but rather arises from the structural alterations attending intermolecular linkage.

On the basis of infrared spectroscopy of insulin fibrils and the assumption that the strong 4.8 Å. meridional reflection is a backbone spacing, Ambrose and Elliott[19] suggest that the polypeptide chains are in the β-configuration and lie perpendicular to the fibril axis. The data presented are not decisive and, as the authors note, could be interpreted also to indicate folded polypeptide chains parallel to the fibril axis. The X-ray diffraction pattern of insulin fibrils bears little resemblance to the cross β-pattern given by "denatured" insulin.[20] The 4.8 Å. meridional reflection is not a backbone spacing, as they assumed, since it is not only larger (4.8 *vs.* 4.65 Å.) but more intense than the usual backbone spacing. Rather for the reasons given above, we suggest that it is associated with the fibril period.

Moreover, the reversibility of the monomer–fibril transformation and the usual seeding conditions under which fibril elongation will take place suggest that monomeric insulin enters the fibril structure with, at most, slight distortion, and without the far reaching changes accompanying transformation to the β-form. It seems highly probable, therefore, that in insulin fibrils the polypeptide chains retain their normal folded condition, with axes predominantly parallel to the fibril axis.

Acknowledgment.—The work reported here was generously supported by Armour and Company, Chicago, Ill., and S. E. L. Manduro and Sons, Inc., of Chicago.

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Mechanism of Desoxyribonuclease Depolymerization: Effect of Physical and Enzymatic Depolymerization on the Affinity of Methyl Green and of Desoxyribonuclease for Desoxyribonucleic Acid[1a]

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Desoxyribonucleic acid (DNA) was partially depolymerized by heat. The products were characterized by methyl green binding capacity, viscometry, dialysis and ultracentrifugation. The effect on the rate of depolymerization by desoxyribonuclease (DNase) was studied by means of methyl green affinity and viscometry. It was found that heat depolymerization of a degree insufficient to change the elementary composition of the DNA or to give rise to dialyzable products, greatly reduced the affinity for DNase. The change in enzymatic susceptibility paralleled the reduction in methyl green affinity produced by heat. Both DNase affinity and methyl green affinity are less extensively influenced by DNase depolymerization than by heat depolymerization, when the same reduction in viscosity of the DNA solution is produced by both. The implications as to the structure of polymerized DNA are considered.

The specific requirements in the desoxyribonucleic acid (DNA) molecule for optimal susceptibility to depolymerization by desoxyribonuclease (DNase) are not known. Tamm, *et al.*,[2] have suggested that alterations in the macromolecular structure of DNA, which do not involve change in atomic composition ("denaturation"), do not influence affinity for DNase. They report, however, that the removal of purine groups by acid hydrolysis results in marked reduction in enzyme affinity. The present study is concerned with the effect of "denaturation" on the susceptibility of the DNA molecule to depolymerization by DNase.

Previous studies on the action of depolymerizing agents on DNA have employed techniques to follow the reduction in viscosity, reduction in rate of sedimentation in the ultracentrifuge, increase in ultraviolet absorption, lowering of *p*H, formation of acid-soluble and dialyzable products. Because the affinity of DNA for methyl green appears to be influenced by the molecular size and steric configuration of the nucleic acid,[3,4] we have applied this tool, together with those mentioned above, to a study of the effect of "denaturing" agents on DNA.

Experimental

Materials. (a) Preparation of DNA.—The sodium desoxyribonucleate (DNA) used in these experiments was prepared by a modification of the method of Kay, Simmons and Dounce.[5] Fresh calf thymus (obtained directly from the slaughter-house) was blended in a Waring Blendor with approximately 5 volumes of a solution containing 0.14 *M* NaCl. and 0.01 *M* sodium citrate per liter, plus 1 volume ice (sufficient to keep the temperature at 0–2° during the blending). After blending 9 minutes, the sediment obtained on centrifugation at 2,000 r.p.m. (all procedures at 0–2°) was washed several times with cold sodium chloride–sodium citrate solution. The sediment was then resuspended in this solution, an equal volume of 2 *M* NaCl added, and blended. The very viscous, opalescent solution was centrifuged for 2–4 hours at 18,000 r.p.m. at 2° (Sorvall SS-2 centrifuge), the small sediment discarded, and the clear viscous supernatant treated 3 times with sodium dodecylsulfate according to Kay, Simmons and Dounce.[5] The fibrous DNA was collected, following precipitation by alcohol, washed with 95% alcohol and acetone. The acetone was evaporated off at room temperature, and the DNA stored in a $CaSO_4$ desiccator. It contained 8.1% P, 13.3% N (N/P =

(1) (a) This work was aided by grants from the American Heart Association; the National Heart Institute (H-714), United States Public Health Service; the American Cancer Society (recommended by the Committee on Growth, National Research Council) and the Life Insurance Medical Research Fund. Presented at the 123rd meeting of the Am. Chem. Soc., Abstract No. 58, p. 23C, March 15–19, 1953, Los Angeles. (b) V. A. Hospital, Long Beach 4, Calif.

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1.65), 10% H_2O. Corrected for water content, the content of P is thus 9.0%, and of N, 14.8%.

(b) **Other Reagents.**—A stock solution of crystalline beef pancreatic DNase[6,7] in 0.2 *M*, *p*H 5.0, acetate buffer was made, containing 200 μg./ml. The stock solution was stored at 0–2° and discarded after 1 week.

A 0.4% aqueous solution of methyl green[8] was exhaustively extracted with $CHCl_3$ before use, in order to remove the contaminating violet stain (presumably crystal violet).

(c) **Properties of the DNA and Methyl Green–DNA Complex.**—The DNA combines with methyl green in the proportions expected[9] and sediments with it in the analytical ultracentrifuge (Fig. 1b). The methyl green–DNA complex (0.1% DNA, 0.0075 *M* $MgSO_4$, 0.02% methyl green in *p*H 7.5, 0.05 *M* tris-methylolaminomethane buffer[10] sediments at a rate of 6.5 svedbergs (52,640 r.p.m.) with a very sharp boundary. The sedimentation rate for a 0.25% solution of DNA (0.05% methyl green, $MgSO_4$ and buffer as above) is 5.3S. The sedimentation velocities are the same without methyl green, and are similar to those reported by Conway, Gilbert and Butler[11] for their preparation. The absorption spectrum shows a maximum at 259 mμ (extinction coefficient per gram-atom phosphorus per liter, P/l. = 6640) and a minimum at 231 mμ (extinction coefficient per gram-atom P/l. = 2415). These values may be compared to those of Chargaff and Zamenhof,[12] who found the maximum at 260 mμ of 6056–6210 and the minimum at 232 mμ of 2485.

The specific viscosity of a 2 mg./ml. solution of DNA in 0.05 *M* tris-methylolaminomethane buffer (*p*H 7.5) measured in an Ostwald–Fenske viscometer (#3, Table II) at 30°, is about 35. Under the same conditions, a 5 mg./ml. solution does not flow at all, even in a viscometer with a viscosity gradient of 2500 for water (#6). On prolonged storage of the solution at 0–2°, the viscosity falls to approximately 8. The viscosity may be reduced rapidly to this point by only brief blending in the Waring Blendor.

Methyl green increases the viscosity of DNA slightly, as shown in Table I. The effect on viscosity was determined by diluting solutions of known concentration of DNA in 0.05 *M*, *p*H 7.5, tris-methylolaminomethane buffer with an equal volume of water or an equal volume of 0.04% methyl green in water and comparing the viscosities (in the same viscometer for each pair). The viscosities of the solutions are the same for a given concentration of DNA whether prepared by serial dilution with buffer of a concentrated methyl green–DNA solution (0.25% DNA, 0.02% methyl green) or by the addition of methyl green solution to diluted DNA solutions as above, and are thus independent of the concentration of methyl green (always in considerable excess of the stoichiometric amount).[13,14]

TABLE I

EFFECT OF METHYL GREEN ON VISCOSITY OF DNA SOLUTIONS

Note: Because of the anomalous viscosity of the DNA solutions, the results (both relatively and absolutely) vary with the viscometry pipet used. The concentration and viscosity gradient ($\bar{\beta}$) dependence is greater with the more viscous solution. Thus, in pipet #7 (Table II), the values for the 0.25% solution were 172 with methyl green and 86 without (ratio 2.0); in pipet #6, the values were 24.8 and 17.1, respectively (ratio 1.45).

	Concentration of DNA, % 0.25	0.125	0.0625	0.01
DNA, η_{sp}	39.4	5.16	1.55	0.219
(Methyl green–DNA), η_{sp}	66.6	5.81	1.70	0.233
Ratio	1.69	1.13	1.10	1.06
Pipet used	#3	#3	#3	#4

Procedure.—A 0.2% solution of DNA was prepared in 0.05 *M*, *p*H 7.6, tris-methylolaminomethane buffer with gentle stirring, to avoid mechanical depolymerization. Ten-ml. samples of this solution were heated at 80° or at 100° in loosely stoppered tubes for periods of 5 minutes to 7 hours. After cooling in running tap water, the original volume (reduced by evaporation) was restored with distilled water. The absorption spectra of the original solution and of treated solutions were measured in the Beckman DU quartz spectrophotometer using path lengths suitably reduced by quartz inserts[15] or after dilution with water (1:50). Total nitrogen (by Nesslerization[16] following H_2SO_4–H_2O_2 digestion), phosphorus[17] and viscosity were determined. Viscometry was performed in Ostwald–Fenske pipets without external pressure. The characteristics of the pipets used in this study are given in Table II.[18]

TABLE II

CHARACTERISTICS OF VISCOMETRY PIPETS

Pipet no.	Total vol., ml.	Vol. flowing through capillary	Mean *h*, cm.	Length of cap., cm.	Radius of capillary, mm.[a]	Time, sec., H_2O 30°	$\bar{\beta}$[b] H_2O, 30°, sec.$^{-1}$
1	3	2.1	15	9.7	0.27	50.8	1683
2	3	2.1	13.5	9.1	.26	64.2	1578
3	6	3.1	10.2	7.5	.32	47.0	1747
4	6	3.1	9.7	7.0	.20	272	1150
5	6	3.3	10.1	7.6	.33	50	1772
6	6	2.9	10.0	7.0	.45	9.2	2490
7	0.35	0.2	14.2	13.7	.23	14.2	970

[a] From Poiseuille's equation. [b] After Kroepelin.[23]

Two to five ml. portions of the original and heated nucleic acid solutions were placed in Viscose tubing and dialyzed for 24 hours at 0–2° against 5 volumes of buffer or distilled water. The phosphorus content and the absorption spectra of the dialysis baths were determined. The nucleic acid solutions were then dialyzed against running tap water (15°) for 24 hours, and against several changes of distilled water (0–2°) for 24 hours. The contents of the dialysis bags were analyzed for phosphorus and nitrogen, and the ultraviolet absorption spectrum was determined.

Samples of the original and of the heated nucleic acid solutions were diluted to 0.1% with 0.05 *M*, *p*H 7.6, buffer and centrifuged at 52,640 r.p.m. in the Spinco Analytical Ultra-centrifuge. Another sample of each DNA solution was diluted with the buffer and 0.4% methyl green solution to a final concentration of 0.1% DNA, 0.05 *M* buffer, 0.02% methyl green, and similarly centrifuged. Schlieren (and methyl green) patterns were photographed when full speed was attained and at 32-minute intervals for 128 minutes.

(6) Purchased from Worthington Biochemical Laboratory, Freehold, N. J.

(7) M. Kunitz, *J. Gen. Physiol.*, **33**, 349 (1949).

(8) National Aniline Division, Allied Chemical and Dye Corp., Methyl Green (Ethylated Hexamethyl Pararosaniline), C. I. No. 685, Certification No. NG 26.

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(16) J. A. Kolmer and F. Boerner, "Approved Laboratory Technic," D. Appleton-Century Co., New York, N. Y., 1941.

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(18) Since, for the most viscous solutions, the viscosity gradient, $\bar{\beta}$, was as low as 100 sec.$^{-1}$, the η_r values measured for these solutions are higher than the limiting value at which η_r is independent of $\bar{\beta}$,[19] and increase with anomalous rapidity with concentration.[20] On the other hand, extrapolation to $\beta = 0$[21] (*i.e.*, the viscosity in the absence of orientation) would greatly increase the values over the measured viscosities.[22] However, as will be apparent, the conclusions to be drawn are not affected significantly by the anomalous structural viscosity.

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From each of the heated and unheated 0.2% DNA solutions a DNA–methyl green substrate solution for desoxyribonuclease activity determination was prepared.[24] The final concentration of enzyme in the substrate–enzyme solution was approximately 0.02 μg./ml. Depolymerization of the DNA was followed by change in viscosity and in optical density at 640 mμ of the methyl green–DNA solutions. Solutions were also prepared with DNA and methyl green concentrations 10 times that ordinarily used[24] in order to provide sufficient material to give a visible pattern in the ultracentrifuge and to permit more accurate viscometric analysis of the enzymatic depolymerization. With the concentrated substrate solution, 1.75 μg./ml. enzyme was used because of the inhibitory effect of high DNA concentration (*cf.*[25,26]). In the figures, the rate of DNase depolymerization is given in terms of the reduction in optical density in 240 minutes, and is referred to as "DNase affinity."[26]

The methyl green binding capacity of both heated and unheated DNA solutions was determined by measuring the optical density at 640 mμ of the methyl green–DNA substrate in the absence of enzyme.

Results

Effects of Heat Depolymerization.—The effects of heat depolymerization of DNA in solution with respect to specific viscosity (η_{sp}), molar extinction coefficient (referred to P) before and after dialysis, and the sedimentation constant are summarized in Table III. Marked reduction in specific viscosity is apparent. However, no dialyzable nitrogenous bases were produced by heating under the conditions used, as indicated by the absence of ultraviolet absorbing material in the dialysis baths and the agreement of the absorptions of the dialyzed solutions with the pre-dialysis values. This result is in agreement with that of Tamm, Hodes and Chargaff,[27] who found the same negligible change in elementary composition of the dialyzed DNA after subjecting it to 100° for 1 hour at *p*H 4.45 as at 37° *p*H 4.0 or *p*H 2.9. The absorption coefficient rises as noted in the table, but the maximum absorption remains at 259 to 260 mμ and the minimum at 231 mμ. The sedimentation constant falls only after very marked reduction in viscosity. Indeed, the sedimentation constant rises slightly at first. The sedimentation boundary becomes broader, however.

TABLE III

EFFECT OF HEAT DEPOLYMERIZATION ON DNA

Treatment	η_{sp} 0.2% soln.	$\epsilon_P^{260m\mu}$ before dialysis	E_{260}/E_{231}	$\epsilon_P^{260m\mu}$ after dialysis	$S \times 10^{-13}$ of 0.1% soln. with 0.02% methyl green
Untreated	19.2	6640	2.75	6460	6.62
180′ 80°	4.5	6750	2.80	6780	
420′ 80°	1.41	7060	2.71	6900	7.68
20′ 100°	0.635	7210	2.71	7360	
60′ 100°	0.125	7270	2.71	7570	4.30
120′ 100°	0.09	7560	2.60	7790	3.00

In Fig. 1, are shown the sedimentation patterns of 0.1% solutions of heated and unheated DNA complexed with methyl green after 32 minutes of centrifugation. Sedimentation of methyl green with the DNA (*i.e.*, as a complex) in all samples is apparent. Shown in Fig. 1a is the sedimentation of a 0.1% solution of untreated DNA (without methyl green) to demonstrate the hyper-sharp boundary, which coincides with the sharp methyl green boundary.

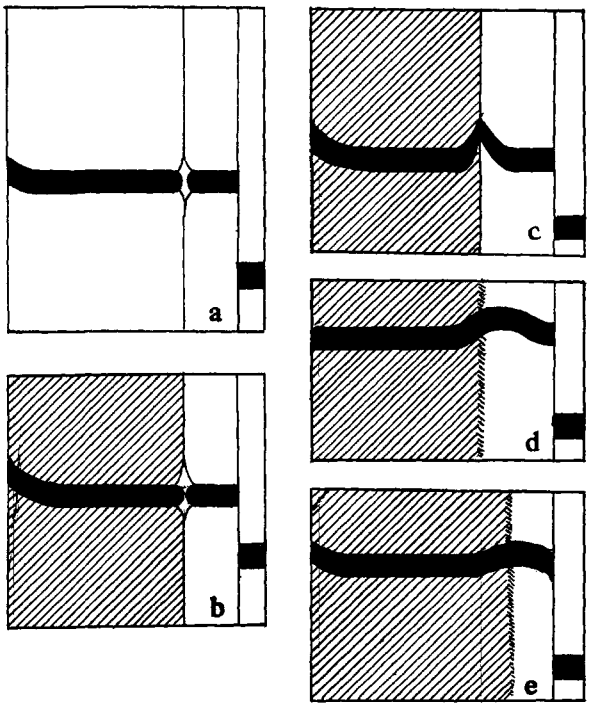

Fig. 1.—Ultracentrifugation patterns after 32 minutes at 52,640 r.p.m. Sedimentation is from right to left. Shaded areas represent light absorption by the methyl green–DNA complex: a, 0.1% untreated DNA; b, 0.1% DNA with 0.02% methyl green, untreated; c, same as b, heated to 80° for 7 hours; d, same as b, heated to 100° for 1 hour; e, same as b, heated to 100° for 2 hours.

The data on the effect of heat on η_{sp} methyl green affinity ($E°_{640}$) and DNase affinity are shown in Fig. 2. Figure 2 illustrates the progressive reduction in specific viscosity of DNA solutions toward zero during heating. The affinity for methyl green and DNase is reduced less rapidly. At 100°, both of these functions reach a steady state within 20 minutes. The molecule characteristic of this steady state still shows slight susceptibility to the action of DNase and binds considerably more methyl green than the products of complete enzymatic digestion (which bind no measurable dye: *cf.* solid circles and the triangles in Fig. 2). In Fig. 3, the curves from which the DNase affinity (dE/dt) were calculated are shown. The dotted projections onto the curve of DNase attack on untreated DNA illustrate that whereas reduction in methyl green binding capacity of DNA to about 50% by DNase is not associated with change in rate of further DNase depolymerization, similar reduction in dye binding capacity by heat results in significant reduction in the rate of enzymatic attack. This is equally well demonstrated when the rate of depolymerization by DNase is measured by change in viscosity as by change in dye binding capacity (Fig. 4). The reduction in DNase affinity is correlated with the reduction in dye binding capacity produced by heat (Fig. 5), but proceeds

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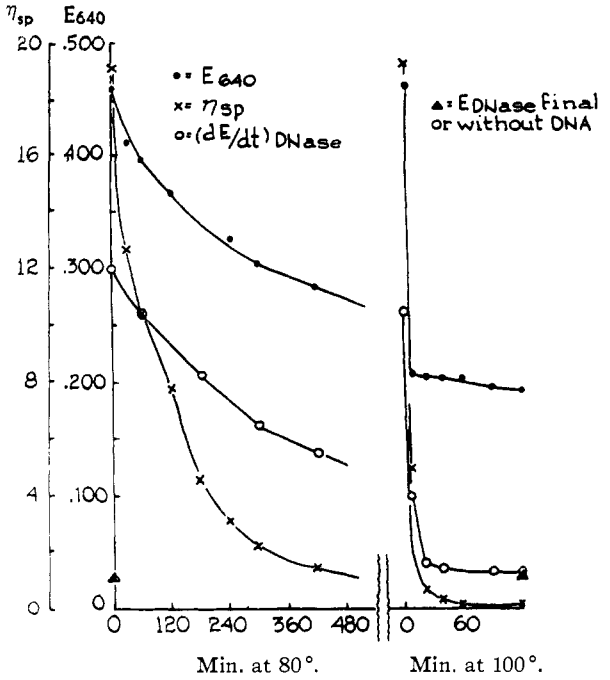

Fig. 2.—Effect of heat on viscosity (×), rate of depolymerization by DNase (O) and affinity for methyl green, E_{640} (●). The ordinates for rate of DNase depolymerization are the same as E_{640}, and are expressed as ΔE_{640} per 240 minutes (= DNase affinity).

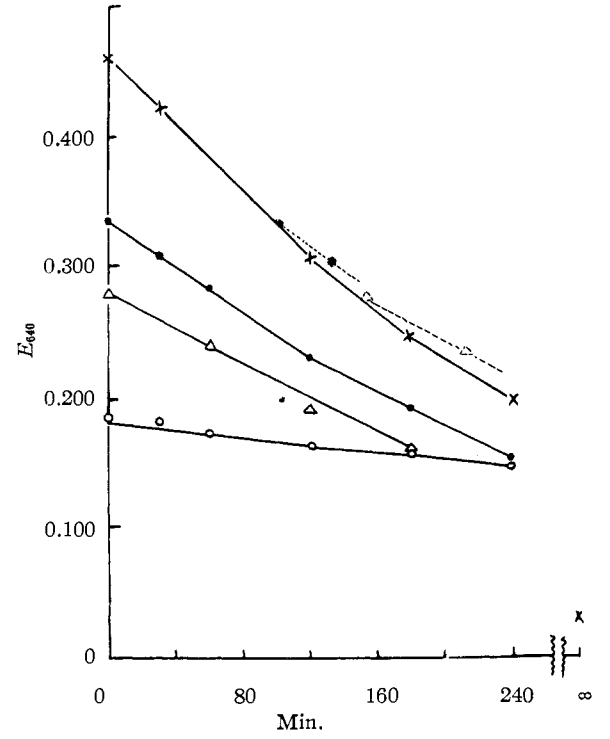

Fig. 3.—Effect of heat depolymerization on rate of attack by DNase (dE/dt), measured by methyl green method.[24] Substrates as in [24], pretreated as indicated below. Interrupted lines are projections of corresponding solid lines, for easier comparison: ×, untreated, η_{sp} = 19; ●, 180″–80°, η_{sp} = 4.5; △, 420″–80°, η_{sp} = 1.4; O, 120″–80°, η_{sp} = 0.09.

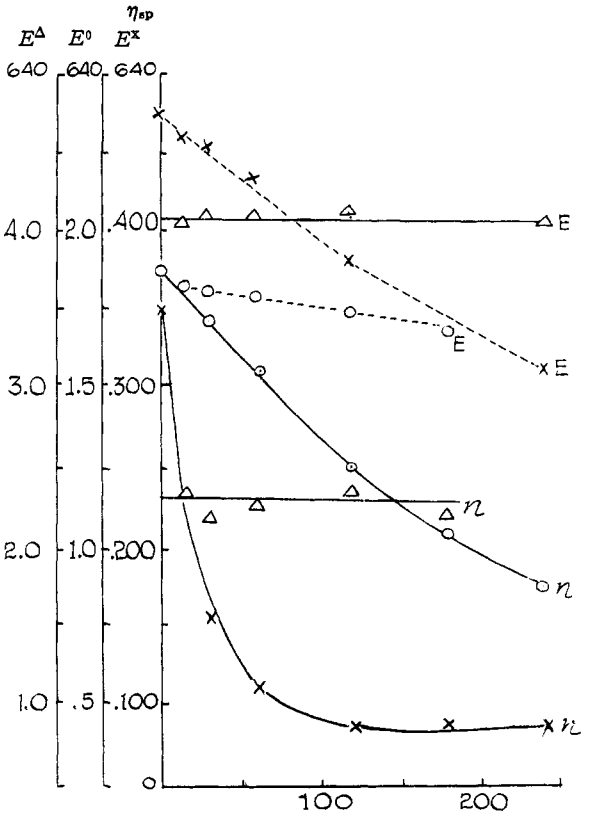

Fig. 4.—Effect of heat depolymerization on rate of attack by DNase, as measured by the reduction in methyl green affinity (interrupted lines) and viscosity (solid lines). Final DNase concentration is 0.03 μg./ml. ×, untreated DNA (substrate as in ref. 24); O, DNA treated 5 hours at 80° (DNA and methyl green 3 times concentration of ×, in order to make initial η_{sp} similar to above); △, DNA treated 40 minutes at 100° (DNA (0.1%) and methyl green 6.7 times concentration of ×).

Note that the ordinate scales for the optical densities at 640 mμ differ by factors of 5 and 10 for the 80 and 100° heated substrates, respectively. The scale for specific viscosity is the same for all (coincides with E_{640} scale for the untreated DNA). Viscosity was measured on the DNA–methyl green–enzyme–citrate mixture (*i.e.*, same solutions as were used for optical density determination). Quartz inserts were used to reduce the light paths to 2 and 5 mm. for the heated solutions (*i.e.*, E_{640} shown is calculated for 10 mm. light path).

much less rapidly than the reduction in specific viscosity (Fig. 6). Both properties are significantly affected, however, before the specific viscosity approaches zero.

Effects of Enzymatic Depolymerization.—In Fig. 7, the data on the effect of DNase depolymerization on methyl green affinity (E_{640}) and η_{sp} are presented for several enzyme concentrations (in geometrical progression by 2 starting at 0.02 μg./ml.). Because of the great dilution (0.01% DNA), $\bar{\beta}$ is high (only slightly less than for water); the values for η_{sp} obtained are, therefore, practically independent of $\bar{\beta}$. It is apparent that the rate of enzymatic depolymerization, as measured by

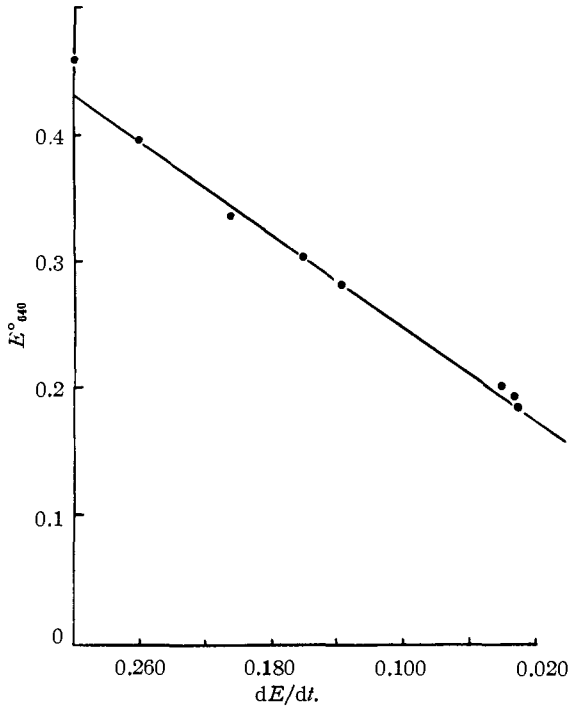

Fig. 5.—Relationship between effect of heat depolymerization on methyl green affinity (E°_{640}) and DNase affinity (as measured by rate of depolymerization, dE/dt), derived from Fig. 2.

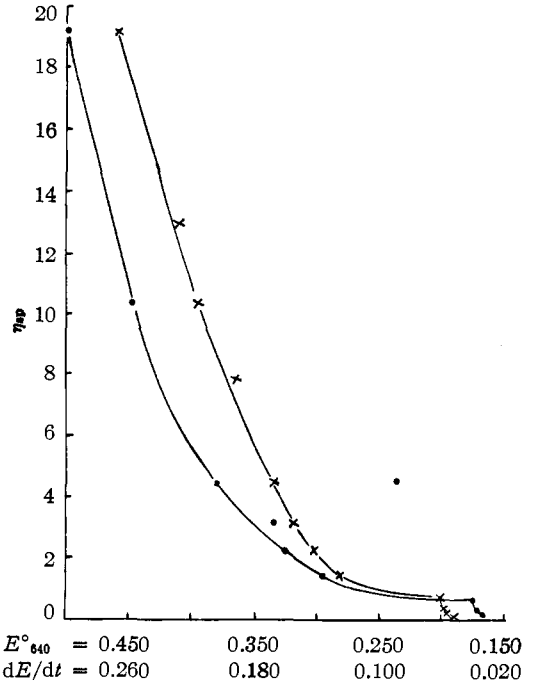

Fig. 6.—Effect of reduction ofvi scosity by heat on methyl green affinity (×) and rate of attack by DNase (●); derived from Fig. 2.

the methyl green method ($^{de}/_{dt}$), does not diminish until well after the viscosity has been reduced to its limiting value. Reduction in dye binding capacity is a function of reduction in viscosity regardless of the enzyme concentration (*i.e.*, rate)

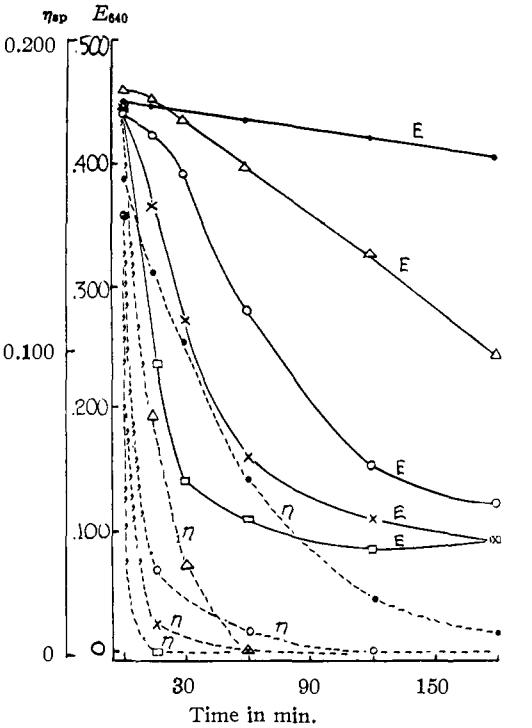

Fig. 7.—Effect of DNase depolymerization on η_{sp} (dotted lines) and E_{640} (affinity for methyl green) (solid lines). Substrate as in ref. 24; η_{sp} measured as in Fig. 4: ●, 0.02 μg. DNase/ml.; △, 0.04; ○, 0.08; ×, 0.16, □, 0.32.

with which depolymerization is performed (Fig. 8). However, the specific viscosity reaches zero long before the dye binding capacity does; and a 50% reduction in η_{sp} is associated with only 4% reduction in methyl green affinity.

Figure 9 presents a series of ultracentrifugal patterns after 32 minutes of centrifugation at 52,640 r.p.m. of methyl green–DNA exposed to DNase faction for 0, 120 and 240 minutes prior to centriugation. In Table IV η_{sp}, sedimentation constants and methyl green affinity are given for these solutions. The high DNA concentration used to permit recording of the ultracentrifugal patterns has inhibited enzymatic depolymerization[24,26] so that the methyl green binding capacity (E_{640}) is reduced at a low rate. Specific viscosity is greatly reduced, however, while the sedimentation constant changes only little (although the boundary broadens).

TABLE IV

EFFECT OF DNASE ON VISCOSITY, SEDIMENTATION RATE AND METHYL GREEN BINDING OF DNA

Final DNase concn., before addition of citrate = 1.75 μg./ml.; DNA concn., 0.118%; methyl green concn., 0.024%; magnesium concn., 0.0059 *M*.

Length of exposure to DNase, min.	η_{sp}	$S^{a} \times 10^{-13}$ 0.1% soln. with methyl green	E_{640} (dil. 1:20)
0	7.9	6.5	0.805
120	0.6	6.5	.720
240	0.05	5.9	.650

[a] Concentration of DNA reduced to 0.1% by addition of sodium citrate (final sodium citrate concentration 0.047 *M*).

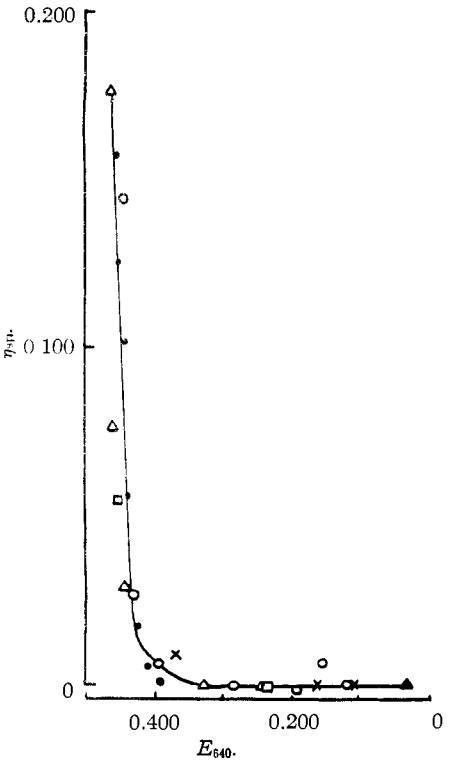

Fig. 8.—Relationship between effect of DNase on viscosity and on methyl green affinity. DNase concentrations and symbols as in Fig. 7, from which Fig. 8 is derived. O, DNase concn. × 1; △, × 2; O, × 4; ×, × 8; □, ×16.

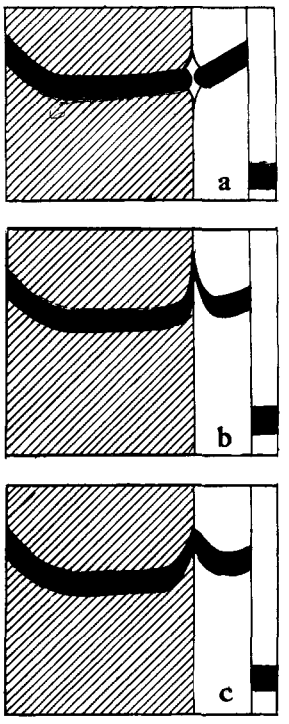

Fig. 9.—Ultracentrifugation patterns of methyl green–DNA complex following DNase treatment—see Table IV. Substrate as in ref. 24, but 10 times as much DNA and methyl green. Enzymatic action stopped with citrate as in ref. 24, no pretreatment of substrate: a, untreated, as in Fig. 1b; b, after 120 minutes exposure to 1.75 μg. DNase/ml.; c, after 240 minutes exposure to 1.75 μg. DNase/ml. The curvature of the base line is identical to that obtained with the buffer alone, and is presumed to be due to strain in the cell.

Discussion

Similarities between Effects of Heat and Enzymatic Depolymerization.—Heat and enzymatic depolymerization of DNA produce certain effects in common: both reduce the viscosity of the solution rapidly (*cf.* Figs. 2 and 7), increase the ultraviolet absorption (Table III and refs. 7, 26) and reduce methyl green affinity.[26] These changes are maximal before any dialyzable, acid-soluble oligonucleotides are formed (*cf.* 28–33) and nearly maximal before reduction in sedimentation rate appears (*cf.* Tables III and IV). It is possible that reduction in sedimentation rate is compensated to some extent by reduction in viscosity of the solution,[34] however, which may account for the increase in sedimentation rate observed after partial heat depolymerization (Table III). The products of heat and initial enzymatic digestion (and of sonic depolymerization)[31–35] are large molecules which, as judged by their sedimentation velocity, are probably still quite asymmetric rods. This hypothesis is in better accord with the unaltered sedimentation rate (despite marked reduction in viscosity) than the alternative suggestion that heat has produced collapsed molecules of the original molecular weight.[20,36] Such collapsed molecules would show a great reduction in viscosity, but would also show considerable change in sedimentability. The broadening of the sedimentation boundary (Figs. 1 and 9) which accompanies depolymerization may represent reduction in homogeneity. However, increased rate of diffusion of the smaller molecules, without increase in heterogeneity may, also account for the less sharp boundary.[37]

Differences between Effects of Heat and Enzymatic Depolymerization. (a) **End Products.**—Certain differences between the effects of heat and enzymatic depolymerization are apparent. Whereas even at low enzyme concentration, breakdown of the molecule proceeds in time to tetra- and dinucleotides,[30] the data (Fig. 2) suggest that the steady state which is reached at moderate temperatures represents much larger molecules. Thus, at 100°, a molecule with very low intrinsic viscosity, but of a considerable size as characterized by its non-dialyzability, sedimentability and affinity for methyl green (Fig. 2) is stable. This suggests that the increased bombardment by the solvent molecules at elevated temperature snaps the rigid molecule into still large units, but of such lesser size as to be stable when subjected to the bombardment characteristic of this temperature. On the other hand, the enzyme attacks specific sites in a preferential order, producing first large fragments, which are broken into smaller fragments only after the preferred size is less abundant or absent.

(b) **Effect on DNase Affinity: Effect of Changes in Structure of DNA on Rate of DNase Depolymerization.**—The rate of depolymerization of DNA by DNase as measured by the methyl green method

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falls off only very slowly with time (Fig. 7) and only late in the process (some time after viscosity has reached its limiting value and only as methyl green binding capacity approaches its limit). However, preliminary heat depolymerization greatly reduces the rate of enzymatic depolymerization (*i.e.*, "DNase affinity") *pari passu* with the reduction in methyl green binding capacity and long before viscosity has been reduced to its lowest value (Figs. 2, 5, 6). Since, unlike enzymatic attack (when the substrate is in excess), thermal energy attacks all the molecules simultaneously, the molecular population may be expected to be homogeneous throughout the heating procedure. The lesser susceptibility of the heat-depolymerized molecules to DNase attack tends to confirm the hypothesis, already proposed on the basis of delayed production of small fragments by the enzyme, that the enzyme attacks large molecules preferentially (*cf.* 25, and the analogous situation with proteolytic enzymes.[38] These results also suggest that, in addition to breaking the molecule, thermal energy alters the internal configuration of the fragments (coiling), thereby reducing the number of sites (or suitable spacings) for DNase attack and methyl green binding. On the other hand, the internal organization of the initial large fragments produced by DNase acting on highly polymerized DNA, may be unaffected. Thus, further enzymatic activity would not be significantly reduced and the methyl green binding sites would be affected only at the point of cleavage.

These observations indicate that both methyl green binding capacity and susceptibility to depolymerization by DNase are functions of the size and configuration of the DNA molecule, and are subject to modification without alteration in elementary composition of the DNA molecule. We do not imply, however, that the sites of binding for the dye and enzyme are the same. Indeed, the absence of competition, as indicated by the equal susceptibility to enzymatic depolymerization of DNA in solutions with and without methyl green (regardless of the concentration of the latter within wide limits), indicates that the sites of linkage are not identical.

Whereas our data suggest that the size and configuration of the DNA molecule (possibly maintained by H-bonds[20]) influence its susceptibility to DNase attack as much as does its elementary composition, Tamm and Chargaff[1,2] have reported reduction in affinity for DNase only after the splitting off of purines from the DNA molecule. They observed no change in DNase susceptibility following "denaturation" of the DNA by acid and alkali without change in its complement of bases. However, it may be noted that their method of determining DNase attack is based upon the production of dialyzable phosphorus by large amounts of enzyme (5 to 10% of the substrate concentration or about 100 mg./ml.; contrast our use of 0.03 μg./ml., representing 0.02% of the substrate concentration). This is a late phenomenon[7,28,31,32,39,40] occurring at a time when DNase affinity is already greatly reduced (note that the curves of affinity for methyl green in Fig. 7 diminish in slope late in the course of DNase attack, as after 120 minutes with 0.08 μg./ml. DNase and even sooner with higher concentrations). With 0.08 μg./ml. DNase (0.07% enzyme : substrate ratio) the viscosity and the methyl green affinity are reduced to their limiting values in 2 and 4 hours, respectively (Fig. 7). Using approximately 100 times as much enzyme, Tamm, Shapiro and Chargaff[2] made their first determination of acid-soluble P after 10 hours. By this time, the substrate has been so extensively altered by the enzymatic hydrolysis as to render the changes we have produced by heat comparatively negligible. Indeed, Laland, *et al.*,[32] have reported that when DNA "denatured" by ultrasonic irradiation was incubated with DNase, "no further decrease in viscosity occurred, while formation of acid-soluble material commenced immediately." On the other hand, with intact DNA, no acid-soluble material appears until after the relative viscosity has reached a constant value.[28] Therefore, only such extensive alterations, as to render the DNA molecule essentially insensitive to the second ("hydrolytic") phase of attack by large concentrations of DNase, will be expected to show reduction in DNase affinity by the method of Tamm, *et al.* We do not agree, therefore, that depolymerization with preservation of the full complement of purines does not affect DNase affinity. Rather, we find that reduction in affinity for DNase results, without change in elementary composition, if a sufficiently sensitive test is applied to the early phase of enzymatic depolymerization. Tamm, *et al.*,[2] ignored the slight reduction in the rate of production of dialyzable P (which is shown in their Fig. 1, curves B-2 and C-3) when the substrate had been "denatured" by brief treatment at *p*H 11.8 or 2.95, which "caused interference with the physical properties, but not with the chemical composition of the parent DNA."

(c) Effect on Methyl Green Binding Capacity.—Reduction in viscosity of DNA solutions by different methods is not associated with uniform reduction in methyl green affinity. Thus 50% reduction in viscosity by DNase reduced methyl green affinity 4% (Fig. 8); the same reduction in viscosity by heat reduced methyl green affinity 16% (Fig. 6); Errera[41] noted a 30% reduction in methyl green affinity with 26% loss in viscosity produced by X-radiation. These results suggest, as Errera[41] and we[4] have pointed out, that changes in the intramolecular configuration may alter methyl green affinity independently of changes in molecular weight. Various depolymerizing agents may vary in the degree of intra-molecular change produced for a given reduction in molecular weight (viscosity). Thus, it appears that X-ray produces a more profound change in molecular configuration than does heat, while DNase produces the least alteration in molecular configuration. It may be expected that X-ray depolymerized DNA will be even less susceptible to DNase than heat depoly-

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merized DNA, since internal configuration seems to influence DNase affinity as well as methyl green affinity. It is suggested that DNase acts only upon the point of cleavage of the DNA molecule, causing loss of affinity for methyl green at this point only. The configuration of the rest of the molecule, unlike the effect of heat and X-ray, is thought to be unaffected until depolymerization has progressed to small fragments.

Effect of Methyl Green Binding on Viscosity of DNA Solution.—Methyl green was found to slightly increase viscosity (Table I) without significantly altering sedimentation velocity. This suggests that the methyl green radical, which attaches to the DNA molecule at two sites,[4,9,13] may produce some linking between adjacent DNA molecules. This type of change in structure of DNA, however, does not appear to alter susceptibility to enzymatic depolymerization.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF ARKANSAS]

On the Soluble Nucleotides of Liver and Muscle[1,2]

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Ion-exchange chromatography of the barium-insoluble fraction of the acid-soluble nucleotides of liver has shown the presence of a mononucleotide which appears to be a pyrimidine derivative different from cytidylic or uridylic acid. Its phosphate group is liberated by enzymes which are specific for monophosphates and 5'-derivatives. This nucleotide has been found in both rat and rabbit liver, but is absent from rabbit muscle. Evidence has been obtained by ion exchange chromatography for the presence in liver and muscle of di- and polyphosphate nucleotide derivatives different from ADP and ATP.

The work to be reported here is a study that was made as preliminary to an investigation of the time-course of the distribution of tracer phosphate between the individual phosphate groups of ADP and ATP in liver and muscle. The procedure used initially for the separation and isolation of these two compounds was that described by Cohn and Carter[3] for the separation of the components of commercial preparations of ATP. This consists of the adsorption of the nucleotide derivatives from ammoniacal solution onto a column of Dowex-1 anion exchange resin, the height of which is equal to the diameter, followed by elution successively with 0.003 *N* HCl, 0.01 *N* HCl plus 0.02 *M* NaCl, and 0.01 *N* HCl plus 0.2 *M* NaCl. These solutions elute AMP, ADP and ATP, respectively.

Columns of these dimensions failed to effect the desired separation of the soluble nucleotide fractions of trichloroacetic acid extracts of tissues. Increasing the ratio of height to diameter of the column of resin did lead to the desired separations, and also revealed the presence of other nucleotides and their polyphosphate derivatives. Most of the experiments have been carried out with columns of 250–400 mesh Dowex-1 resin, 18 cm. high by 1.1 cm. in diameter. Three additional fractions have been obtained from both liver and muscle extracts; these appear to be mixtures of nucleotide di- and polyphosphates. The composition of these fractions is still under investigation.

Liver extracts contain, in addition to these fractions, a substance which shows the properties of a pyrimidine nucleotide, but does not correspond in elution characteristics or absorption spectrum to either cytidylic or uridylic acid. These four nucleotide fractions from liver account for about 20% of the total specific absorption at 260 mμ shown by the crude extracts; the three fractions from muscle account for about 10% of such absorption.

These studies have been carried out on rabbit muscle and liver, and on rat liver. Qualitatively similar findings were obtained from the livers of both species.

Experimental

The rats were anesthetized with pentobarbital, the livers excised and dropped into a mixture of Dry Ice and ether. The rabbits were anesthetized with pentobarbital supplemented with ether. The thigh muscles were dissected as free as possible without cutting any major blood vessels, excised rapidly and immediately dropped into the freezing mixture. The liver was then excised and frozen in the same way. All subsequent operations except the determinations of optical density of the solutions were carried out in a cold room maintained at 1–2°. The tissues were broken up into small pieces in a tissue crusher chilled with Dry Ice, and the pieces then placed in a Waring blendor with 5 volumes of 10% trichloroacetic acid. The blendor was run until a homogeneous suspension was obtained; this required 2 to 3 minutes with liver and 5 minutes with muscle. The suspension was centrifuged immediately, and the residue washed once with 3 volumes, and once with 2 volumes of 5% trichloroacetic acid. Glycogen was precipitated from the combined supernatants by the addition of somewhat more than an equal volume of 95% ethanol. This precipitate was removed by centrifugation half an hour after the addition of the ethanol. The fraction of barium-insoluble compounds was separated from the clear supernatant by the use of barium hydroxide and calcium acetate, as described previously.[4] It was found that the fraction of alkaline-earth soluble compounds, obtained by the addition of 4 volumes of ethanol to the combined supernatants from the precipitations with barium and calcium, contained appreciable quantities of material showing absorption at 260 mμ. This material was dissolved in a small volume of 0.2 *N* HCl, the solution made just alkaline to phenolphthalein by cautious addition of powdered barium hydroxide, calcium acetate solution added, and the mixture allowed to remain in

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(2) These experiments were initiated while J. S. and L. L. were at Brookhaven National Laboratory, Upton, N. Y.

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