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*THE ENZYMATIC SYNTHESIS OF A CIRCULAR DNA-RNA HYBRID**

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It was first noted by Doerfler[1] and his colleagues that RNA synthesized on a single-stranded DNA template yielded a product markedly resistant to RNAase, suggesting[2] the appearance of a DNA-RNA hybrid in the reaction mixture. This supposition was confirmed by Warner *et al.*[3] who characterized the density and thermal transitions of the product. Recently, two groups[4, 5] have used the single-stranded[6] DNA of the bacteriophage ϕX174 in a similar study. They confirmed the conversion of the single-stranded templates to DNA-RNA hybrids and showed that free RNA does not appear until the composition of the hybrid approaches a limiting value of 1:1.

The virus ϕX174 has proved to be a potent tool for the experimental analysis of genetic transcription. With its aid it has been shown[7] that RNA messages found in the infected cell are complementary only to the complement of the mature strand. The development[8] of a chromatographic procedure provided pure preparations of the double-stranded replicating form (RF-DNA) of ϕX174 which were shown[9] to consist of over 95 per cent intact circular structures. With the aid of these it was possible to reproduce[10] in the test tube the strand selection mechanism observed in the cell.

The ready availability of pure double-stranded circular RF-DNA and the single-stranded component of the virus particle permitted an informative comparison of the RNA polymerase reaction with these two sorts of templates, both intact and fragmented. We report here some of our results with the single-stranded DNA since they both confirm and augment the available data. The experiments to be described show that during the course of the reaction, RNA appears in three different density regions of a Cs_2SO_4 gradient. One can be identified with a ribonuclease-resistant hybrid structure. The other two are both sensitive to ribonuclease, one corresponding to free RNA and the other found in the density region of single-stranded DNA. It will further be shown by electron microscopy that the collapsed coil of the single-stranded DNA template is gradually converted in the course of the reaction to a hybrid structure possessing circular morphology.

Materials and Methods.—(a) *Preparation of nucleic acid:* The single-stranded DNA was isolated from virus particles and purified chromatographically as described previously.[8] All DNA preparations were monitored for nuclease by examining for acid-soluble products with radioactive substrates after extensive (20 hr) incubation at 37°C. The material employed in the experiments described showed negligible nuclease activity.

(b) *Preparation of P^{32}-riboside triphosphate:* UTP labeled with P^{32} in the alpha phosphorus was synthesized according to the detailed procedure described by Haruna *et al.*[11] The initial specific activity was about 3.3×10^8 cpm/mM. Counting was done in a Packard liquid scintillation spectrometer. Acid-precipitable material was washed with 10% TCA and dried on Schleicher and Schuell (coarse) membrane filters. Tritium-labeled ϕX174 DNA was prepared as described by Hayashi *et al.*[7]

(c) *The enzymatic synthesis of DNA-RNA hybrids:* The RNA polymerase was isolated from log-phase cells of *E. coli* (C-122) according to the procedure of Chamberlin and Berg[12] as detailed by Hayashi *et al.*[10] The reaction mixture (1 ml) contained 24 μg of template DNA, 40–100 μg of enzyme, and the indicated number of μmoles of the following: 40 of Tris buffer at pH 7.9, 1 of $MnCl_2$, 4 of $MgCl_2$, 46 of KCl, 12 of β-mercaptoethanol, 0.5 each of ATP, CTP, GTP, and UTP^{32}. The extent of hybrid synthesized was varied by the time and temperature of incubation.

(d) *Standard RNAase treatment of DNA-RNA hybrids:* Pancreatic RNAase (Sigma Chemical Co., St. Louis, 5 × crystallized 49 Kunitz units/mg, lot no. R92B-89) is treated to eliminate possible DNAase by heating (80°C for 10 min) in pH 5 SSC (0.15 *M* sodium chloride, 0.015 *M* sodium citrate adjusted to pH 5 by adding 1 *M* citric acid) at a concentration 2 mg/ml. The RNAase, thus treated, is added at 30 μg/ml to the DNA-RNA mixtures dissolved in 2 × SSC and incubated at 26°C for 30 min. When estimates of the RNA hybridized as an RNAase-resistant structure are required, an aliquot of the reaction mixture is precipitated with cold 10% TCA filtered onto membrane filters, washed, dried, and counted. Analysis of the product before and after RNAase treatment in Cs_2SO_4 gradients is as described by Doi and Spiegelman.[13]

Results.—(a) *Ribonuclease resistance as a function of extent of synthesis:* RNA was enzymatically synthesized as described under *Methods* (*c*) using the single-stranded DNA isolated from the bacteriophage ϕX174 as the template. The course of the synthesis was followed by the incorporation of UTP^{32} in the presence of the other unlabeled riboside triphosphates. Since the amount of template DNA and the specific activity of the UTP^{32} are both known, the ratio of RNA to DNA in the product can be readily estimated.

At the termination of the reaction, the nucleic acids were isolated by the phenol method and dialyzed against 2 × SSC. An examination was made of the ribonuclease-resistant fraction [*Methods*, (*d*)] found after varying amounts of RNA were synthesized. Every test included an internal control of H^3-23S ribosomal RNA to monitor the effectiveness of the nuclease digestion. The results obtained with products ranging in DNA to RNA from 1:0.3 to 1:1.3 are given in Figure 1. Only

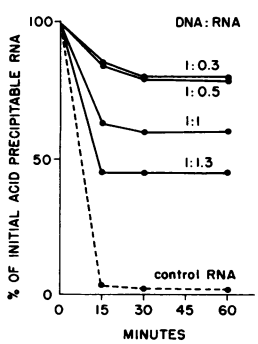

FIG. 1.—RNAase resistance at different stages of synthesis. The reaction mixtures (1.0 ml) contained the components listed in *Methods* (*c*) except that 10 μg of ϕX174 was added. Incubation was carried out at 25°C for 7 min (1:0.3), at 36.5°C for 10 min (1:0.5), for 20 min (1:1), and 90 min (1:1.3). The reaction was terminated by adding water-saturated phenol, the nucleic acid extracted from the water phase, and dialyzed against 2 × SSC. RNAase treatment was carried out as in *Methods* (*d*). Each test included an internal control of free H^3-RNA, only one of which is shown.

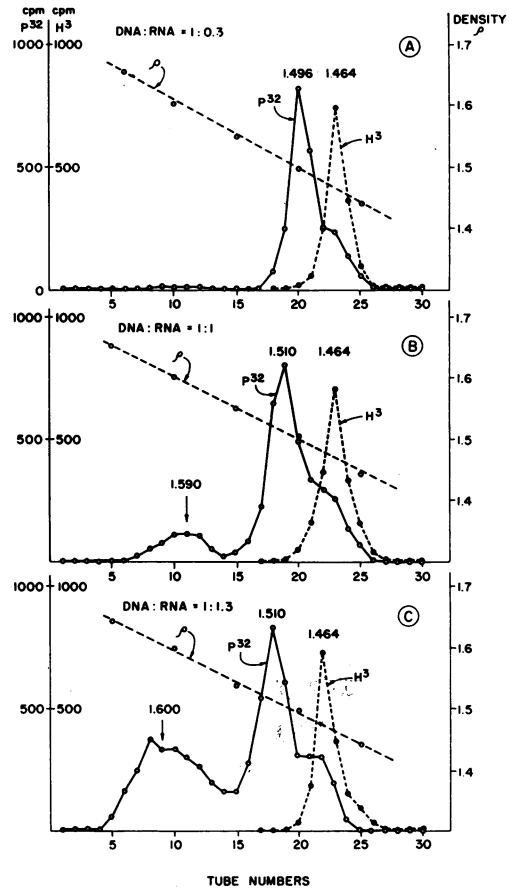

FIG. 2.—Equilibrium density gradient centrifugations. Cs_2SO_4 was added to the purified products corresponding to the indicated extents of RNA synthesis. The input density was 1.52 in a total volume of 3.0 ml. H^3-ϕX174 DNA was added as a density marker. The mixture was centrifuged at 33,000 rpm for 3 days in a Spinco model L using a SW 39 rotor held at 21°C. Radioactivity of acid-precipitable material was measured on fractions collected from the bottom of the tube through a syringe needle.

one of the internal controls is included since they all behaved identically. It will be noted that when the synthesis is limited to 30 or 50 per cent of the DNA, the proportion of RNA resistant to enzymatic digestion is high (about 80%). As soon, however, as the synthesis approaches the 1:1 ratio the per cent resistance drops to 60 per cent, and at a ratio of 1:1.3 it falls to 45 per cent.

It would appear that with increasing time and extent of synthesis, more and more of the RNA becomes susceptible to RNAase treatment, reflecting the accumulation of unhybridized RNA.

(b) *Profiles in Cs_2SO_4 gradients as a function of extent of synthesis:* The distribution of the synthesized RNA was examined in a density gradient. As the synthesis progressed, one would expect that increasing amounts of the RNA would band in the position characteristic of free RNA. Further, this sort of analysis could shed some light on the significance of the 20 per cent found to be sensitive to

ribonuclease (Fig. 1) even when the synthesis is limited to ratios of RNA to DNA of less than 1.

The results of gradient examinations of the various products are shown in Figure 2. It will be noted that when the extent of synthesis was 30 per cent of the DNA, virtually no free RNA is found in the corresponding density region. When, however, the reaction is allowed to proceed to a 1:1 ratio of RNA to DNA, approximately 20 per cent of the RNA bands in the density region characteristic of free RNA. The amount of unhybridized RNA increases to approximately 50 per cent when the synthesis proceeds to a ratio of RNA to DNA of 1.3.

The profiles of Figure 2*B* and 2*C* indicate that three identifiable RNA components are to be found in the final reaction mixture. One occupies the position characteristic of free RNA. Another bands in the region which would be assumed by a DNA-RNA hybrid, and the third, seen in all the reaction mixtures, appears in a density position normally assumed by the single-stranded DNA.

To characterize the three components further, an aliquot of the reaction mixture of Figure 2*C* was treated with RNAase and then banded in Cs_2SO_4 with results as shown in Figure 3. Predictably, all of the RNA found in the free RNA density region is completely eliminated. In addition, however, the shoulder of RNA seen in the region of the single-stranded H^3-DNA also disappears. The RNAase sensitivity of the latter suggests that this RNA represents incomplete synthesis involving hybrids of very short regions of the DNA, making the complex sensitive to the ribonuclease under the conditions of our test. This could be explained by an enzyme starting a synthesis on a strand but then, for some reason, stopping.

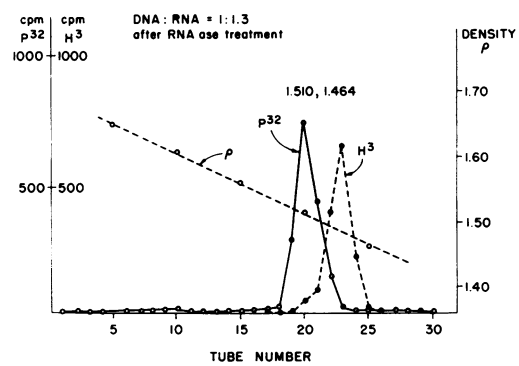

FIG. 3.—Effect of RNAase pretreatment on the P^{32}-RNA profile in a Cs_2SO_4 gradient. A portion of the 1:1.3 product of Fig. 2 was treated with RNAase under standard conditions, and the enzyme removed by the phenol method. After dialysis, the centrifugation in Cs_2SO_4 was carried out as in Fig. 2.

The presence of these aborted syntheses even in limited reactions, where no free RNA is produced, may explain the presence of the 20 per cent RNA-sensitive material observed in such products. All the available data indicate that under the conditions of our standard test for ribonuclease resistance, the true hybrid peak is resistant.

It should be noted that a similar type of examination with identical results was made[14] by a chromatographic procedure which readily identifies hybridized RNA, double-stranded DNA, and single-stranded DNA.

(c) *Electron microscopic morphology as a function of the extent of synthesis:* One does not observe in Figure 2*A* the free RNA, or hybrids of the final limiting density, found in the later samples of Figure 2*B* and 2*C*. It would appear, therefore, that the reaction is quite well synchronized. One might, therefore, hope to follow the progress of the reaction directly by electron microscopy. The random coil characteristic of the initial single-stranded DNA template yields a collapsed amorphous

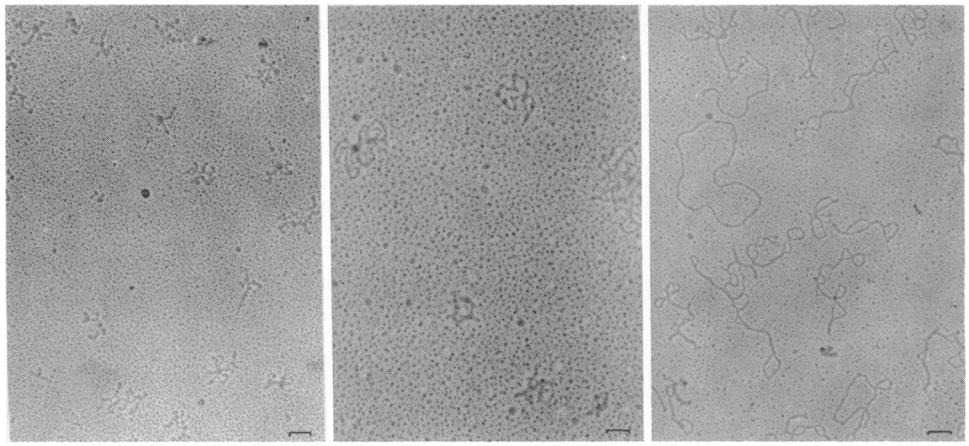

FIG. 4.—Changes in electron-microscopic morphology during synthesis. The reaction mixtures are detailed under *Methods;* plates *A*, *B*, and *C* represent samples taken at 0, 10, and 130% synthesis and treated with RNAase. Grids were prepared according to the method of Kleinschmidt *et al.*[28] as detailed by Chandler *et al.*[9] Pictures were taken with a Siemens-Elmiskop I at 60 kv. The indicated magnification line is 0.1 μ.

body in the electron microscope. As the reaction progresses, one might hope to see the conversion of the initial material into a structure recognizable as double-stranded material. In addition, there is the added feature stemming from the observations of Fiers and Sinsheimer[15–17] suggesting that the single-stranded DNA of ϕX174 is a covalently linked ring structure. If this is the case, the ultimate product of the enzyme reaction should be a double-stranded ring.

Samples taken at various intervals during the synthesis were purified by the phenol procedure and subjected to ribonuclease treatment to focus attention on the morphology of the ribonuclease-resistant structures. Typical results of such an experiment are given in Figure 4 which gives the zero time sample (*A*) and two others corresponding to 10 per cent (*B*) and to 130 per cent (*C*) synthesis of RNA. It would appear that the expectation of observing the evolution of an annular double-stranded structure is fully confirmed. Figure 5 shows some typical well-opened ring structures found in samples allowed to run to completion.

The annular hybrid structures look very much like the electron microphotographs of the duplex RF-DNA.[9, 10, 18] However, comparison of sizes revealed an interesting difference. The circumference of the circular RF-DNA fell within a narrow range (1.80–1.92 μ) with a mean of 1.86 for 20 rings. On the other hand, the DNA-RNA hybrids yielded an average of 1.57 μ with a range of 1.35–1.85 μ in 18 measurements. In general, the circumference of the hybrid structure is smaller and tends to approach the average of the RF-DNA as an upper limit. This would be explained if, in many instances, the polymerase skips small segments of the ring template. The unpaired portions of the DNA would, as a consequence, collapse yielding a shorter circumference for the apparently completed ring. Finally, these pictures represent the first ones of RNA-DNA hybrid structures and provide convincing evidence that they are indeed similar in their morphology to a DNA-DNA duplex.

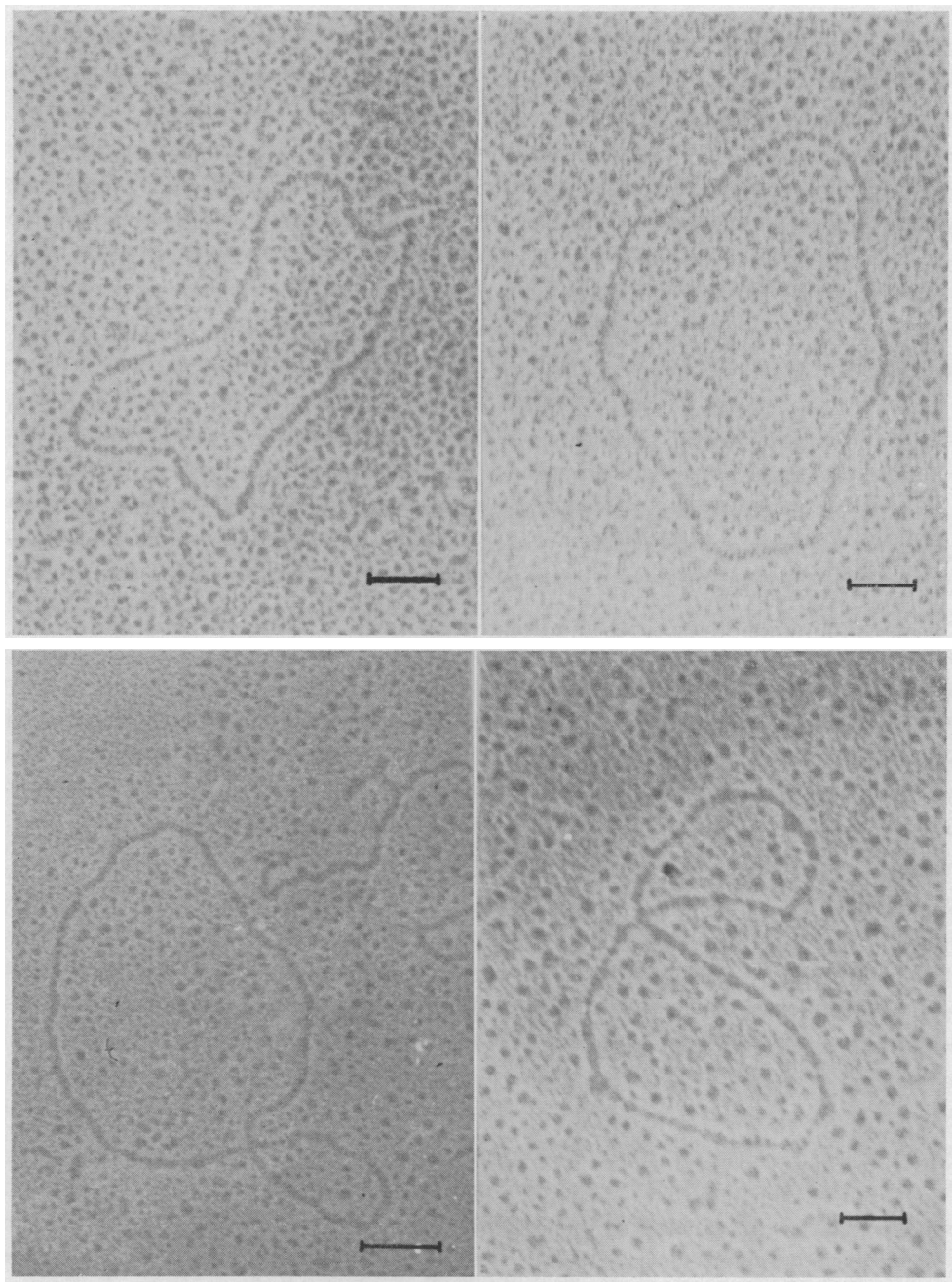

FIG. 5.—Selected open circular DNA-RNA hybrids. Some well-opened DNA-RNA hybrids representative of the various size classes observed when synthesis is allowed to go to completion. The indicated magnification line is 0.1 μ.

Discussion.—(a) *On the nature of the enzymatically synthesized DNA-RNA complex:* The data presented are in accord with previous studies[3-5] which concluded that a hybrid DNA-RNA structure is formed when RNA is synthesized on a

single-stranded DNA template. Although somewhat sensitive at low salt concentrations,[4] the hybrid is resistant to RNAase (free of DNAase) in 0.3 *M* NaCl at 30°C. Its maximal density in Cs_2SO_4 is achieved when the ratio of RNA to DNA becomes unity.

The most significant advance in our understanding of the nature of the RNA-DNA complex emerged from the electron microscopic examinations of the present study. The ribonuclease-resistant hybrid structures were shown to have the same circular double-stranded appearance as the homologous double-stranded DNA replicating form of ϕX174. Further, the *maximum* linear dimensions of the RNA-DNA hybrid are in excellent agreement with those found for the RF-DNA.[9] These findings clearly eliminate the possibility that this DNA-RNA complex is an unpaired aggregate held together by protein[19] or some other device.

(b) *A comparison of in vitro and in vivo DNA-RNA complexes:* It is natural to raise the question of the significance of the hybrid structures described here to the normal process of *in vivo* transcription. The first suggestion of naturally occurring DNA-RNA complexes in cells came with the observations of Sibatani *et al.*[20-22] of a rapidly labeled RNA associated with DNA found in the phenol-H_2O interphase. Using P^{32} and H^3 to label viral DNA and RNA, respectively, in *E. coli* infected with T2, Spiegelman *et al.*[23] located a DNA-RNA complex in a CsCl gradient as a peak containing both isotopes and occupying a density position between that of *E. coli* and T2-DNA. The quantities found (involving less than 0.1% of the RNA synthesized) discouraged further attempts at characterizing this complex.

Fortunately, other biological materials (amphibian eggs,[24] *Neurospora crassa*,[25] and *Drosophila melanogaster*[26]) yielded DNA-RNA complexes by similar procedures and in sufficient quantities to permit more illuminating studies of their chemical nature. In all three instances, the composition was consistent with a triplex structure containing two DNA strands to one of RNA. Further, both Mead[26] and Schulman and Bonner[25] showed that the complex could be destroyed by either DNAase or RNAase. Finally, the triplex possessed[25] a distinctly heavier density in CsCl than native DNA, a difference which disappeared on treatment with RNAase. The weight of the evidence cited would appear to favor the existence of *in vivo* DNA-RNA complexes, which can survive protein denaturants, despite the recently reported failure of Konrad and Stent[27] to find them.

The 2:1 composition and sensitivity to ribonuclease clearly distinguishes the *in vivo* DNA-RNA complexes from the 1:1 hybrids observed in the present investigation with single-stranded DNA templates. Further, in all cases examined, the triple complexes survived extensive deproteinization and lengthy centrifugations in CsCl of high molarity. This stability against protein removal makes it unlikely that they are analogous to the DNA-RNA-protein complexes detected[19] in an *in vitro* system using double-stranded DNA as a template. The latter complexes are readily disrupted by very brief exposures to protein denaturants.

The *in vitro* systems represent fruitful experimental devices to further our understanding of the details of the transcription mechanism. Two models have been proposed for transcription from native DNA. One would assume local opening of the DNA duplex to permit limited and transient hybrid structures.[29, 30] The other requires that the DNA helix remain closed, the specification of the RNA being carried out by base pairs with the formation of new types of hydrogen bonds.[31] The first

model implies that transcription from single- and double-stranded DNA is basically the same. The second suggests that they are fundamentally different. A continued study of transcription from double- and single-stranded DNA using more subtle criteria of comparison is clearly required for a decision among the possible models. For this, the ϕX174 system provides obvious experimental advantages.

Summary.—The synthesis of RNA on single-stranded DNA of the bacteriophage ϕX174 has been examined by ribonuclease resistance, density gradient centrifugation, and electron microscopy. During the course of the reaction, RNA appears in three regions of the density gradient. One component has been identified with an RNAase-resistant hybrid structure. The other two components are both sensitive to RNAase, one corresponding to free RNA and the other to abortive syntheses. Electron microscopic examination revealed the gradual conversion of the collapsed coil of the single-stranded DNA template to a double-stranded hybrid structure possessing circular morphology. These photographs establish that this RNA-DNA hybrid possesses the appearance and dimensions of a hydrogen-bonded duplex and eliminates the possibility of unpaired aggregates held together by unspecified devices.

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EFFECTS OF VARIOUS POTASSIUM SALTS AND PROTEASES UPON EXCITABILITY OF INTRACELLULARLY PERFUSED SQUID GIANT AXONS

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The ability of the squid giant axon to develop action potentials can be preserved for hours under continuous intracellular perfusion if solutions of the proper salts are chosen for perfusion.[1, 2] In the early stage of the development of the perfusion technique, a great emphasis was placed on the importance of the proper choice of intracellular cations for maintenance of axonal excitability. Recently, however, it became increasingly clear that intracellular anions exert a great influence upon the resting and action potentials of the axon.

The first half of the present article deals with the results of a systematic study of the effects of various anions in the intracellular perfusion fluid on the bioelectric potentials of the axons. It is shown that the anions examined can be arranged according to their effects in an order similar to the classical lyotropic series in protein chemistry[3] and in muscle physiology.[4]

The second half of this article deals with the effects of various proteolytic (and other) enzymes in the perfusion fluid upon the resting and action potentials. As recently observed by Rojas and Luxoro,[5] intracellularly administered proteases produce strong injurious effects upon giant axons while these enzymes are totally ineffective when applied externally. These and other findings described in this article suggest the existence of a close relationship between the polypeptides in the membrane and the excitability of the giant axon.

Methods.—Partially cleaned giant axons of *Loligo pealii* were used in the present study. The details of our technique of intracellular perfusion were described in a previous article of this series.[1] The glass pipette for introducing the perfusion fluid into the axon was approximately 160 μ in outside diameter, and the glass drainage pipette was roughly 300 μ. The length of the perfusion zone varied between 12 and 25 mm. The resting and action potentials of the axons were determined under continuous perfusion with a movable glass pipette electrode which was introduced into the axon through the drainage pipette. The electrode was approximately 100 μ in diameter, and was filled with isotonic KCl solution. Stimulating current pulses were delivered to the axon near the end of its perfused zone. A Bak unity-gain electrometer (Electronics for Life Sciences), a Tektronix oscillograph (type 502), and a Grass camera were used for recording bioelectric potentials.

Perfusion fluid was prepared by mixing 12.5 vol per cent glycerol solution with various isotonic salt solutions. The pH of the solution was adjusted to 7.2–7.3