A FLOW BIREFRINGENCE METHOD FOR THE DETERMINATION OF DEOXYRIBONUCLEASE ACTIVITY*

by

WALTER J. FRAJOLA, JACOB G. RABATIN AND HAROLD C. SMITH

The Herman A. Hoster Research Laboratory of the Department of Medicine and the Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio (U.S.A.)

Though KUNITZ[2] in 1950 reported that deoxyribonuclease (DNase)** caused complete disappearance of the double refraction of flow of deoxyribonucleic acid (DNA), flow birefringence studies have not been utilized for quantitative kinetic measurements of DNase activity. The emphasis, rather, in flow birefringence studies has been directed toward the determination of the size and shape of the DNA molecule[3–7] and toward the effect of acid, alkali and various salts on the birefringence of DNA solutions[8–16]. This report describes a new method for the determination of DNase activity based upon the ability of the enzyme to decrease the flow birefringence of DNA or calf thymus nucleoprotein (DNP) solutions. TODESCO in 1928[17] added a photoelectric cell and a densitometer to the usual birefringence of flow apparatus. Accurate quantitative measurement of the decrease in transmission of elliptically polarized light resulting from enzyme action upon DNA solutions located in the annular gap of the birefringence of flow apparatus is consequently possible. For detailed information on the theory, apparatus and applications of flow birefringence the excellent reviews by EDSALL[18] and CERF AND SCHERAGA[19] are recommended.

EXPERIMENTAL

Materials

DNP was prepared from calf thymus according to the procedure of MIRSKY AND POLLISTER[20]. The final precipitate was dissolved in 1 *M* NaCl pH 7.0. DNase (from beef pancreas, once crystallized) and DNA were purchased from Worthington Biochemical Sales Company, Freehold, New Jersey. The DNase was dissolved in 1 *M* NaCl for DNP experiments and in H_2O for DNA experiments. DNA was dissolved in water.

Methods

A schematic diagram of the flow birefringence apparatus is shown in Fig. 1. The construction of the apparatus was based on the design of EDSALL *et al.*[21]. The outer rotor had a diameter of 2.54 cm. The inner cylinder diameter was 2.34 cm. The annular gap was 0.10 cm. The length of the annular space through which the polarized light passed was 7.0 cm. To fill the annular space required 7.5 ml of solution. When the polaroids were crossed and the annular space filled with

* This investigation was supported in part by research grants #C-1084(C4) and #C-2330 Bio from the National Cancer Institute of the National Institutes of Health, Public Health Service and from the Dorothy H. and Lewis Rosenstiel Foundation. Part of the data was presented to the Division of Biological Chemistry of the American Chemical Society meeting at Cincinnati, Ohio, March 29 – April 1, 1955[1].

** The following abbreviations will be used in this paper: DNase = deoxyribonuclease; DNA = deoxyribonucleic acid; DNP = calf thymus nucleoprotein.

References p. 544.

a DNP or DNA solution, the area between the concentric cylinders brightened as the outer cylinder was rotated. The amount of elliptically polarized light reaching the photocell was measured as % transmission by the deflection on the scale of the photovolt apparatus (Photometer model

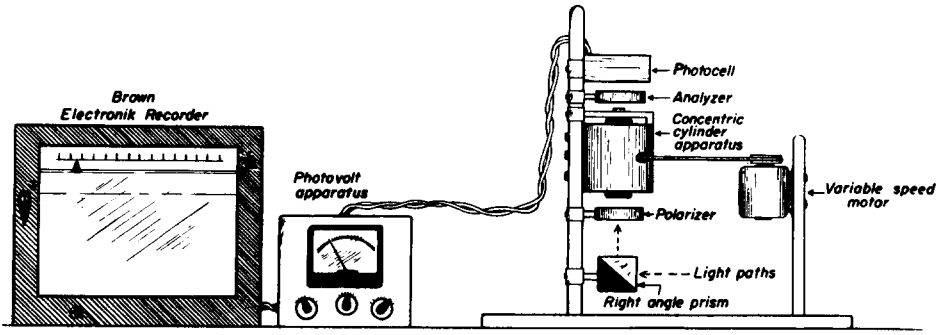

Fig. 1. Flow birefringence apparatus for measurement of DNase activity.

501-A, Photovolt Corporation, N.Y.). When the annular space contained only 1 *M* sodium chloride or water or when the outer cylinder was stationary, practically no light reached the photocell and thus % transmission was approximately zero depending on the light source diaphragm setting. During enzymic action the large initial % transmission gradually diminished until it approached zero when the reaction was complete. For some experiments automatic recording of the changes in the transmission of elliptically polarized light as a function of time was obtained by connecting a Brown Electronik recorder to the photovolt apparatus. For greatest accuracy, however, readings were not made while the rotor was in continuous operation but at stated time intervals between which the rotor was stopped. This reduced the heating of the solutions due to friction. All enzymic reactions and flow birefringence measurements were conducted in a 20°C constant temperature room.

DNP solutions were made by dilution of a 0.85% DNP stock solution with 1 *M* NaCl. For comparison of flow birefringence changes with viscosity changes, 5 ml aliquots of the reaction mixtures were added to 1 ml of 1 *M* sodium citrate to inhibit the enzyme. All viscosity measurements were made at 25°C with an Ostwald viscosimeter.

RESULTS

Because the transmission of light at zero velocity gradient varied from 0–5% depending upon the solutions used and upon the extent of the opening of the light source diaphragm some results are expressed as Δ % transmission. This was obtained by subtracting the % transmission at zero velocity gradient from that observed at the velocity gradient under study. Fig. 2 presents the results obtained for the study of the effect of the rate of flow upon the birefringence of DNA solutions of four different concentrations. It is apparent that an increased velocity gradient produced an increased birefringence of DNA. The relationship was

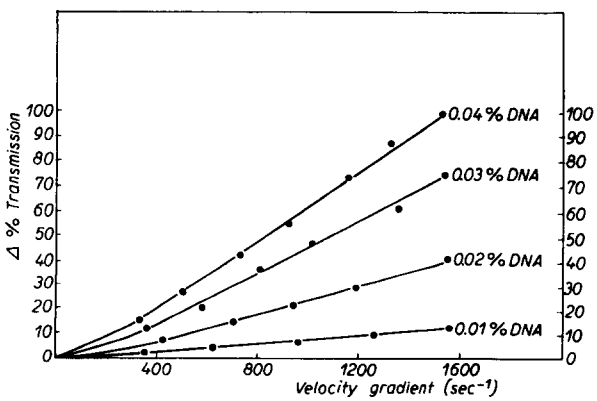

Fig. 2. Effect of concentration of DNA and its rate of flow on the percent transmission of elliptically polarized light (flow birefringence) by the DNA.

References p. 544.

linear for DNA solutions from 0.01 to 0.04% for velocity gradients from about 300 sec^{-1} to 1600 sec^{-1}. Graphs of Δ % transmission per unit velocity gradient versus concentration of DNA also indicated the direct relationship between flow birefringence, velocity gradient and concentration of the DNA. Similar results were obtained for solutions of DNP in 1 M NaCl except that at higher concentrations (above 0.19%) the Δ % transmission reached a maximum at about 1100 sec^{-1} and then decreased when the flow rate was increased. Maximum birefringence was observed at lower flow rates when the concentration of the DNP solutions was increased.

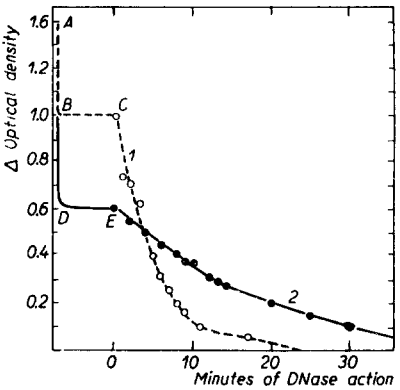

Fig. 3. The change in flow birefringence of DNA (optical density units proportional to the concentration of the birefringent DNA) as a function of the time of DNase action. DNA = 0.04%; Mg^{++} = 0.044 M; DNase = 0.039 γ/ml; G = 524 sec^{-1}

It may be assumed on the basis of flow birefringence studies that DNA or DNP solutions may contain elongated rigid particles in a distribution of sizes or semi-rigid, moderately flexible, kinked rods in various stages of contraction or elongation[6]. The Δ % transmission as determined above is thus directly proportional only to the concentration of those DNA or DNP particles which are birefringent for the given conditions. Then, since Δ % transmission is directly proportional to the concentration of the birefringent DNA the results of DNase action upon DNA at a given velocity gradient can be expressed as changes in the concentration of the birefringent DNA. In kinetic studies of enzyme action it is customary to plot the logarithm of the substrate concentration versus time to determine the order of the reaction. In these birefringence experiments this is simply accomplished by recording the optical densities directly from the densitometer rather than the % transmission. The term, ΔOD, as hereafter used, therefore, refers to the logarithm of the concentration of birefringent DNA.

Fig. 3 illustrates the effect of DNase and also of NaCl and $MgSO_4$ upon the bi-

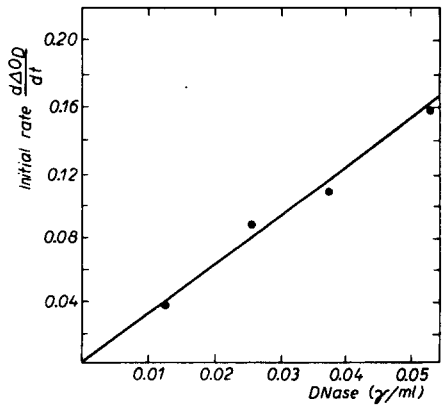

Fig. 4. The rate of change of the concentration of birefringent DNA as a function of the enzyme concentration. DNA = 0.04%; Mg^{++} = 0.044 M; G = 524 sec^{-1}

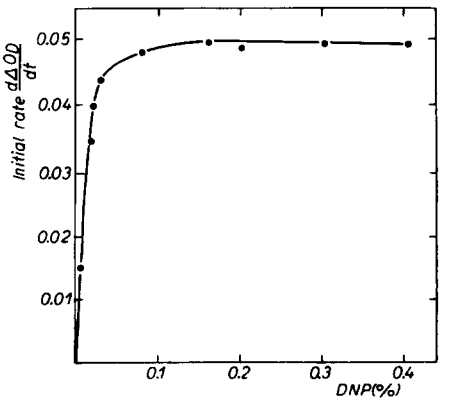

Fig. 5. The rate of change of the concentration of birefringent DNP as a function of the concentration of the DNP. DNase = 0.0008 mg/ml; Mg^{++} = 0.031 M; G = 524 $sec.^{-1}$.

References p. 544.

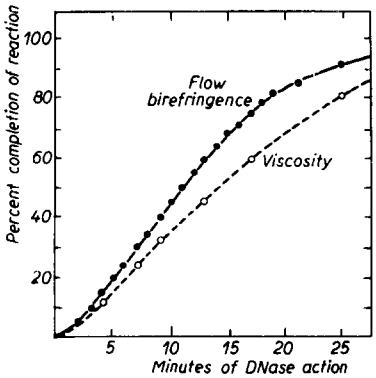

Fig. 6. A comparison of DNase action as measured by changes in the viscosity of DNP with changes in the concentration of birefringent DNP. DNP = 0.17%; DNase = 0.00095 mg/ml; Mg^{++} = 0.035 M; G = 524 sec^{-1}.

refringence properties of DNA. The addition of $MgSO_4$ to a 0.04% water solution of DNA resulted in an immediate 37.5% decrease in the ΔOD (part AB of curve 1, Fig. 3). No further change occurred (BC curve 1, Fig. 3) until DNase was added. Birefringence of flow then decreased as a first order reaction. When a mixture of NaCl and $MgSO_4$ was added to the DNA solution (final concentrations 0.33 M NaCl and 0.044 M $MgSO_4$) a 62.5% decrease in ΔOD was observed. The rate of the reaction for the water system (curve 1) was 0.11 ΔOD/min as compared to 0.03 ΔOD/min for the NaCl system (curve 2).

The initial rate of the reaction between DNase and DNA was directly proportional to the concentration of DNase for the concentrations measured (0.01 to 0.05 γ/ml). (Fig. 4). Similar results were obtained for the DNase-DNP system for DNase concentrations between 0.001 and 0.003 mg/ml. Fig. 5 is a typical Michaelis-Menten curve obtained by the flow birefringence method for the action of DNase on DNP in molar salt solution.

A comparison of the changes in flow birefringence and viscosity of solutions of DNP was obtained by inhibition of the reaction mixtures with sodium citrate at various time intervals. Inspection of Fig. 6 reveals that the flow birefringence changes occurred somewhat more rapidly than the viscosity changes.

DISCUSSION

The variation of the streaming birefringence of DNA solutions as a direct function of the concentration of the DNA or of the velocity gradient was not an unexpected finding. LAUFFER AND STANLEY[22] reported that the amount of elliptically polarized light transmitted by a solution of tobacco mosaic virus increased as the concentration of the virus increased. EDSALL[18] and others[4,5,30] observed a direct relationship between the velocity gradient and the flow birefringence of sodium thymonucleate solutions. Likewise, the observed decrease in flow birefringence of DNA solutions is in accord with results of other investigators[8,9,11,12].

The flow birefringence method for the determination of DNase activity is relatively simple to perform, fast, accurate and reproducible. Kinetic studies are possible with one enzyme-substrate mixture by recording the optical densities at various times during the continuous reaction. Because of the time required to make the measurements, viscosimetric[23], ultracentrifugal[24] and other methods for measuring DNase action require inhibition of the reaction at certain time intervals and thus involve the use of several aliquots or reaction mixtures.

Theoretically this method for the study of DNase action should be applicable to the study of any enzyme-substrate system in which the substrate initially exhibits flow birefringence but loses this property after enzyme action. Application of this method to the study of the DNase activity of various sera, serum protein fractions, white blood cells and various tissue extracts is presently being investigated.

References p. 544.

ACKNOWLEDGEMENT

The authors wish to thank Mr. EARL SCHOFIELD and Mr. FRANK ZIMMERMAN of the Ohio State University Medical Shop for the construction of the flow birefringence apparatus.

SUMMARY

A quantitative method for the study of deoxyribonuclease activity by means of a photocell-flow birefringence apparatus is described. The amount of elliptically polarized light transmitted by DNA or DNP solutions varied directly as the velocity gradient and the concentration of the DNA or DNP solutions. Salts decreased the flow birefringence. The decrease in flow birefringence due to the action of DNase is comparable to the data obtained by viscosimetry.

RÉSUMÉ

Une méthode quantitative pour l'étude de l'activité DNase au moyen d'un appareil à cellule photo-électrique pour la mesure de la biréfringence est décrite. La quantité de lumière polarisée elliptiquement transmise par des solutions de DNA ou DNP varia directement avec le gradient de la vitesse et les concentrations des solutions de DNA ou DNP. Les sels diminuèrent la biréfringence d'écoulement. Le décroissement de la biréfringence d'écoulement dû à l'action de la DNase est comparable aux données obtenues par viscosimétrie.

ZUSAMMENFASSUNG

Eine quantitative Methode zur Untersuchung der DNase-Aktivität mittels eines Photozelle-Strömungsdoppelbrechungs-Apparates wurde beschrieben. Die Menge des elliptisch polarisierten Lichtes welche die Lösungen von DNA oder DNP durchlassen, variierte mit der Geschwindigkeitssteigung und der Konzentration der DNA- und DNP-Lösungen. Salze verminderten die Strömungsdoppelbrechung. Die Abnahme der Strömungsdoppelbrechung die von der Wirkung der DNase herrührt, ist mit den Angaben vergleichbar, die man durch Viskosimetrie erhalten hatte.

REFERENCES

1 W. J. FRAJOLA, J. G. RABATIN AND H. C. SMITH, *Absts. Division of Biol. Chem., 127th meeting Am. Chem. Soc.*, Cincinnati, Ohio, March 29–April 1, 1955.
2 M. KUNITZ, *J. Gen. Physiology*, 33 (1950) 363.
3 O. SNELLMAN AND G. WIDSTROM, *Arkiv Kemi. Mineral Geol.*, 19A (1945) No. 31.
4 R. SIGNER AND H. SCHWANDER, *Helv. Chim. Acta*, 32 (1949) 853.
5 H. SCHWANDER AND R. CERF, *Helv. Chim. Acta*, 32 (1949) 2356.
6 M. GOLDSTEIN AND M. E. REICHMANN, *J. Am. Chem. Soc.*, 76 (1954) 337.
7 A. R. MATHIESON AND M. R. PORTER, *Nature*, 173 (1954) 1190.
8 J. M. CREETH, J. M. GULLAND AND D. O. JORDAN, *J. Chem. Soc.*, (1947) 1141.
9 H. SCHWANDER AND R. CERF, *Helv.Chim. Acta*, 34 (1951) 1344.
10 R. JEENER, *Compt. rend. Soc. Biol.*, 140 (1946) 1138.
11 R. L. REY, *Compt. rend.*, 237 (1953) 1570.
12 R. L. REY, *J. chim. phys.*, 51 (1954) 85.
13 H. SCHWANDER AND R. SIGNER, *Helv. Chim. Acta*, 34 (1951) 1344.
14 J. P. GREENSTEIN AND W. W. JENRETTE, *J. Nat. Cancer Inst.*, 1 (1940) 77.
15 A. R. MATHIESON AND M. R. PORTER, *Biochim. Biophys. Acta*, 14 (1954) 288.
16 J. W. ROWEN, *Biochim. Biophys. Acta*, 10 (1953) 913.
17 G. TODESCO, *Atti accad. Lincei*, 7 (1928) 394.
18 J. EDSALL, *Advances in Colloid Science*, 1 (1942) 269.
19 R. CERF AND H. SCHERAGA, *Chem. Rev.*, 51 (1952) 185.
20 A. E. MIRSKY AND A. W. POLLISTER, *J. Gen. Physiol.*, 30 (1946) 117.
21 J. T. EDSALL, A. RICH AND M. GOLDSTEIN, *Rev. Sci. Instruments*, 23 (1953) 695.
22 M. A. LAUFFER AND W. M. STANLEY, *J. Biol. Chem.*, 123 (1938) 507.
23 J. P. GREENSTEIN AND W. V. JENRETTE, *J. Nat. Cancer Inst.*, 1 (1941) 845.
24 J. G. RABATIN, R. FRIEDLAND AND W. J. FRAJOLA, *J. Biol. Chem.*, 203 (1953) 23.

Received July 30th, 1955