[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Chromatographic Detection of Differences between Bacteriophage-related Deoxyribonucleic Acids[1]

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A method is presented for comparing two deoxyribonucleic acid (DNA) species with respect to their content of various polynucleotides. One species was labeled with P^{32} and then mixed with 20 times its amount of the other species. Polynucleotides were obtained from the mixture by various means, separated on ion-exchange columns, and their specific activities determined. Differences in specific activities of the isolated polynucleotides would reflect differences between the two DNA's. By this method, differences were detected between bacteriophage T2 DNA and DNA isolated from T2-infected, proflavin-inhibited *Escherichia coli*.

After infection of *Escherichia coli* B with bacteriophage T2, a hundredfold increase in the phage population occurs before lysis of the host bacterium. In order to meet the demands of phage growth, the infected host must vigorously synthesize phage DNA. The presence of hydroxymethylcytosine[2] and the absence of cytosine distinguish this newly synthesized DNA from normal bacterial DNA.

If proflavin is present during phage infection, lysis of the host bacteria eventually occurs but little or no infectious progeny phage are liberated.[3] The infected cells synthesize DNA at about the same rapid rate observed in the absence of proflavin[4] (but see 5) and the DNA may be phage DNA since it contains hydroxymethylcytosine[6] instead of cytosine. We are extending these studies to examine the possibility that DNA synthesized in the proflavin system may differ from phage DNA in nucleotide sequences.

In this paper we present a method for comparing the nucleotide sequences in two samples of DNA, and report the results of a comparison between phage DNA and DNA obtained from the proflavin system. For this comparison, DNA synthesized in the presence of proflavin was labeled with P^{32} and then mixed with 20 times its amount of authentic phage DNA. The mixed DNA's were treated to yield polynucleotide fragments that could be partially separated into several fractions by ion-exchange chromatography. In each chromatographed fraction, the chemically determined phosphate is predominantly a measure of material from authentic phage DNA, whereas radioactivity measures only the contribution from proflavin system DNA. A difference between the intact DNA's would be detected when a fraction was found whose specific activity differs from the average.

Experimental

T2 DNA.—$T2r^+$ bacteriophage was grown on *E. coli* B and purified according to the directions of Herriott and Barlow.[7] Bacteriophage DNA was obtained by the method of Mayers and Spizizen,[8] modified to include the additional deproteinization step of shaking with a chloroform and octyl alcohol 4:1 mixture.[9]

Radioactive Nucleic Acids from Phage-infected, Proflavin-inhibited Bacteria (Proflavin System).—Radioactive cells of *E. coli* B were grown from a small inoculum in P^{32}-labeled synthetic medium. At the time of phage infection, the cell and medium phosphorus should have the same specific radioactivity. One liter of medium contained 0.5 g. each of NH_4Cl, $(NH_4)_2SO_4$ and KCl; 5 g. of NaCl; 7.5 g. of tris-(hydroxymethyl)-aminomethane (TRIS); 0.125 g. of Na_2HPO_4; 1 mmole of $MgCl_2$; 0.1 mmole of $CaCl_2$; 0.01 mmole of $FeCl_3$; 2 mc. of P^{32} added as neutralized orthophosphate (7.5 mc. at start of experiment), *p*H 8.0; and 10 ml. of 40% glucose, the last sterilized separately. Log phase cells, at a concentration in this medium of 3–4 $\times 10^8$ cells/ml. were infected with ten times their number of bacteriophage T2. Two minutes after infection, proflavin (3,6-diaminoacridine) was added to a final concentration of 3 mg./liter and aeration at 37° was continued for two hours.

Cell nucleic acids were isolated by the following general procedure. After the cooled suspension was acidified and centrifuged to remove acid-soluble material, the acid-insoluble precipitate was neutralized and then extracted three times at 45° with a 3:1 mixture of ethanol and ether in order to remove lipid material. The remaining precipitate was extracted twice at 45° with 1.7% aerosol OT[10] in 0.7 *M* sodium acetate (*p*H 7.8). By this treatment nucleic acids were dissolved free of protein. One preparation, however, required the further deproteinization accomplished by shaking with a 4:1 mixture of chloroform and octyl alcohol.[9] The nucleic acids were then precipitated at 0–5° by addition of 2.5–3 volumes of ethanol, dissolved and dialyzed against distilled water. In one representative experiment, the nucleic acids isolated from 1 liter of culture fluid contained 1.3×10^6 cts./sec. and 52 μmoles of phosphorus with a millimolar extinction coefficient of 9.2 at 260 mμ.

Mixed DNA's.—T2 DNA, containing 180 μmoles of phosphorus, was mixed with radioactive proflavin system nucleic acids containing 30 μmoles of phosphorus. The proflavin system ribonucleic acid (RNA) was hydrolyzed after four hours' treatment of the mixture with 0.1 *N* NaOH at 45°. The mixture was then chilled and adjustment of the *p*H to 1.5–2.0 precipitated the total DNA. The RNA supernatant contained 65–70% of the added radioactivity. From this, we calculated that the DNA precipitate contained 15–20 parts of authentic T2 DNA to each part of proflavin system DNA. The DNA precipitate was washed with cold 0.02 *N* HCl, dissolved in 0.02 *N* $NaHCO_3$ and then dialyzed against cold distilled water.

Polynucleotide Preparations.—Four methods were used to prepare polynucleotides of different types for chromatography: (a) hydrolysis by DNAse, (b) hydrolysis by snake venom enzymes (diesterase plus 5′-monoesterase) after a preliminary hydrolysis by DNAse, (c) hydrolysis by snake venom enzymes after a heat treatment consisting of incubating the DNA in a boiling water-bath for one hour, and (d) heat treatment alone. Hydrolysis by DNAse was carried out for six hours at room temperature in a system

(1) Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.
(2) G. R. Wyatt and S. S. Cohen, *Nature*, **170**, 1072 (1952).
(3) R. A. C. Foster, *J. Bacteriol.*, **56**, 795 (1948).
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(5) L. A. Manson, *Federation Proc.*, **13**, 503 (1954).
(6) S. E. Luria, quoted in ref. 4.
(7) R. M. Herriott and J. L. Barlow, *J. Gen. Physiol.*, **36**, 17 (1952).
(8) V. L. Mayers and J. Spizizen, *J. Biol. Chem.*, **210**, 877 (1954).
(9) A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.*, **30**, 117 (1946).
(10) Dioctyl sodium sulfosuccinate, a detergent obtained from Fisher Scientific Co.

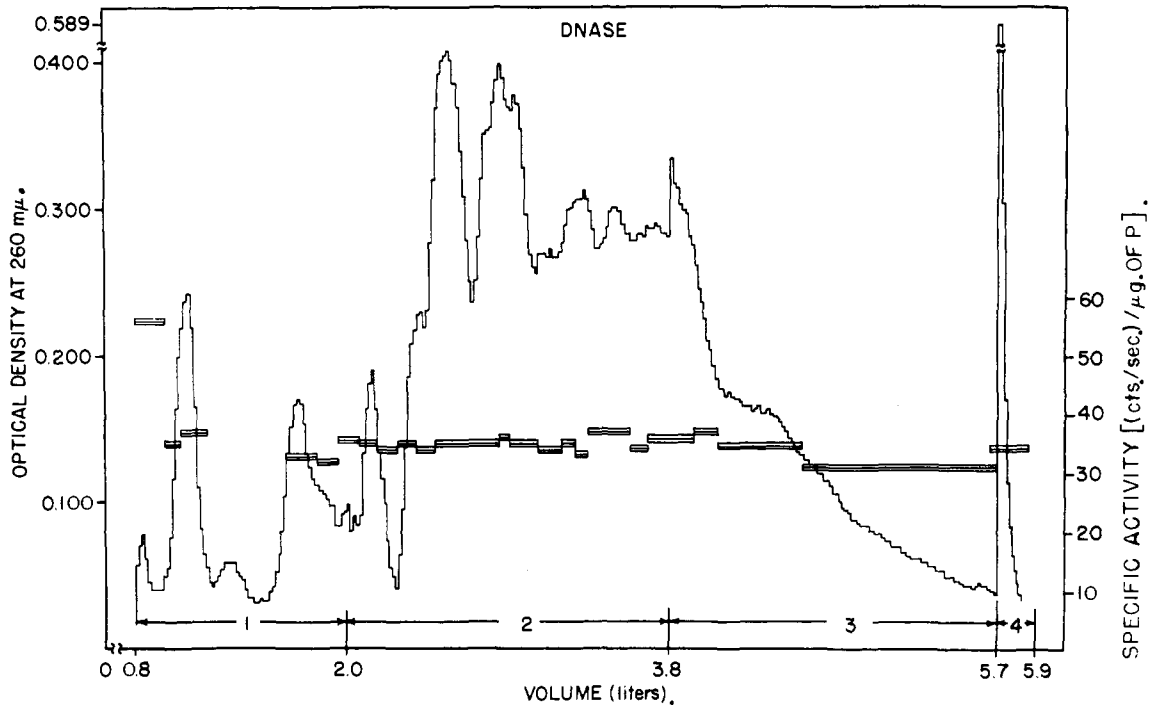

Fig. 1.—Chromatography of DNAse hydrolytic products. Left ordinate, elution pattern; right ordinate, specific activities of pooled fractions (represented by bars). Anion exchanger, Dowex-1-Cl^-, 12 cm. × 0.78 sq. cm. Eluents: (1) gradient, 0.002 *N* HCl to 0.002 *N* HCl + 0.04 *M* NH_4Cl, 1 liter each; (2) gradient, 0.002 *N* HCl + 0.04 *M* NH_4Cl to 0.005 *N* HCl + 0.2 *M* NH_4Cl, 1 liter each; (3) gradient, 0.005 *N* HCl + 0.2 *M* NH_4Cl to 0.005 *N* HCl + 0.4 *M* NH_4Cl, 1 liter each; (4) 2 *N* HCl. At very beginning, 0.5 liter of eluent was lost. Recovery: 96% of phosphorus, 103% of radioactivity.

containing 0.02 *M* *p*H 7.5 buffer (TRIS or bicarbonate), 0.004 *M* $MgCl_2$, 100 γ/ml. of DNAse, and 3–4 μmoles/ml. of DNA phosphorus. When snake venom enzymes were used after DNAse action, incubation at *p*H 7.5 was for one hour only. Hydrolysis by snake venom enzymes was carried out for 22 hours at 37° in a system containing 0.02 *M* NH_4Cl buffered to *p*H 9.0 with NH_4OH, 0.002 *M* $MgCl_2$, 3–4 μmoles/ml. of DNA phosphorus, and 1 ml. of a snake venom enzyme solution for every 10–15 ml. of total volume. The enzyme solution was prepared by centrifugation of the insolubles from a 2% mixture in water of *Crotalus adamanteus* lyophilized venom,[11] and then exhaustively dialyzing the supernatant.

Chromatography.—The DNA hydrolytic products were partially separated on ion-exchange columns. Fragments produced by DNAse plus snake venom enzymes, or DNAse alone, were chromatographed on Dowex-1 anion-exchange resin, and fragments produced by heat treatment plus snake venom enzymes, or heat treatment alone, were chromatographed on ECTEOLA,[12,13] a cellulose triethanolamine weak-base anion exchanger. Gradient elution, with eluents listed in figure legends, was used extensively. Of those products released by DNAse plus snake venom enzymes, only the dinucleotides were well separated on Dowex-1 resin regardless of whether the resin was in the chloride, sulfate or perchlorate forms. The optical absorption of each fraction was measured at 260 and 280 mμ in a Beckman DU spectrophotometer. Fractions were pooled on the basis of peaks at 260 mμ, 280–260 ratios, and radioactivity. Each pool was measured for optical absorption, phosphorus[14] and radioactivity. A Tracerlab scaler was used to measure the radioactivity of samples dried on glass planchets. Appropriate corrections were made for decay of P^{32}. A few fractions were assayed for deoxyribose.[15]

(11) Obtained from Ross Allen Institute, Silver Springs, Florida.

(12) We are indebted to Drs. Peterson and Sober for the gift of ECTEOLA to our laboratory.

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Results

The first method chosen to produce polynucleotides from the mixture of phage DNA and radioactive proflavin system DNA was hydrolysis with pancreatic DNAse. From the work of Sinsheimer[16] it is known that the products released by DNAse range in complexity from a minute amount of mononucleotides through larger amounts of di- and trinucleotides up to an appreciable amount of polynucleotides estimated to be of the order of hepta- and octanucleotides. The average size of the products, calculated from a variety of experimental techniques (cited by Sinsheimer[16]) is approximately that of a tetranucleotide. Hydrolysis with DNAse therefore offers a method for comparing phage DNA with proflavin system DNA at a low to intermediate level of organization.

Figure 1 presents the ion-exchange separation of the products released by DNAse. All but the first of the 27 fractions collected had, within experimental error, the same specific activity. The first fraction had a higher specific activity but the significance of that value was somewhat diminished by subsequent analyses that indicate that the fraction was not wholly derived from DNA. The ultraviolet absorption spectrum was not that of nucleic acid since the ratio of optical density at 250 mμ to that at 260 mμ was 1.6 and the millimolar extinction coefficient at 260 mμ, based on phosphorus content, was 15.7. Deoxyribose analysis, by a method[15,17] that detects pyrimidine de-

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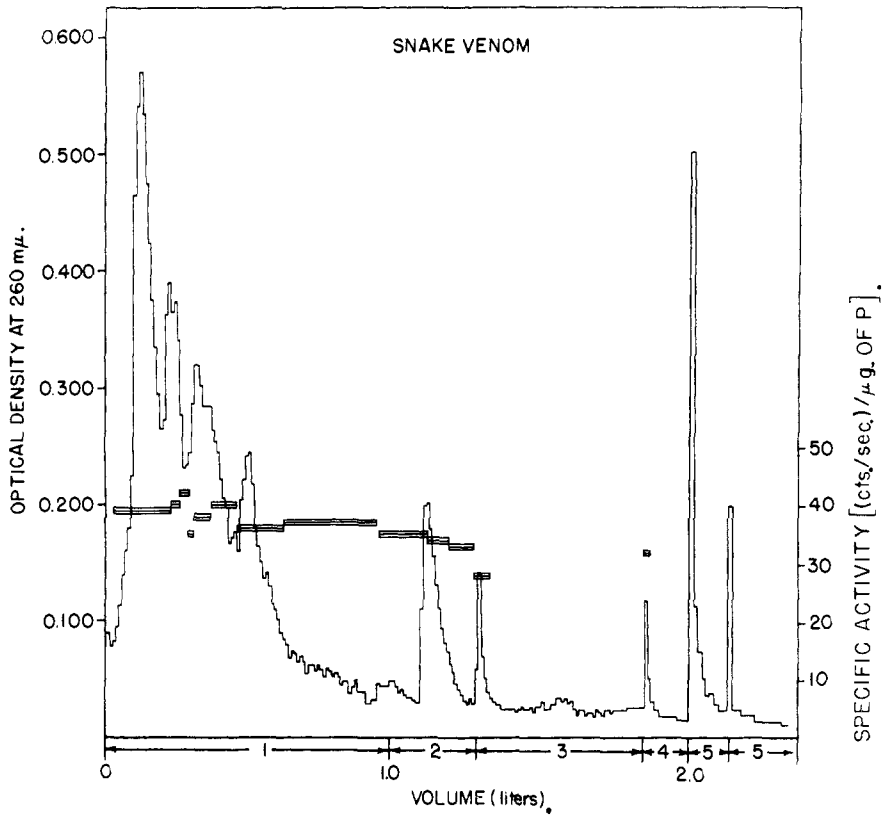

Fig. 2.—Chromatography of snake venom enzyme hydrolytic products. Left and right ordinates as in Fig. 1. Bars represent specific activities of pooled fractions. Anion exchanger, ECTEOLA-HCO_3^-, 10 cm. × 0.78 sq. cm. Eluents: (1) gradient, 0.005 *M* HCO_3^- (*p*H 7.0) to 0.005 *M* HCO_3^- (*p*H 7.0) + 0.05 *M* NaCl, 1 liter each; (2) gradient, 0.01 *M* HCO_3^- (*p*H 7.0) + 0.025 *M* NaCl to 0.01 *M* HCO_3^- (*p*H 7.0) + 0.25 *M* NaCl, 0.5 liter each; (3) gradient, 0.01 *M* HCO_3^- (*p*H 7.0) + 0.25 *M* NaCl to 0.01 *M* HCO_3^- (*p*H 10) + 0.25 *M* NaCl, 0.5 liter each; (4) 2 *M* NH_4OH; (5) 0.5 *N* NaOH. Recovery: 82% of phosphorus and radioactivity.

oxynucleotides equally as well as purine deoxynucleotides, indicated a molar ratio of one deoxyribose to two phosphates. It can therefore be concluded that the oligo- and polynucleotides from authentic phage DNA, obtained by DNAse hydrolysis and Dowex-1 separation, are equally represented in proflavin system DNA. It is probable, however, that not every sequence liberated at the various levels of organization was separated. The possibility therefore exists that some sequences had greater representation in one or the other of the DNA samples, but averaged out in all the fractions to give the same specific activity.

Larger polynucleotides were obtained by heating the DNA mixture and then hydrolyzing with snake venom diesterase plus 5′-monoesterase. Attempts to separate the polynucleotides by chromatography on Dowex-1 failed since most of the material could not be eluted. We therefore chromatographed the polynucleotides on a cellulose triethanolamine weak-base anion exchanger (ECTEOLA),[13] a material that has been used successfully by Bendich, *et al.*,[18] to separate fractions from whole DNA. Figure 2 shows the separation of the polynucleotides obtained by hydrolysis with snake venom enzymes. With the possible exception of one fraction, which by statistical tests had a significantly lower specific activity, the polynucleotides had a uniform specific activity equal to the original mixture. There is no bar across the fraction eluted with 0.5 *N* NaOH (eluent 5) because of loss before assay. On repetition of the experiment, material eluted with 0.5 *N* NaOH had a low specific activity, indicating greater representation from the phage DNA.

A difference between the two DNA's became apparent when we produced polynucleotides by merely heating the mixed DNA's for an hour in a boiling water-bath. Figure 3 presents the ion-exchange separation of the polynucleotides on ECTEOLA. Starting from an original average specific activity of 44 cts./sec./γ of phosphorus, the specific activities of the isolated fractions varied from 10 to 90. A low specific activity indicates that a polynucleotide is present to a greater extent in the cold authentic phage DNA than in the labeled proflavin system DNA and a high specific activity indicates the reverse. From these data, we conclude that DNA isolated from a proflavin-inhibited, phage-infected *E. coli* is not the same as phage DNA. Both DNA's apparently have the same amounts of the sequences obtained at the levels of DNAse hydrolytic products, but at higher levels of organization differences appear.

Discussion

With the finding that phage DNA and proflavin

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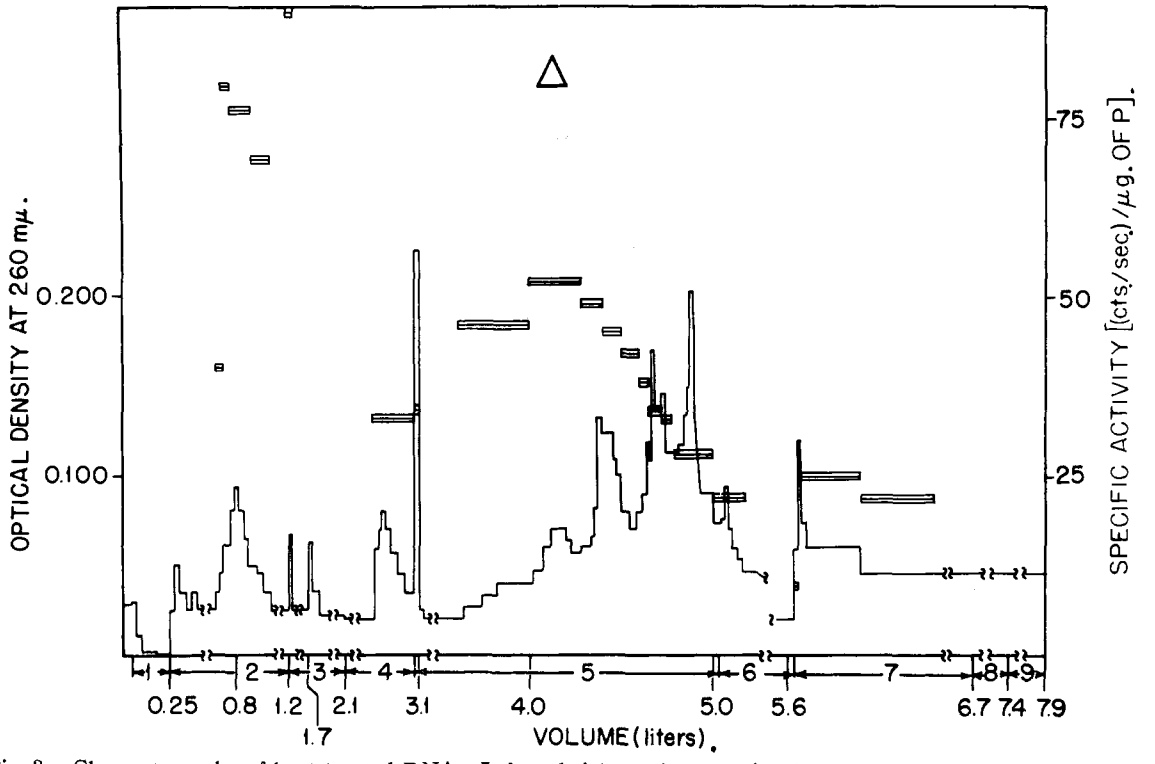

Fig. 3.—Chromatography of heat-treated DNA. Left and right ordinates as in Fig. 1. Bars represent specific activities of pooled fractions. Anion exchanger, ECTEOLA-OH^-, 10 cm. × 0.78 sq. cm. Eluents: (1) 0.005 *M* HCO_3^- (*p*H 7.0); (2) gradient, 0.005 *M* TRIS (*p*H 7.5) to 0.005 *M* TRIS (*p*H 7.5) + 0.5 *M* NaCl, 0.5 liter each; (3) gradient, 0.01 *M* TRIS (*p*H 7.5) + 0.1 *M* NaCl to 0.01 *M* TRIS (*p*H 9.8) + 0.1 *M* NaCl, 0.5 liter each; (4) gradient, 0.01 *M* TRIS (*p*H 8.7) + 0.1 *M* NaCl to 0.001 *N* NaOH + 0.1 *M* NaCl, 0.5 liter each; (5) gradient, 0.0001 *N* NaOH + 0.1 *M* NaCl to 0.005 *N* NaOH + 0.1 *M* NaCl, 1 liter each; (6) gradient, 0.005 *N* NaOH + 0.1 *M* NaCl to 0.01 *N* NaOH + 0.1 *M* NaCl, 1 liter each; (7) gradient, 0.01 *N* NaOH + 0.1 *M* NaCl to 0.1 *N* NaOH + 0.1 *M* NaCl, 1 liter each; (8) gradient, 0.06 *N* NaOH + 0.1 *M* NaCl to 0.25 *N* NaOH + 0.1 *M* NaCl, 0.5 liter each; (9) 0.5 *N* NaOH. Recovery: (in pooled fractions) 68% of phosphorus and radioactivity.

system DNA are different, it becomes of interest to examine mechanisms that might account for the results. One possible explanation arises from the fact that the two DNA species were in part isolated separately. The various procedures used for preliminary purification might have resulted in partial degradation of the two DNA's to differing extents. These differences must be limited, however, since the isolation procedures for the two DNA's were very similar with respect to any heat treatments.

It is also possible that the DNA isolated from the proflavin system was a mixture of phage DNA and normal bacteria DNA. Tests were made for the presence of bacterial DNA by a technique that involved hydrolysis of the mixed DNA's with DNAse plus snake venom enzymes. This treatment completely hydrolyzes bacterial DNA to nucleosides. After chromatography on Dowex-1, we isolated inorganic phosphate and dinucleotides of hydroxymethylcytosine with adenine, guanine and thymine, all of which had the same specific activity as the original mixture. Since our methods for measurement of specific activities were accurate to 10–15%, we can conclude that the proflavin system DNA contained less than 10–15% of bacterial DNA.

It may be noted in the Experimental section that the conditions for producing proflavin system DNA involved infection of the labeled cells in labeled medium with a multiplicity of 10 unlabeled phage particles for each cell. The amount of unlabeled DNA thereby added to the system is 10–30%[19] of the DNA finally isolated from the proflavin system. If this unlabeled DNA were incorporated as large polynucleotides into the proflavin system DNA, we would expect to isolate from the heat-treated DNA mixture a number of polynucleotides with very low specific activities. Although we intend to investigate this intriguing possibility, by a modification of the same technique reported here, there are some reasons for considering this possibility to be of little numerical significance to the present problem. First of all, the contribution from the infecting phage DNA must be less than 10–30% of total proflavin system DNA, since losses of the latter must have occurred owing to the observed extensive lysis of the infected cells during incubation. Secondly, Watanabe, *et al.*,[20] have reported that during T2 infection of *E. coli*, at least 40% of the parental T2 phosphorus becomes acid soluble at some time and

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(20) I. Watanabe, G. S. Stent and H. K. Schachman, *Biochim. Biophys. Acta*, **15**, 38 (1954).

therefore could not be incorporated into new DNA as large polynucleotides.

There are several other possible explanations for the differences observed between the two DNA's. The first is that real sequence differences are present in a few places and that they become distinguishable when larger polynucleotides are isolated. Another explanation would be that the fractions with high specific activities are phage DNA precursors isolated together with phage DNA from the infected bacterial cell but not present in viable phage. However, this would not explain the isolation of polynucleotides with very low specific activities. A third possibility is that phage DNA and the proflavin system DNA are made up of several polymers but the amounts of these polymers in the two systems are different although they have the same structure. Whichever possibility is correct, phage DNA and DNA isolated from proflavin-inhibited, phage-infected *E. coli* are not the same.

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The Disulfide Bonds of Human Serum Albumin and Bovine γ-Globulin[1]

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The structural role of disulfide bonds in human serum albumin (HSA) and bovine γ-globulin (BγG) has been evaluated by investigating the conditions of their reduction and its effect on the optical rotation and viscosity of the proteins. Reduction was effected with β-mercaptoethylamine HCl with and without sodium decyl sulfate. HSA has one free sulfhydryl group and 17 disulfide bonds, only one of which is reduced in the absence of detergent. Complete reduction of the disulfide bonds in the presence of detergent increases the reduced viscosity indicating disorganization of the tertiary structure. The concomitant large drop in the levorotation is interpreted as a gain in secondary structure. BγG has also one sulfhydryl group and 17 disulfide bonds, 5 of which can be reduced in the absence of detergent. Reduction of these 5 bonds does not change the viscosity and the optical rotation of this protein in the neutral *p*H range. Complete reduction in the presence of detergent causes little change in these properties beyond that brought about by the detergent alone. It is concluded that in HSA the disulfide bonds provide cross-links between distant portions of the polypeptide chain which stabilize the tertiary structure, and also prevent the maximum degree of secondary structure. Reduction releases the strain imposed by these bonds and permits the molecule to attain a higher degree of secondary structure with the accompanying loss of the original tertiary organization. In BγG, on the other hand, disulfide bonds contribute little to the cross-linking within the molecule as shown by the constancy of the high viscosity and levorotation after complete reduction in the presence of detergent. The bearing of these results on the problem of the intrinsic stability of α-helical structures in aqueous solution is considered.

During the past few years the α-helix of Pauling and Corey[2] has received increasing acceptance as the common and basic pattern for the organization of soluble proteins. This development has brought forth the recognition that the analysis of the structural organization of the protein molecule requires a conceptual distinction, as well as a corresponding experimental one, between secondary and tertiary structures.[3,4] The secondary structure arises from the stabilization achieved by the formation of hydrogen bonds between peptide groups relatively close to each other along the polypeptide chain. In the case of the α-helix each group is bonded to the third one beyond it. The tertiary structure emerges as a result of the interactions between the side chains of the amino acid residues. It is clear that the existence of the secondary structure will limit the tertiary organization. However, we know very little of the extent of their mutual dependence. It may be the case that the most stable tertiary structure precludes the full utilization of the peptide groups for intrahelical hydrogen bonding. It is quite possible, too, that for some proteins the integrity of the secondary structure can be maintained only by the additional stability provided by the tertiary interactions.

Among the various possible side chain interactions, the disulfide bond would be expected to be of particular importance. It appears to be the only commonly occurring covalent bond between side chains and it can provide a degree of stabilization of the tertiary structure far beyond that of any other interaction. Since this bond can be formed readily and specifically under physiological conditions and during the last stages of protein synthesis, it is well suited for the formation and stabilization of the selective configurations associated with the biological specificity of proteins. That the disulfide bond may play such a role is indicated by the fact that reduction causes the loss of biological activity of insulin[5] and of crotoxin.[6]

This paper describes the results of our investigations of the disulfide bonds of human serum albumin and bovine γ-globulin. We have sought to evaluate the structural role of these bonds in these two different types of proteins. Primarily, this has been done by relating the extent of reduction to changes in the specific rotation and reduced viscosity of the proteins. The results have been interpreted in terms of their secondary and tertiary structures.

(1) These studies were aided by a grant from the National Science Foundation and by a research grant (H-869) from the National Heart Institute of the National Institutes of Health, Public Health Service.

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