† Biochemistry Division, Department of Chemistry.

‡ Department of Microbiology.

§ Department of Botany.

[1] Nirenberg, M. W., and J. H. Matthaei, these PROCEEDINGS, **47**, 1588 (1961).

[2] Nathans, D., G. Notani, J. H. Schwartz, and N. D. Zinder, these PROCEEDINGS, **48**, 1424 (1962).

[3] Tsugita, A., H. Fraenkel-Conrat, M. W. Nirenberg, and J. H. Matthaei, these PROCEEDINGS, **48**, 846 (1962).

[4] Ofengand, J., and R. Haselkorn, *Biochem. Biophys. Res. Commun.*, **6**, 469 (1962).

[5] Ohtaka, Y., and S. Spiegelman, *Science*, **142**, 1 (1963).

[6] Spiegelman, S., in *Informational Macromolecules*, ed. H. J. Vogel, V. Bryson, and J. O. Lampen (New York: Academic Press, Inc., 1963), pp. 27–53.

[7] Reichmann, M. E., these PROCEEDINGS, **52**, 1009 (1964).

[8] Rothman, F., and R. Byrne, *J. Mol. Biol.*, **6**, 330 (1963).

[9] Beaudreau, G., B. Bassel, and S. Spiegelman, in preparation.

---

*SPECIFIC DEGRADATION OF THE COLLAGEN MOLECULE BY TADPOLE COLLAGENOLYTIC ENZYME**

BY JEROME GROSS AND YUTAKA NAGAI†

DEPARTMENT OF MEDICINE, MASSACHUSETTS GENERAL HOSPITAL AND HARVARD MEDICAL SCHOOL, BOSTON

Communicated by John T. Edsall, August 31, 1965

Recently the production of a collagenolytic enzyme by anuran tadpole tissues in culture was demonstrated by lysis and degradation of reconstituted mammalian collagen gels upon which tissue fragments were cultivated *in vitro* under physiologic conditions.[1, 2] The culture chamber acts as a closed reservoir in which the enzyme accumulates over a period of time long enough to reach detectable levels. There is no appreciable amount stored in the tissues at any one moment,[1, 3] nor has the enzyme been obtained by extraction of fresh living or autolyzing dead tissues.

By incubating thin strips of sterile living tadpole tail fin or back skin on filter paper disks floating in amphibian Tyrode solution at 37°C for several days, relatively large amounts of a collagenolytic enzyme appear in the culture medium. The details of preparation, purification, and description of some of the properties of this enzyme will appear elsewhere. In brief, the enzyme has been purified 300-fold from the lyophilized dialyzed culture medium by sequential ammonium sulfate fractionation, Sephadex filtration, starch gel electrophoresis, and DEAE-cellulose chromatography. It has a pH optimum of 6–8, loses activity at 50–60°C after 10 min, is inhibited by ethylenediaminetetraacetate and cysteine, but not by diisopropylfluorophosphate, and has very little activity against casein.[4]

In this report we describe some aspects of the effect of this enzyme on the collagen molecule, the products of which are far different from those resulting from the action of bacterial collagenase.

Methods.—Several different purified acid-extracted calf skin collagen preparations and neutral extracted collagen from the skin of normal and lathyritic guinea pigs and rats and from tadpole tail fins were used in these experiments. Two samples of acid-extracted calf skin collagen were prepared as described by Gross and Kirk[5] and another sample obtained from the laboratory of

Dr. F. O. Schmitt had been purified as described by Rubin *et al.*[6] Neutral extracted guinea pig skin collagen was purified by the method of Gross[7] and that from rat skin was isolated by the procedure of Martin *et al.*[8]

Stock substrate solutions were prepared by suspending 200 mg of lyophilized collagen in 50 ml of cold phosphate buffer, pH 7.6, $\Gamma/2$ 0.4, and agitating on a wrist shaker overnight at 4°C in the cold. The viscous solutions were then dialyzed against 0.25 *M* NaCl for 24 hr with two changes of salt solution, followed by centrifugation at 100,000 *g* for 90 min to remove undissolved material. Solutions were diluted to final collagen concentrations ranging between 0.2 and 0.3 mg/ml.

Purified tadpole collagenase had been kept cold in solution containing 0.1 *M* NaCl and 3×10^{-3} *M* $CaCl_2$ after final elution from DEAE Sephadex. Because of the very small amounts obtained, the enzyme had not been prepared in the dry state; lyophilization proved to be destructive.

A typical reaction mixture consisted of the following: 10 ml of 0.05 *M* Tris, pH 7.6, 5×10^{-3} *M* $CaCl_2$; 10 ml of 0.2% collagen in 0.4 *M* NaCl plus 0.05 *M* Tris, pH 7.6, plus 5×10^{-3} *M* $CaCl_2$; 1 ml of enzyme solution, 0.05 *M* Tris plus 5×10^{-3} *M* $CaCl_2$.

The control solution was prepared in the same manner except that the enzyme was heated at 90°C for 5 min.

An aliquot of the reaction mixture (1–3 ml) was transferred to an Ostwald viscometer and the remainder incubated in the viscometer bath in Erlenmeyer flasks at 20°C.

The change in viscosity was followed in Ostwald viscometers whose flow times for water at 20°C ranged from 30 to 90 sec; generally, no appreciable increase of opacity occurred up to 15 hr. Occasionally slight gelation and increased opacity would appear in the control at 24 hr.

Optical rotation was followed simultaneously with viscosity measurements in a Rudolph photoelectric spectropolarimeter equipped with an oscillating polarizer. Temperature was controlled in the 10-cm jacketed cell by circulating water from a refrigerated constant-temperature bath.

Denaturation temperature was measured in the polarimeter in reaction mixtures dialyzed to pH 4.8 in 0.15 *M* potassium acetate, at a temperature increment of 1°C every 20 min.

Samples were taken periodically during the reaction for analysis by disk electrophoresis on acrylamide gels using the Canalco apparatus. They were denatured at 45°C for 10 min at pH 4.8 and 0.1 ml of the solutions was run at pH 4 at room temperature under conditions described elsewhere.[9]

Samples of reaction mixtures were dialyzed exhaustively against 0.05 *M* acetic acid until free of salts. To 0.5 ml of the reaction mixture containing approximately 1 mg of collagen per ml, 0.2 ml of a 1% solution of free adenosine triphosphoric acid was added; the solutions became cloudy in a few seconds and after 10–60 min standing at room temperature or in the cold the suspension was applied in droplets to carbon-coated parlodion films on standard 200-mesh grids. These were positively stained with 1% phosphotungstic acid at pH 3.5. Specimens were examined and photographed in a RCA EMU 3G electron microscope at magnification of 26,000–50,000 times.

Results.—Purified enzyme solution reduced the viscosity of collagen solutions to about 40–60 per cent of the control values after 2 hr. Addition of fresh enzyme had no further effect. During this period there was no significant change in optical rotation (Fig. 1).

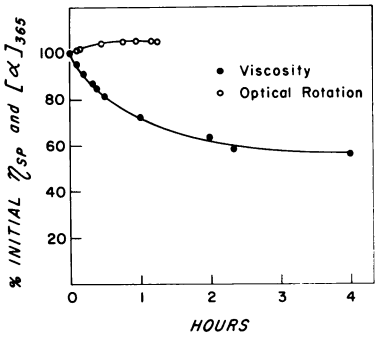

FIG. 1.—Effect of tadpole enzyme on viscosity and optical rotation at 20°C. Purified acid-extracted calf skin collagen, 0.1% in Tris, 0.05 *M*; NaCl, 0.1 *M*; $CaCl_2$, 0.001 *M*; pH 7.5. Control viscosity remained unchanged during 4-hr period. Optical rotation measurements discontinued after 1 hr and 20 min because of slight increase of opacity. In other experiments no change in rotation was observed during 6 hr.

The denaturation temperature (T_m), midway between maximum and minimum optical rotation, of the reaction products was about 5°C lower than that for native collagen (Fig. 2). The shape of the melting curve for the digested samples differed from that of the control in the presence of a small hump at the end and a somewhat broader melting range.

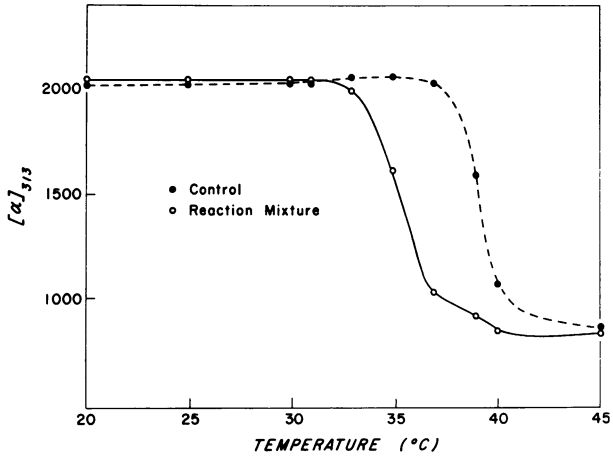

FIG. 2.—Melting curve of enzyme-collagen reaction mixture and that of collagen plus heat-inactivated enzyme. Purified acid-extracted calf skin collagen, 0.05% in acetate buffer, pH 4.8, Γ/2 0.15.

Disk electrophoresis: Figure 3 shows the disk electrophoretic patterns of the purified enzyme, the reaction mixture at time 0, and the changes in the reaction products at sequential times after incubation. The enzyme activity had been localized to the single band seen in the first tube by slicing an unstained gel and measuring activity in the eluted protein. The reaction mixture at 0 time showed only the usual α and β bands[9] plus material concentrated at the top of the running gel which

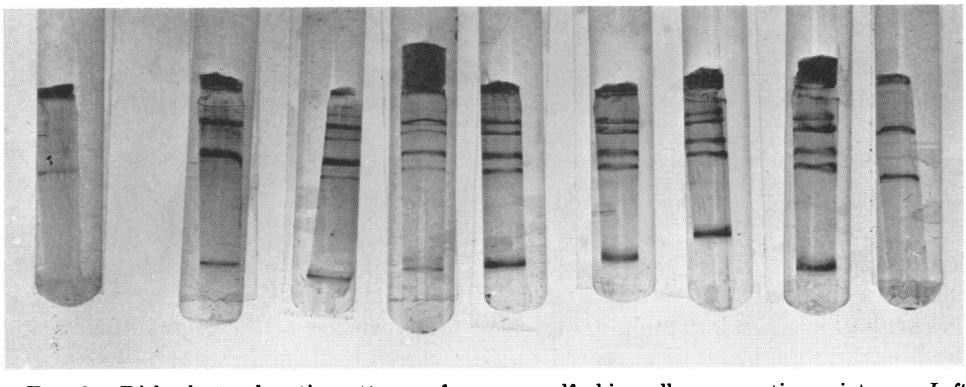

FIG. 3.—Disk electrophoretic patterns of enzyme-calf skin collagen reaction mixture. *Left to right:* purified enzyme, 0 time reaction mixture, 10-min incubation period, 20-min, 40-min, 2-hr, 4-hr, 8-hr, 24-hr. Sample buffer, acetate 0.06 *M*, pH 4.6; lower gel, pH 4.2, 0.06 *M*; upper gel, pH 5.8, 0.06 *M*; 5 ma/tube for 40 min.

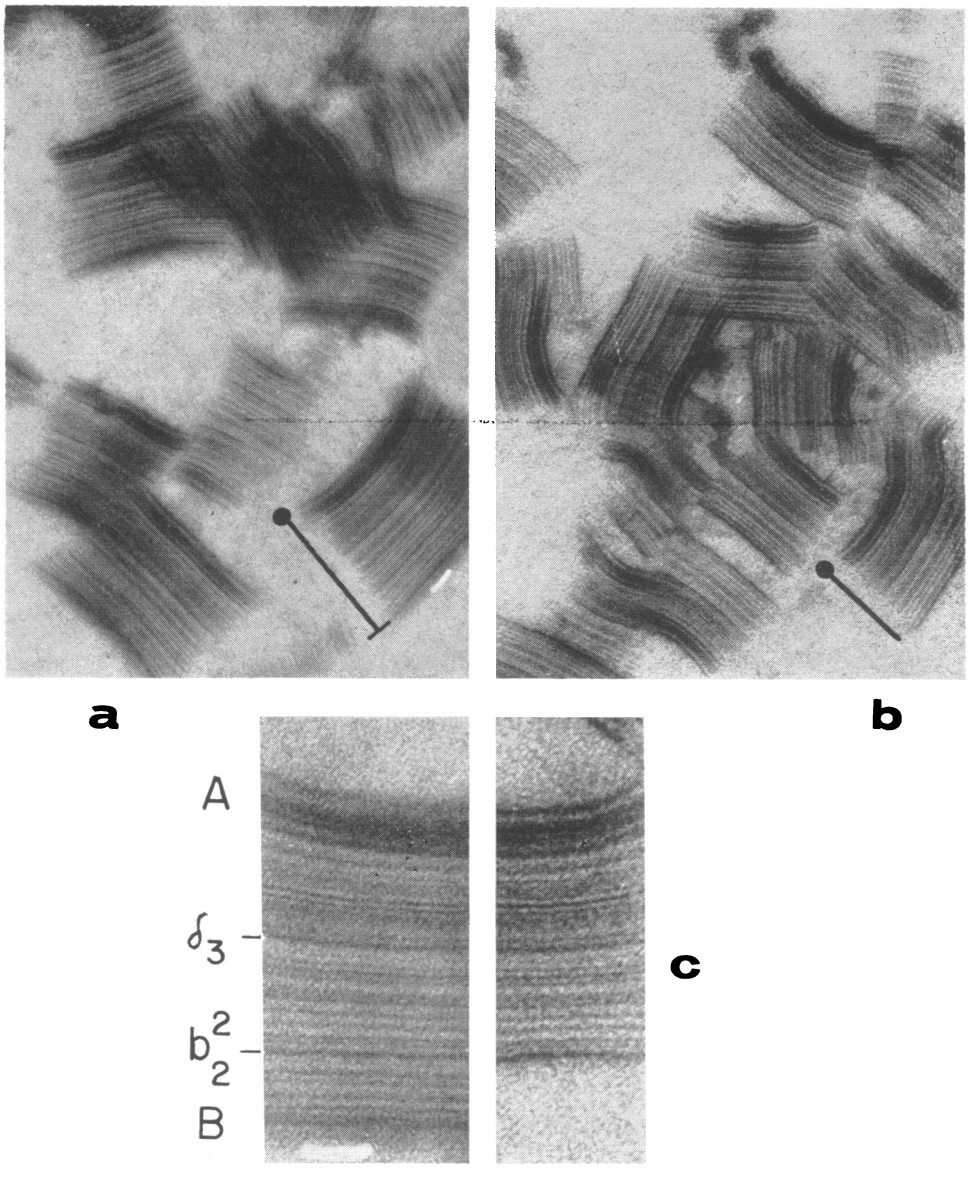

FIG. 4.—(*a*) SLS of normal calf skin collagen; (*b*) SLS of three-quarter-lengths TC^A fragments from reaction mixture. Magnification, × 66,220; (*c*) SLS of normal collagen compared with that of TC^A. Magnification, × 180,000.

is probably γ and higher aggregates. After 10 min incubation the reaction products showed one new band below each of the three original ones. With increased time of incubation these became more intense at the expense of the original bands and within 24 hr the latter disappeared, to be replaced completely by the three faster-moving components. There was often an apparent increase in staining at the solvent front although in the 24-hr sample in this experiment it was not seen, probably having run off the end of the gel in this experiment. Following shorter elec-

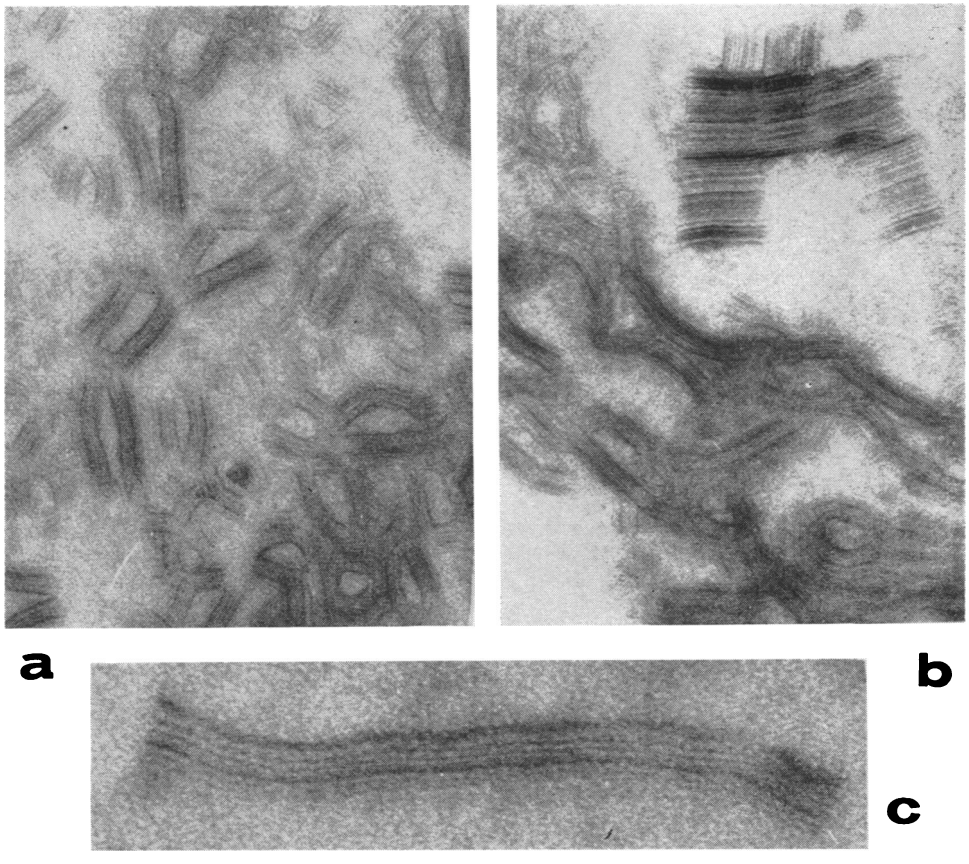

FIG. 5.—(*a*) One-quarter-length SLS of the short fragment TC^B. Magnification, × 56,700; (*b*) a similar field showing segments of TC^B, and in addition a group of segments of the large fragments TC^A aggregated in a dimeric fashion; (*c*) an enlarged picture of a single broad SLS of the short fragment of TC^B. Magnification, × 123,000.

trophoresis time the material near the solvent front was seen to consist of two distinct bands in the experimental samples.

Electron microscopy: Large areas of the grids prepared from ATP precipitates of the reaction mixtures contained only three-quarter-length segment long spacings (SLS) representing the *A* end[10] fragment, called here TC^A. Figures 4*a* and *b* compare typical fields of normal SLS with those formed from TC^A. All the segments in the field of Figure 4*b* are missing one quarter the molecular length at the *B* end. Cuts at any other locations have not been observed in any of many preparations. Figure 4*c* shows more clearly the point of severance at the b_2^2 band of Hodge and Schmitt.[10] Results with acid and neutral extracted collagens from calf, guinea pig, normal and lathyritic rat skins, and tadpole tail fin were indistinguishable. Segments of TC^A tended to aggregate as dimers with the cut ends in apposition as seen in Figure 5*b*. On occasion they formed fairly long fibers with alternating apposed severed ends and apposed normal *A* ends.

In comparing the length of segments TC^A with the normal, corrections for differences in magnification and distortion in the different electron micrographs were

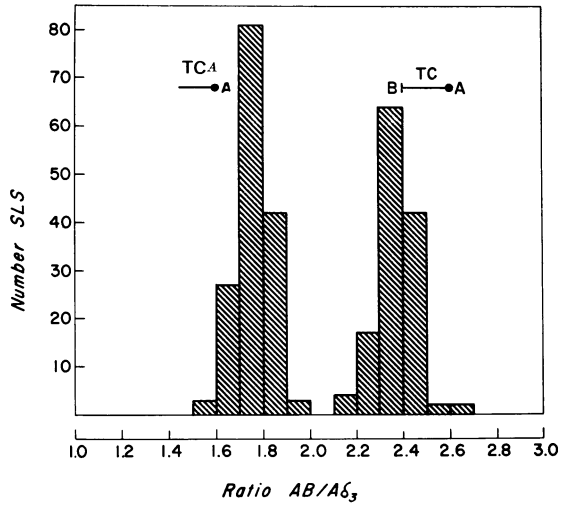

FIG. 6.—Frequency distribution curves of ratios $AB/A\delta_3$ for segments of normal collagen molecules and those prepared from the long fragments TC^A.

made by comparing the length of each segment with a well-defined landmark within the same segment, the δ_3 band.[10] Thus, in Figure 6 the distribution of the ratio $AB/A\delta_3$ is plotted for normal SLS and for those from TC^A.

The ratio of $AB/A\delta_3$ of the TC^A fragment SLS to that for normal segments indicates the former to be 74.4 ± 0.1 per cent of the normal segment or molecular length.

In other fields there were found large areas of short segments of varying width representing one-quarter lengths from the B end, called here TC^B, and illustrated in Figure 5. In Figure 5*b* there is a rare example of segments of TC^A in the same field with TC^B. Figure 5*c* is an enlargement of a segment of TC^B. These short TC^B segments frequently aggregate to form stacks of as many as six or even eight. The structure within the TC^B segments is always the same and always resembled that of the B end of normal SLS. Detailed fine structural studies will be reported elsewhere.

Discussion.—The rapid but limited fall in viscosity of the enzyme-collagen reaction mixture without significant change in optical rotation suggested that the enzyme caused either a reduction in collagen molecular length or an increase in flexibility without disturbing the general conformation of the polypeptide chains. Disk electrophoresis confirmed that the tadpole enzyme made a limited and specific attack on the collagen molecule. Unlike the action of bacterial collagenase, which produces large numbers of various-sized peptides seen on disk electrophoresis in the form of many bands (Nagai and Gross, unpublished), the tadpole enzyme yields discrete, new, faster-moving α, β, and γ bands from acid-extracted collagen and an increase of material moving close to the solvent front. No other bands were formed even after 24-hr digestion time nor was there an increase in the new α at the expense of new β, suggesting little or no disruption of intramolecular crosslinks.

Electron micrographs of SLS prepared from the reaction mixture revealed the presence of only two forms; segments from the A end, three quarters the length of the normal with the point of severance at the b_2^2 band, and one-quarter-length frag-

ments from the B end. Very recent experiments by Sakai and Gross (to be published elsewhere) in which the reaction mixture has been fractionated cleanly with ammonium sulfate into large (TC^A) and small (TC^B) fragments show that no dialyzable peptides are produced nor are there any other collagen fragments remaining in the precipitating solution. Disk electrophoresis of the isolated TC^B short fragments under conditions of higher resolving power have shown that the material at the solvent front derives from this portion of the molecule.

Fragmentation of the collagen molecule without apparent conformational changes was first accomplished by Nishihara and Doty using sonic energy.[11] Hodge and Schmitt[12] prepared SLS from the fragments, finding halves and quarters. It may be that the quarter from the B end in sonically disrupted fragments corresponds to the portion severed by the tadpole enzyme. The enzyme, however, does not produce halves nor does it attack the A portion of the molecule. Recently, Olsen[13] has described slow tryptic action on the B end of the collagen molecule during a 70-hr period of incubation, obtaining molecules the same length as TC^A. This appears to be an etching away of the molecule from the B end, since Olsen does not describe any one-quarter-length SLS. The locus of rupture at the b_2^2 band may represent a susceptible region because of local alteration in the helical structure permitting access to the tadpole enzyme.

Preliminary analysis of the tadpole enzyme reaction mixture[14] indicated the release of about 2.5 moles of N-terminal leucine and isoleucine and the same number of C-terminal glycine residues per mole of collagen. However, further studies by more than one set of methods are needed for adequate documentation—these are in progress.

Although the helical structure of the collagen fragments seems to remain intact, there is a significant loss of stability as measured by a fall in denaturation temperature. This effect may have physiologic significance in that reduced stability at body temperature causing partial denaturation would increase the susceptibility of collagen to attack by the tissue proteases.[15, 3]

Summary.—An enzyme isolated from the culture medium of tadpole tissue cultures cleaves native collagen molecules extracted from a variety of mammalian skins and tadpole fin at neutral pH and physiologic temperatures. Enzymatic attack occurs at one point, severing the molecule at the b_2^2 locus into two fragments, one being three quarters the molecular length from the A end and the other one quarter the length from the B end, without disrupting the helical structure of either fragment.

The specificity of the attack was manifested by a limited fall in viscosity of the reaction mixture, unchanged optical rotation, appearance of new faster-moving bands in disk electrophoresis directly referable to the original subunits, and the formation of segment long spacing (SLS) from each of the fragments.

Reduction of the denaturation temperature of the reaction products suggests the manner in which this enzyme might prepare collagen for physiologic degradation.

* This is publication no. 399 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Massachusetts General Hospital, Boston, Massachusetts. These investigations were supported by the National Science Foundation grant 24307 and U.S. Public Health Service research grants AM 3564 and AM 5142 from the National Institutes of Arthritis and Metabolic Diseases.

† Present address: Tokyo Medical and Dental College, Institute for Hard Tissue, Yushima, Tokyo, Japan.

[1] Gross, J., and C. M. Lapiere, these PROCEEDINGS, **48**, 1014 (1962).

[2] Lapiere, C. M., and J. Gross, in *Mechanisms of Hard Tissue Destruction*, ed. R. Sognnaes (New York: Academic Press, 1962), p. 663.

[3] Eisen, A. Z., and J. Gross, *Develop. Biol.*, in press.

[4] Nagai, Y., C. M. Lapiere, and J. Gross, *Federation Proc.*, **22**, 648 (1963).

[5] Gross, J., and D. Kirk, *J. Biol. Chem.*, **233**, 355 (1958).

[6] Rubin, A. L., M. P. Drake, P. F. Davison, D. Pfahl, P. T. Speakman, and F. O. Schmitt, *Biochemistry*, **4**, 181 (1965).

[7] Gross, J., *J. Exptl. Med.*, **107**, 247 (1958).

[8] Martin, G. R., K. A. Piez, and M. S. Lewis, *Biochim. Biophys. Acta*, **69**, 472 (1963).

[9] Nagai, Y., J. Gross, and K. A. Piez, *Ann. N. Y. Acad. Sci.*, **121**, 494 (1964).

[10] Hodge, A. J., and F. O. Schmitt, these PROCEEDINGS, **46**, 186 (1960).

[11] Nishihara, T., and P. Doty, these PROCEEDINGS, **44**, 411 (1958).

[12] Hodge, A. J., and F. O. Schmitt, these PROCEEDINGS, **44**, 418 (1964).

[13] Olsen, B. R., *Z. Zellforsch.*, **61**, 913 (1964).

[14] Nagai, Y., C. M. Lapiere, and J. Gross, *6th Intern. Cong. Biochem.*, **II**, 170 (1964).

[15] Usuku, G., and J. Gross, *Develop. Biol.*, **11**, 352 (1965).

---

*THE ROLE OF HISTONES IN THE MAINTENANCE OF CHROMATIN STRUCTURE**

BY V. C. LITTAU, C. J. BURDICK,† V. G. ALLFREY, AND A. E. MIRSKY

THE ROCKEFELLER UNIVERSITY, NEW YORK

Communicated July 22, 1965

In the nuclei of many interphase cells some of the chromatin is dense; the rest is diffuse. In a thymus lymphocyte, for example, the larger part of the chromatin is in dense masses and only a relatively small part is diffuse (Fig. 1). It is now known that RNA synthesis proceeds mainly in the diffuse chromatin[1] and not in the dense chromatin.[2] Since RNA synthesis is a DNA-dependent process, it is clear that in such a nucleus only a small part of the DNA (that which is in the diffuse chromatin) is active as a template in the RNA polymerase reaction.[3, 4]

What holds chromatin together in clumps? In this paper we present experiments which indicate that histones bind chromatin threads together into dense masses. The experimental procedure was to remove histones selectively from isolated thymus nuclei and after each extraction to examine the nuclei with the electron microscope. It was found that removing the lysine-rich histones (which comprise 20% of the total histone), and only these histones, loosened the structure of the dense chromatin. Removing arginine-rich histones did not have this effect.

The special role of lysine-rich histones in binding chromatin threads together was shown by restoring histones to histone-depleted nuclei, in which chromatin masses had been broken down into a loose fibrous network. Only the lysine-rich histones caused chromatin masses to reappear, although both arginine-rich and lysine-rich histones combined with such nuclei; indeed, if a mixture of the two histones was added to histone-depleted nuclei, presence of arginine-rich histones prevented the lysine-rich histones from clumping the chromatin threads.