ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE GIBBS LABORATORY, DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

The Alkaline Denaturation of Deoxyribose Nucleic Acid

BY PAUL EHRLICH AND PAUL DOTY

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Deoxyribose nucleic acid (DNA) from calf thymus has been examined by sedimentation, viscosity and light scattering methods in the *p*H range from 7 to 12.5. When the ionic strength is 0.15, it is found that the molecular configuration undergoes a sharp transition in the vicinity of *p*H 11.7. Above *p*H 11.9 the transition is complete and the DNA is found to retain its original molecular weight but to have contracted in size. The 10-fold lowering of the intrinsic viscosity, the 12% rise in optical density at 259 mμ, the nearly 3-fold reduction in radius of gyration and the invariance of the mean sedimentation constant are essentially identical with the changes previously observed for acid denaturation (*p*H 3.1) and thermal denaturation. Alkaline denatured DNA undergoes a very large molecular expansion when the ionic strength is lowered to 0.005. Under the same conditions light scattering results show that the configuration of native DNA remains unchanged. All of these observations are consistent with the view that the undenatured DNA is highly extended and relatively rigid because of the continuous hydrogen bonding between the two polynucleotide chains of the Watson–Crick structure, whereas the denatured state consists of randomly coiled, highly flexible chains that remain paired and highly contracted due to the persistence of a substantial number of non-periodically arranged, intramolecular hydrogen bonds.

In 1947 Gulland, Jordan and Taylor[1] showed that the viscosity of deoxyribose nucleic acid (DNA) solutions fell sharply when the solutions were acidified or made alkaline. A study of the effects of acid[2,3] demonstrated that upon careful downward adjustment of the *p*H at 0.2 ionic strength, a sharp transition occurred in the vicinity of *p*H 3.1 and that by *p*H 2.6 a new, relatively stable state of DNA had been attained. Another study[4] showed that at the same ionic strength a comparable transition occurred upon heating in the vicinity of 90°. In both cases the configurational transition was marked by very substantial molecular contraction at constant molecular weight. This was interpreted as a breakdown of the periodically hydrogen bonded, Watson–Crick structure of DNA to form a randomly coiled and much more compact state in which the residual intramolecular hydrogen bonds kept the two polynucleotide chains united.

The investigation reported here was undertaken to complete this study of the denaturation of DNA. In particular we wished to see if it followed the same pattern established for acidic and thermal denaturation, to see if the alkaline conditions might lead to a separation of the two polynucleotide chains thought to make up the native DNA molecule and to examine the effects of diminished ionic strength on the molecular configuration of both the native and undenatured forms.

Experimental

DNA.—The DNA sample used was prepared from freshly frozen thymus glands by Dr. N. Simmons using a procedure that has been outlined elsewhere[4] and is known as sample SB-2. For the determination of concentration an extinction coefficient of 213 was used for neutral solutions (0.15 ionic strength) of undenatured DNA. After alkaline denaturation the optical density at 259 mμ increased 12%.

Physical Methods.—The details of the measurements of intrinsic viscosity and its extrapolation to zero gradient as well as the light scattering techniques are the same as previously described.[2,4,5] Sedimentation velocity measurements were determined using Schlieren optics and 30 mm. path length cells[5] and also using ultraviolet absorption. The results obtained by the latter methods were carried out by Mr. J. Eigner and will be reported later in fuller detail. They do not differ substantially from the technique described by Shooter and Butler.[6]

Exposure to Alkali.—The following standardized procedure was used throughout unless otherwise specified. The DNA solutions were prepared by dissolving DNA in 0.15 *M* NaCl with gentle stirring in the cold room for one or two days. A phosphate buffer solution of ionic strength 0.15 (containing approximately 0.05 *M* phosphate with NaOH) and *p*H 12.3–12.4 was added in approximately equal volume. After mixing the *p*H was observed to be about 12.1–12.2 and steady. The solutions were kept at this *p*H in tightly stoppered bottles for 1 hr. Subsequently they were dialyzed against a standard saline–citrate solution (0.15 *M* NaCl and 0.015 *M* sodium citrate). When viscosity measurements were made of the alkaline solutions, soda lime tubes were used to close all openings.

The *p*H-Dependence of Specific Viscosity: The Display of Alkaline Denaturation

The reduced specific viscosity of a DNA solution (51 μg./cc.) is shown as a function of *p*H in Fig. 1. Each point corresponds to a solution brought to that *p*H with buffer so as to avoid transient exposure to higher *p*H values. It is seen that the viscosity drops sharply in the region of *p*H 11.7 and reaches a lower plateau at *p*H 12.0. This change of viscosity with *p*H follows very closely the forward titration curve.[1]

This decrease in specific viscosity is irreversible if the DNA had been exposed to *p*H 12.0 or higher. When the maximum *p*H is slightly lower than this, where the transition is incomplete, a partial increase in specific viscosity was observed upon reneutralization.

From these observations we conclude that at 0.15 ionic strength DNA undergoes a substantial reduction in specific viscosity in the region of *p*H 11.5 to 12.0 and that change becomes complete and irreversible above *p*H 12.0. Such DNA is referred to as alkaline denatured.

Two other changes brought about by alkaline denaturation have been noted and these are common to acid and thermal denaturation as well. There is an abrupt loss of birefringence of flow and

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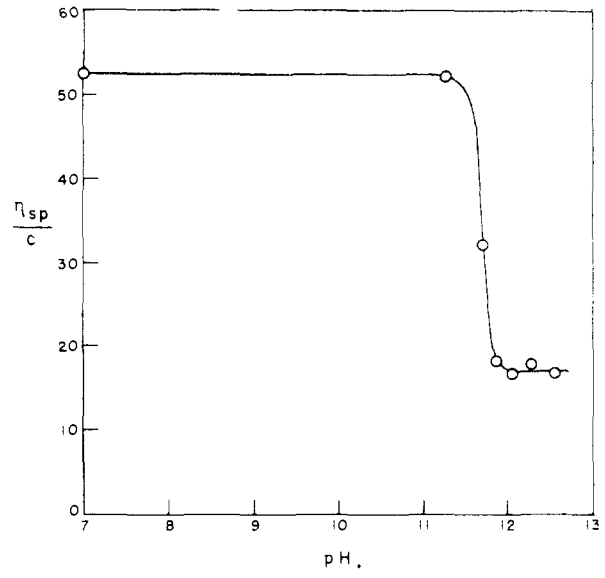

Fig. 1.—Reduced specific viscosity of DNA as a function of *p*H; ionic strength 0.165; gradient approximately 100 sec.$^{-1}$

an increase in optical density in the region of 260 mμ. This hyperchromic effect amounts to 12% increase in optical density at 259 mμ at the ionic strength used here.

Characterization of Alkaline Denatured DNA

Light Scattering.—The light scattering behavior of DNA at neutral *p*H before and after exposure to *p*H 12.1 is shown in Fig. 2 where the data are plotted according to Zimm. These results are typical of a number of experiments. They show that the alkaline treatment has brought about a substantial contraction of the DNA molecules since the mean radius of gyration has fallen from 2600 to 900 Å. as deduced from the limiting slope of the zero-concentration line. The molecular weight has not changed significantly as a result of the exposure to alkali. In this particular experiment the light scattering molecular weight was 5.0 million before and 6.3 million after exposure. The average results for our experiments are shown in Table I where it is seen that the corresponding numbers are 5.1 and 5.7 million, respectively, quantities that are within probable experimental error of each other.

The time duration of exposure to alkali was not critical. In one experiment aliquots of the same solution that had been brought to *p*H 12.2 were kept there for 30 minutes and 24 hours. The molecular weights of the neutralized solutions were determined to be 6.4 and 5.4 million and the radii of gyration 970 and 920 Å., respectively.

Viscosity.—In attempting to determine the reduced specific viscosities and hence the intrinsic viscosity of the neutralized denatured DNA, considerable lack of reproducibility was encountered. It was found eventually that above a concentration of 300 μg./cc. the values of the reduced specific viscosity (η_{sp}/c) increased with the concentration of the DNA solution that was exposed to alkaline *p*H. Values of η_{sp}/c determined on solutions of about 300 g./cc. were reproducible, but since the relative viscosity of such solutions was less than 1.2, measurements could only be made through a onefold dilution. This same dependence on the DNA concentration pertaining at the time of denaturation was also found in the study of thermal denaturation.[4]

When concentrations higher than 300 μg./cc. were avoided and extrapolations made to zero gradient (see Fig. 3), the value obtained for the intrinsic viscosity, $[\eta]$, was 6.1 ± 1.0.

TABLE I

CHARACTERISTIC PARAMETERS OF UNDENATURED AND ALKALI DENATURED DNA IN 0.165 IONIC STRENGTH AT *p*H 7

	Undenatured	Alkali denatured
Molecular weight (L.S.)	5,100,000[a]	5,700,000
Radius of gyration, Å.	2600	950
Intrinsic viscosity $[\eta]$	56	6.1
Sedimentation constant, $S^0_{20,w}$	22	22

[a] This molecular weight is not as high as that of other samples prepared in this Laboratory. This apparently is due to differing degrees of fractionation and degradation during isolation. In all cases the secondary structure of hydrogen bonds is intact.

Sedimentation.—The hypersharp sedimenting boundary characteristic of DNA in Schlieren optics remained unchanged as the *p*H was raised as high as 11.9. Indeed at *p*H 11.9 measurements of the sedimentation constant (s_{20}) at four concentrations in the range of 135 to 270 μg./cc. gave an extrapolated value of s_{20} of 21.0, a value equal to that found for the unexposed DNA. When this sample was returned to *p*H 7 and examined by light scattering, it was found that its radius of gyration had decreased somewhat, that is, to 2000 Å. Moreover, its intrinsic viscosity had fallen to 30 (100 cc./g.). Thus the DNA had undergone some irreversible damage, but this was undetectable by sedimentation behavior.

When the *p*H was increased to 12.2, however, the sedimentation pattern in Schlieren optics was quite changed. At the same concentrations as used above one now observed a broad peak that spread rapidly together with a faster moving peak. These two peaks merged into a single, asymmetric one before disappearing from view.

It seemed likely that the faster peak was due to aggregated material already thought to be responsible for abnormally high specific viscosities when the concentration of DNA during exposure to alkali was too high. Consequently it was considered best to examine the sedimentation pattern with ultraviolet optics which permitted observations to be made at a ten-fold greater dilution.

This was done by Mr. J. Eigner who exposed the DNA solution to *p*H 12.1 for 1 hr. and then dialyzed against the standard saline–citrate solvent. The intrinsic viscosity was found to fall from 61 to 6.0 duplicating quite well measurements that had been made in the work reported above two years previously. The sedimentation results in ultraviolet optics are shown in Fig. 4 where the relative concentration is plotted against the sedimentation rate. It is seen that the results for the unexposed and the exposed samples are almost identical. The mean

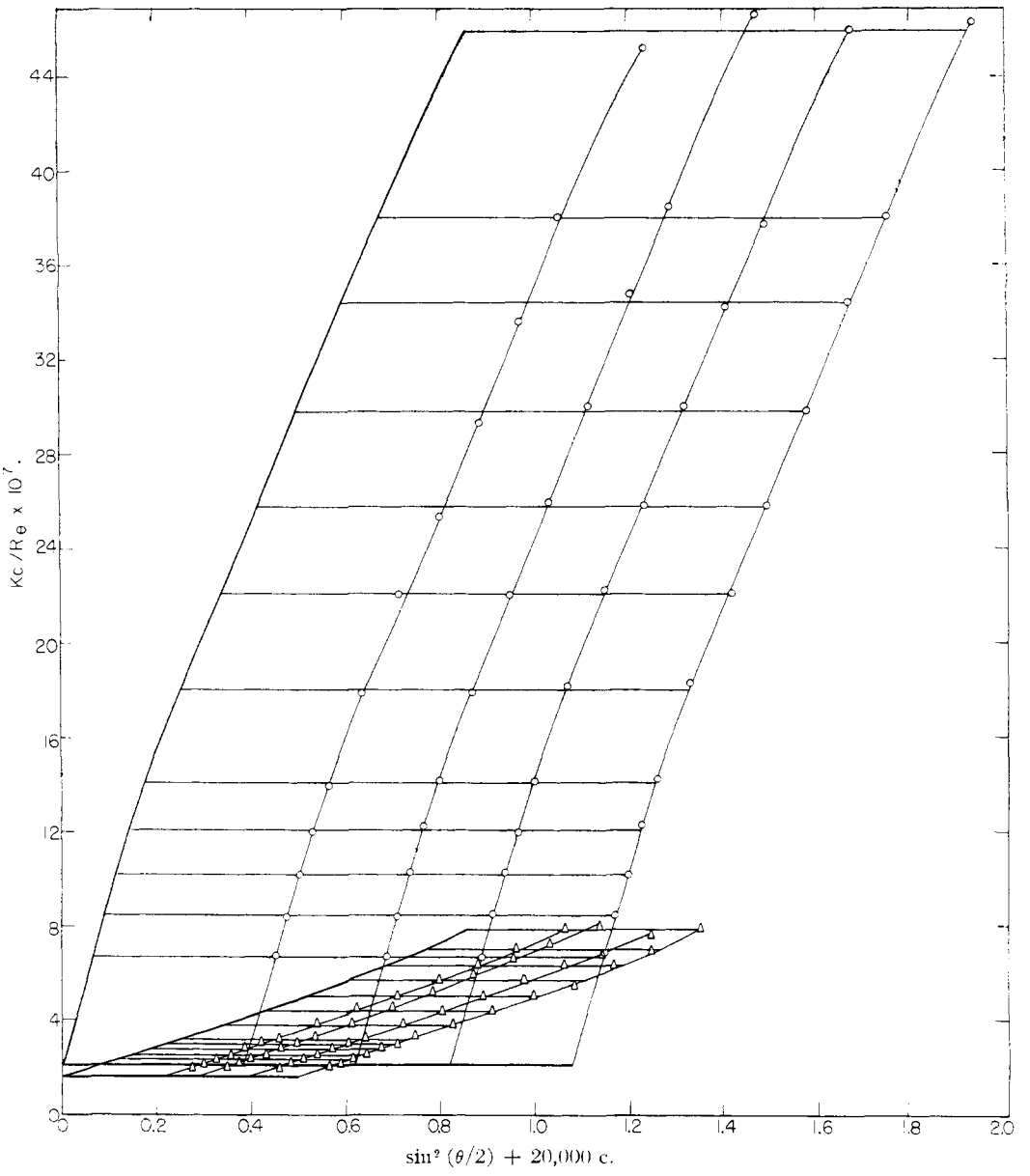

Fig. 2.—Light scattering data of DNA before (O) and after (Δ) exposure to *p*H 12.1 for one hour; measurements made at *p*H 7; ionic strength 0.165.

sedimentation coefficient is 21.5 for both samples. Using the concentration dependence found from Schlieren measurements the s^0_{20} value becomes 23.0.

Thus it appears that when the exposure to alkaline *p*H is done at sufficiently high dilution, as it was in the case of light scattering and viscosity, neither the mean sedimentation constant or the distribution of sedimentation constant is significantly altered.

Summary.—The physical chemical characteristics of the DNA and the alkaline denatured DNA studied here are summarized in the table. The constancy in the molecular weight and sedimentation constant, the threefold drop in radius of gyration and the ten-fold drop in intrinsic viscosity are all essentially the same as have been observed in acid and thermal denaturation. It seems clear therefore that each type of denaturation produces the same kind of disorganized and contracted structure in which the two polynucleotide strands making up the native molecule remain together.

The failure of the sedimentation constant to increase upon denaturation is surprising because the contraction of the molecules without a change in molecular weight should decrease the frictional constant and hence increase the sedimentation constant. The difficulty posed by this situation has been commented on before.[4,7,8] Now with the distribution of sedimentation constants also showing no change upon denaturation the dilemma becomes even more sharply etched.

Although we pose no solution to this difficulty here, it seemed of interest in this connection to examine the response of native and alkali denatured

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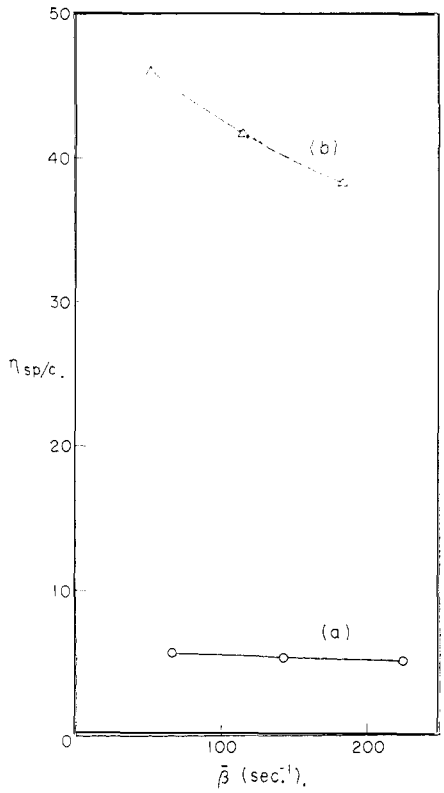

Fig. 3.—Reduced specific viscosity at *p*H 7 of alkali denatured DNA as a function of gradient at two different ionic strengths: upper points, 0.0055 ionic strength; lower points, 0.165 ionic strength.

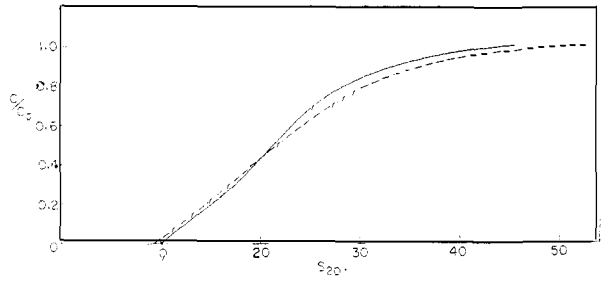

Fig. 4.—Sedimentation distribution of DNA before (full line) and after (dashed line) exposure to *p*H 12.1; DNA concentration; g./cc.

DNA to lowering of the ionic strength. This would indicate the relative rigidity of the two forms and define the lower limit of ionic strength at which measurements might be carried out in an investigation of this problem at lower ionic strength.

The Behavior of Native and Denatured DNA at Low Ionic Strength

The reduced specific viscosities extrapolated to zero gradient for native DNA are shown at two different ionic strengths (0.165 and 0.0055) in Fig. 5. The concentration dependence is quite similar in the two cases and the intrinsic viscosities, 56 and 63, are sufficiently alike to indicate that the mean molecular configuration has been essentially unchanged by this 30-fold lowering of ionic strength.

The examination of the alkali denatured DNA at the same two ionic strengths shows very great differences indeed. An example is shown in Fig. 3 where it is seen that η_{sp}/c is increased about eightfold at the lower ionic strength. That this indicates an expansion of the molecule due to the decreased shielding of the cations and is not due principally to the classical electroviscous effect is shown by the light scattering results. In Fig. 6 the reciprocal scattering envelopes extrapolated to zero concentration are shown for the same two ionic strengths. At 0.0055 ionic strength the radius of gyration is found to be 2.3 times greater than at 0.165. This is sufficient to account for the observed increase in viscosity.

Thus at this lower ionic strength the denatured DNA is expanded due to electrostatic repulsions of the negatively charged phosphate groups to the point where its space-filling properties nearly equal those of the undenatured DNA.

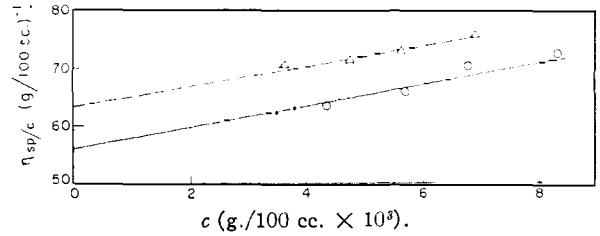

Fig. 5.—Reduced specific viscosities of DNA at two different ionic strengths *p*H 7: full line, 0.165 ionic strength, dashed line 0.0055 ionic strength.

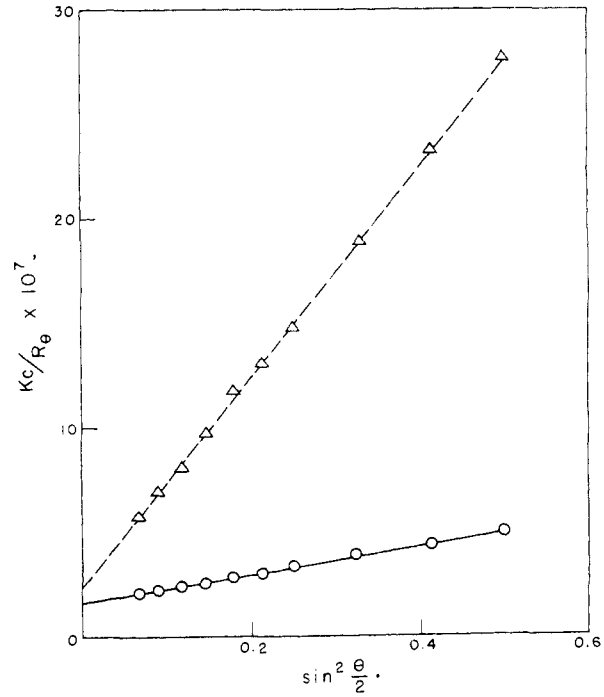

Fig. 6.—Reciprocal scattering envelopes for alkali denatured DNA at two different ionic strengths, *p*H 7. The points shown correspond to zero concentration having been obtained by extrapolation: full line, 0.165 ionic strength; dashed line, 0.0055 ionic strength.

The light scattering of undenatured DNA has also been examined at lower ionic strengths. It was

found that normal Zimm-type plots could be obtained as low as 0.0055 ionic strength. Under this condition we found a molecular weight of 5.0 million and a radius of gyration of 2500 Å. The difference from the 0.165 ionic strength measurements showed up in the value of the second virial coefficient. At the lower ionic strength it had a value of 13.5×10^{-4} (mole-cc./g.). This is about 10 times the value found at the usual ionic strength. Since the molecular size is unchanged this difference in excluded volume is presumably the result of the increased intermolecular electrostatic repulsion.

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CAMBRIDGE 38, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Equilibria in the Fibrinogen–Fibrin Conversion. IV. Kinetics of the Conversion of Fibrinogen to Fibrin Monomer[1,2]

BY SEYMOUR EHRENPREIS, MICHAEL LASKOWSKI, JR.,[3] THOMAS H. DONNELLY AND HAROLD A. SCHERAGA

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The kinetics of the proteolytic action of thrombin on fibrinogen (*i.e.*, step 1 of the fibrinogen–fibrin conversion) have been studied in 1 *M* NaBr, *p*H 5.3, at 0, 15 and 25°. No polymerization occurs under these conditions. The reaction was followed by analysis for fibrin monomer, using *p*-toluenesulfonyl-L-arginine methyl ester as a thrombin inhibitor. Step 1 was found to be initially first order in both thrombin and fibrinogen. The apparent disagreement between this result and those from previous kinetic studies has been resolved in a separate publication. Hence, previously postulated thrombin-inactivation reactions need not be introduced to explain the kinetic data. Step 1 thus appears to be a simple bimolecular reaction to form the enzyme–substrate complex under conditions where the enzyme is not saturated. The effects of *p*H and solvent on the rate constants have been reconciled with the expected behavior of the kinetic constants k_3 and K_m. Finally, in carrying the kinetic runs to their equilibrium position, it was found that step 1 does not go to completion in 1 *M* NaBr at *p*H 5.3. The failure to attain completion is not due to inactivation of any of the protein components present in the reaction but to a reversible equilibrium which will be discussed in greater detail in a forthcoming paper.

Introduction

The conversion of fibrinogen to fibrin may be considered to occur in three reversible steps, only the first of which involves thrombin.[4] In step 1 thrombin catalyzes the hydrolysis of two arginyl-glycine bonds[5-11] in fibrinogen leading to the liberation of at least two peptides and also carbohydrate material.[12] In this process the molecular weight of fibrinogen (330,000)[13,14] is reduced by about 3%, the remaining protein being designated *fibrin monomer*.[15] Under appropriate conditions[4] fibrin monomer polymerizes (step 2) and ultimately forms the fibrin clot (step 3). The three reactions may be written as

$$\text{Proteolysis} \qquad F \overset{T}{\rightleftharpoons} f + P \qquad (1)$$

$$\text{Polymerization} \qquad nf \rightleftharpoons f_n \qquad (2)$$

$$\text{Clotting} \qquad mf_n \rightleftharpoons \text{fibrin} \qquad (3)$$

where T is thrombin, F is fibrinogen, f is fibrin monomer, P represents the peptide and carbohydrate material, f_n designates the intermediate polymers and n and m are variable numbers.

Considerable information already has been obtained about the mechanism of step 2.[4] This paper, and several to follow, report on attempts to understand the mechanism of step 1. In this initial paper the kinetics of the thrombin–fibrinogen reaction are investigated under conditions where only step 1 occurs.[16] If the fibrinogen concentration is less than about 4%, step 1 will occur in 1 *M* NaBr at *p*H 5.3, without the subsequent polymerization of fibrin monomer.[18,19] Hence, this solvent, in which the stability of purified F and T has been fully established,[18,20] was employed in the present study. By using relatively purified thrombin the uncertainties involving the inactivation of fibrin monomer by commercial thrombin preparations[20]

(1) This investigation was supported by research grant No. H-1662 from the National Heart Institute of the National Institutes of Health, Public Health Service.

(2) Presented before the Division of Biological Chemistry at the 131st meeting of the American Chemical Society, Miami, Florida, April, 1957.

(3) Research Fellow of the National Heart Institute, United States Public Health Service, 1952–1956.

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