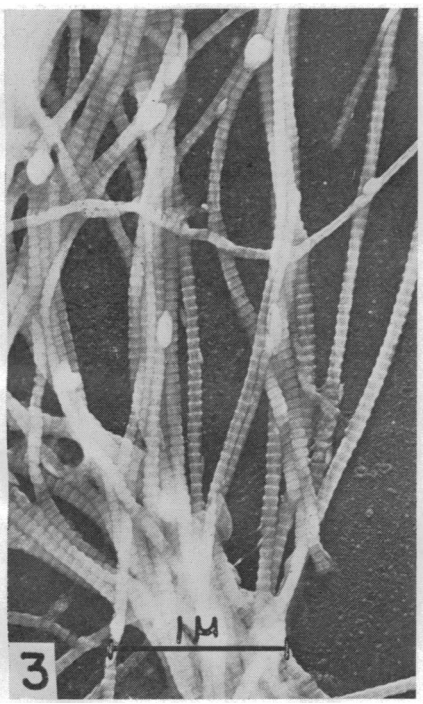
In one experiment in which 400 gm. (dry weight) of calf corium were extracted four times with 10 per cent NaCl and 24 times with 0.15 *M* phosphate pH 8, a total of 38 gm., or 9.5 per cent of the total weight, was extracted. Soluble collagen (as estimated on the basis of hydroxyproline content, assuming 14 per cent for pure collagen) comprised 6.7 per cent of the material extracted and 0.6 per cent of the total corium. Even after 24 extractions the yield per extract was still appreciable although considerably diminished. Experiments of such long duration are complicated by bacterial growth, and one must reckon with the possbility of bacterial attack on the insoluble collagen.

Precipitation of Collagen from Neutral Salt Extracts by Heating.—It had been noted early that phosphate extracts which had been allowed to stand at room temperature for several days formed precipitates which, when examined in the electron microscope, proved to contain well-structured collagen fibrils. Heating these extracts at 37° C. greatly accelerated the precipitation. The extracts at pH 4 did not gel on warming, although fine amorphous precipitates formed.

Nearly all the collagen was precipitated, as indicated by the hydroxyproline and glycine content of the lyophilized precipitates and supernates (Table 2). The collagen thus precipitated from solution was quite pure, as was shown by the analyses and electron micrographs (Figs. 1 and 2).







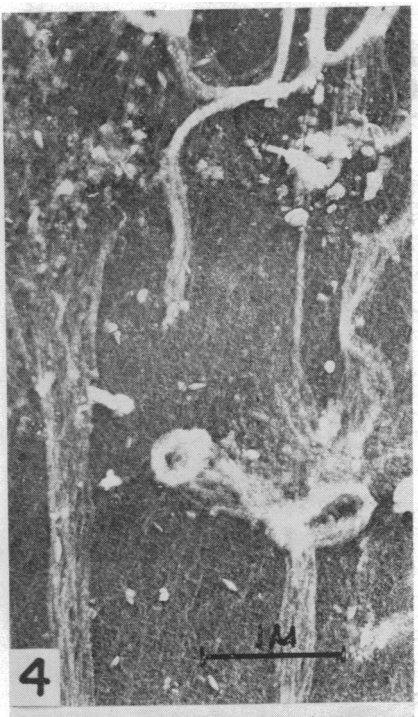


FIG. 1.—Collagen fibrils precipitated from a 0.45 *M* NaCl extract of calf corium by warming to 37° C. ×15,600.

FIG. 2.—Same as Figure 1, except that the source of collagen was the carp swim bladder tunic. × 16,700.

FIG. 3.—Collagen fibrils remaining after extraction of calf corium with 0.45 *M* NaCl. × 22,000.

FIG. 4.—Collagen remaining after extraction of calf corium with acetate buffer (pH 4.0, $\Gamma/2 = 0.45$). Note extensive disorganization of fibrils. × 18,000.

TABLE 2

PRECIPTATION OF COLLAGEN FROM CRUDE SALT EXTRACTS OF CALF CORIUM BY HEATING TO 37° C.

EXTRACTANT	Γ/2	pH	PRECIPITATE		SUPERNATE	
			Mg/Ml*	Gly/Hypro	Mg/Ml*	Gly/Hypro
Na_2HPO_4-NaH_2PO_4	0.45	8.0	0.80	26.4/13.6	6.0	4.2/1.0
Na_2HPO_4-NaH_2PO_4	0.45	7.0	2.12	27.1/13.1	6.1	4.6/1.1
NaCl	0.45	Unbuffered	1.25	24.7/13.1	6.3	4.1/0.1

* Lyophilized weight.

In the early experiments designed to study collagen which had been extracted by salt solutions, the collagen-containing fraction was further purified by dialyzing against water, lyophilizing the precipitate which contained nearly all the collagen, and re-extracting this material with fresh buffer. A considerable amount of the lyophilized material had become insoluble, but all the collagen could be dissolved out in four extractions. The extracts were extremely viscous and still heterodisperse, as was evident by the presence of more than one boundary in the ultracentrifuge. Such extracts, when heated to 37° C., formed stiff opaque white gels within 30 minutes. Electron microscope examination of the washed precipitates revealed typical collagen fibrils and some nonfibrous debris. Hydroxyproline and glycine determinations indicated that the precipitates consisted of fairly pure collagen. The sticky nature of the fibrous precipitates made washing and the preparation of electron microscope specimens difficult. Little hydroxyproline or glycine remained in the supernatant fluids.

Considerable differences were noted in the rate of gel formation on heating the different salt extracts of corium samples, even though they derived from the same animal. Extracts of ionic strength 0.45, 0.7, and 1.0[16] required about 6, 3, and $^1/_2$ hours, respectively, to gel.

The precipitation of collagen from crude salt extracts by heating is irreversible in the sense that cold salt solutions will not redissolve the precipitated collagen. However, weak acidic solutions will partially dissolve it, as they will also dissolve native collagen.

Among the salts tested no specific effects were noticed. Sodium salts of chloride, acetate, phosphate, and oxalate were about equally effective at the same ionic strength and pH. All the phenomena described above were reproduced with rat and guinea pig skin and carp swim bladder tunic.

Examination by electron microscopy of the tissue residues after salt extraction at neutral or near-neutral pH revealed normal unfrayed and unswollen collagen fibrils (Fig. 3). The residues remaining after extraction at pH 4 showed greatly swollen, frayed, and disorganized remnants of fibrils (Fig. 4).

DISCUSSION

It is evident that collagen can be extracted from connective tissues over a wide pH range. However, it is also apparent that the source and properties of the

extracted collagen differ not only with pH of the extraction medium but also with its ionic strength. On the acid side the great swelling and disorganization of the collagen fibrils is consistent with the view that the extracted collagen derives from these fibrils. At low ionic strengths, acid extracts of rat tail tendon or fish swim bladder tunic form clear gels of apparently structureless filaments after dialysis against water, whereas extracts made at the same pH but at higher ionic strength produce a mass of well-striated robust fibrils.

Extracts in the pH range between 5 and 9 differ significantly from those prepared at more acid pH. It is worth noting that this is the isoelectric range of collagen. The collagen fibrils in the residues appear to be unaltered, suggesting that the extracted collagen derived from extrafibrillar sources—either the ground substance or the cells. Practically all the collagen in these extracts may be precipitated in the form of striated fibrils by warming to 30°–37° C.; this is not true of acid extracts. The salt-extractable collagen most probably exists in a dispersed state in the tissue at this temperature. The fact that collagen extracted by cold neutral salt solutions is precipitated irreversibly at body temperature adds further support to the contention that this extractable collagen had not yet been incorporated into the formed fibrils.

It is interesting to speculate that the cell may secrete a precursor or precursors which, at least at one stage, are dispersed in the ground substance in the form of particles of subfibrillar dimensions, perhaps as "tropocollagen." The precipitation of fibrils may then proceed under the influence primarily of environmental factors such as ionic strength and temperature.

The high glycine turnover rate associated with the alkaline phosphate–extracted collagen, as compared to that of the other connective-tissue proteins,[9] strongly suggests a precursor role for this fraction. Whether or not there are metabolic, structural, or chemical differences in the collagen extracted at different ionic strengths remains to be determined.

When a gelatin solution is cooled, relatively larger aggregates are formed; these melt when the temperature is elevated to 28°–40° C.[17] Conversely, elevation of temperature of cold neutral salt solutions of collagen causes irreversible, specific aggregation of collagen chains in the form of cross-striated fibrils.

SUMMARY

Collagen may be extracted from whole intact connective tissues by serum ultrafiltrate and other neutral salt solutions. It is precipitated as typical cross-striated fibrils merely by elevating the temperature to 37° C. Such soluble collagen probably exists in the ground substance of the connective tissue rather than in the fully formed collagen fibrils of the corium. It is suggested that this collagen, possibly in the form of the "tropocollagen" monomer, is a precursor in physiological fibrogenesis.

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[1] K. R. Porter and P. Vanamee, *Proc. Soc. Exptl. Biol. Med.*, **71**, 513, 1949; S. F. Jackson, in J. T. Randall (ed.), *Nature and Structure of Collagen* (New York: Academic Press, 1953), p. 140.

[2] G. Bahr, *Exptl. Cell Research*, **1**, 603, 1950; P. Vanamee and K. R. Porter, *J. Exptl. Med.*, **94**, 255, 1951; H. Noda and R. W. G. Wyckoff, *Biochem. et biophys. acta*, **7**, 494, 1951; J. Gross, *Trans. Fourth Josiah Macy Conf. on Metabolic Interrelationships*, p. 32, 1952; S. F. Jackson and J. T. Randall, in Randall, *op. cit.*, p. 181.

[3] J. H. Highberger, J. Gross, and F. O. Schmitt, these PROCEEDINGS, **37**, 286, 1951.

[4] A. A. Tustanovsky, *Biokhimiya*, **12**, 285, 1947; V. N. Orekhovich, A. A. Tustanovsky, A. A. Orekhovich, and N. E. Plotnikova, *Biokhimiya*, **13**, 55, 1948; V. N. Orekhovich, *Proc. 2nd Internat. Cong. Biochem., Paris*, **1952** (Moscow: Acad. Sci. U.S.S.R., 1952), p. 106.

[5] M. B. McEwen and M. I. Pratt, in Randall, *op. cit.*, p. 158; P. Gallop, in press; S. E. Bresler *et al.* (*Repts. Acad. Sci. U.S.S.R.* [Moscow], **72**, 555, 1950) from ultracentrifuge and diffusion studies report a molecular weight of 70,000 for "procollagen." M. B. Mathews, E. Kulonen, and A. Dorfman (*Arch. Biochem. and Biophys.*, **52**, 247, 1954) report the same figure obtained by osmotic pressure studies on similar solutions. However, neither group determined whether or not the collagen in solution could be reconstituted to form fibrils, and there is reason to believe that in both cases the collagen was degraded to a "parent" gelatin.

[6] F. O. Schmitt, J. Gross, and J. H. Highberger, these PROCEEDINGS, **39**, 459, 1953.

[7] J. Gross, J. H. Highberger, and F. O. Schmitt, these PROCEEDINGS, **40**, 679, 1954.

[8] R. D. Harkness, A. M. Marks, H. M. Muir, and A. Neuberger, in Randall, *op. cit.*, p. 208.

[9] R. D. Harkness, A. M. Marko, H. M. Muir, and Z. Neuberger, *Biochem. J.*, **56**, 558, 1954.

[10] J. H. Highberger, *J. Am. Leather Chemists Assoc.*, **9**, 345, 1936.

[11] J. H. Bowes and R. H. Kenten, *Biochem. J.*, **46**, 1, 1950.

[12] It has been noted that skin which has been frozen solid for more than a week yields very little salt-extractable collagen.

[13] H. N. Christensen, T. R. Riggs, and N. E. Ray, *Anal. Chem.*, **23**, 1521, 1951.

[14] C. J. Martin and A. E. Axelrod, *Proc. Soc. Exptl. Biol. Med.*, **83**, 461, 1953.

[15] R. E. Neuman and M. A. Logan, *J. Biol. Chem.*, **184**, 299, 1950.

[16] It should be realized that these ionic strengths are not the final ones because of dilution by tissue water.

[17] H. Boedtker and P. Doty, *J. Phys. Chem.* **58**, 968, 1954.

TOPOLOGICAL PROPERTIES OF COILED HELICAL SYSTEMS

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In a recent article[1] Max Delbrück proposed a very ingenious way in which a dividing DNA molecule, constructed according to Watson and Crick's helical scheme,[2] can get disentangled and separated into two individual helices. The purpose of the present article is to indicate that there exists another, and in a way simpler and more natural, topological possibility of achieving the same result. In fact, a clean separation of two helices resulting from the division can take place without any breaks if, *prior to the division, the long helical molecule is wound into a coil possessing the same repetition period as the original helix.*

This fact can be easily understood by inspecting the coiling of nonhelical and helical two-stranded systems as shown in Figure 1, *A* and *B*. In the first case (*A*)