THE MOLECULAR WEIGHT AND AGGREGATION OF DNA

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ABSTRACT The effects of enzymatic attack and of shear during the isolation and deproteinization of DNA have been investigated. Different methods of disaggregating DNA have been studied, and conditions under which reaggregation can occur are discussed. It was found that shaking with chloroform-octanol does not degrade DNA from the seven sources studied; that light scattering yields valid weight-average molecular weights for these samples; and that, when disaggregated, the molecular weights of these samples are in the range 1.2-2.4 million and the length-to-mass ratios are high.

Determination of molecular weight is a problem of central importance in the molecular biology of DNA. Although there have been numerous investigations on this subject, no convincing conclusions have been reached. There are two principal reasons for this. The first, and perhaps most important, is protein contamination (1), which leads to aggregation of DNA molecules and consequently to speciously high molecular weights. It must be emphasized that the weight per cent of protein may be very low and yet provide sufficient molecules to produce significant aggregation. The second reason is the possibility of degradation, which might be caused by either enzyme action or mechanical shear (2-4) and, of course, would result in low molecular weights.

In the present paper we show that the extent of aggregation depends on the source of the DNA as well as on the method of isolation, but that methods can be found which permit the preparation of DNA which is neither degraded nor aggregated. Such DNA samples, isolated from diverse sources, possess low molecular weights ($1.2\text{-}2.4 \times 10^6$) and relatively large radii of gyration. These samples can be caused to reaggregate by a number of treatments common in laboratory practice. We also present theoretical and empirical considerations which show that the molecular weights obtained for these samples by light scattering are weight averages.

EXPERIMENTAL METHODS

All molecular weights reported in this paper were determined by light scattering. Light-scattering measurements were carried out according to procedures described in detail

elsewhere (5), with the modification that centrifugation to remove dust before light scattering was carried out at 35,000 *g* for one-half-hour. The dust-free DNA solution (0.05 to 0.15 mg/ml) was withdrawn from the centrifuge tube automatically, with a clean pipette, at the rate of 2 ml/minute. The solvent was cleaned by filtration through millipore. Essentially dustless solutions were obtained by this procedure. The scattering was observed at angles from 25° to 135°. Measured molecular weights were reproducible to within 0.1 × 10^6. The Zimm plot for disaggregated *pneumococcus DNA* (sample 22, Table I) is shown in Fig. 1; this is typical of the light-scattering data. There is no concentration dependence, in the range studied, for any of the samples in Table I.

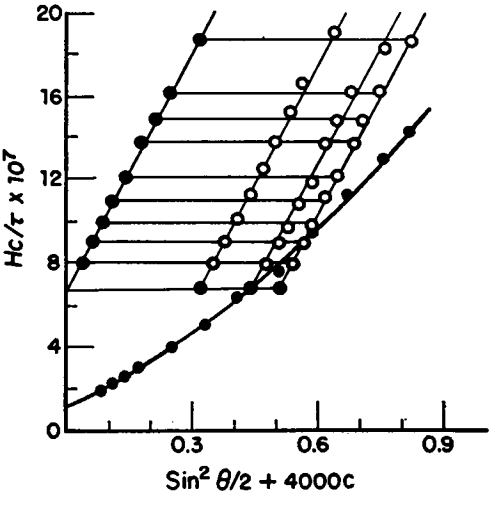

FIGURE 1 Zimm plot of the light-scattering data for pneumococcus DNA (sample 22), in 0.2 M sodium acetate. The concentrations were 0.080, 0.110, 0.127 mg/ml. The lower curve is the zero concentration line from the Zimm plot for aggregated *E. coli* B DNA (sample 14), in 0.2 M sodium chloride. All the DNA samples studied have a second virial coefficient of zero.

Sedimentation experiments were performed in the Spinco model E ultracentrifuge or in the model L preparative ultracentrifuge. Helix-coil transitions (melting points) were carried out in a Beckman DU spectrophotometer with an apparatus designed (6) and constructed in this laboratory.

Isolation Procedures. DNA was isolated from various sources by one of three methods: (*a*) a modification (5) of the Kay, Simmons, and Dounce (7) duponol procedure; (*b*) the saturated sodium bromide method of Emanuel and Chaikoff (8); (*c*) a modification of the sodium acetate method of Meyers and Spizizen (9).

Pneumococcus DNA was prepared by the third method from 12-liter overnight cultures of *D. pneumoniae* R-6. The bacteria were centrifuged and resuspended in 5 ml of 0.1 M EDTA-0.1 M sodium chloride, pH 7.5. The mixture was warmed to 60° to help denature intracellular enzymes before lysis; then 50 ml more of solvent, which also contained 10 ml of 5 per cent deoxycholate, was added. Fifty ml of saturated sodium acetate was added to the resulting gel and the mixture was gently stirred with a mag-

netic bar at 60° for 15 minutes. From this point the directions of Meyers and Spizizen were followed. Pneumococcus DNA was also prepared by the sodium bromide method.

DNA was prepared from *E. coli* B as described elsewhere (10). *E. coli* 15_{T^-} were lysed by suspending in 0.02 M Tris, 0.02 M EDTA, pH 9.5, and adding lysozyme and duponol. Brief incubation at 37° produced a thick, clear gel, which was then cooled to 4° and treated according to the duponol procedure.

Sea urchin sperm (11) DNA was isolated by the duponol procedure, after dissolving 10 gm. of the sperm in 50 ml of 1 M sodium chloride at 4°. We wish to thank Dr. K. A. Stacey for his gift of the salmon sperm DNA, which had been isolated by repeated extraction of the sperm with 1 M sodium chloride.

DNA samples from calf thymus and from mouse sarcoma-180 were prepared from nuclei as well as from whole tissue (see Table I). Nuclei were isolated in one of two ways: the sucrose method of Allfrey *et al.* (12) or the citric acid method of Dounce, (13) modified in that a pH of 2.5 rather than 4.0 was used. No denaturation of the DNA was thereby incurred. DNA was isolated by the duponol method, in all cases using solvent volumes prescribed for the original weight of whole tissue.

Removal of RNA. RNA was removed from crude bacterial DNA by treating it with pentex 5 times recrystallized ribonuclease (RNase). The DNA was adjusted to a concentration of about 1 mg/ml in 0.1 M phosphate buffer, pH 7.0, containing approximately 0.01 mg/ml RNase. The solution was incubated several hours at 37°, while dialyzing against phosphate buffer. It was then dialyzed against 2 M salt, and finally against 0.2 M salt, at 4°. The DNA concentration of all samples was adjusted to 0.35 mg/ml and alcohol was added in the proportion 55:45. (At higher DNA concentrations, some very low molecular-weight material occasionally coprecipitated.) The fiber thus obtained was redissolved in 0.002 M NaCl and further deproteinized without prolonged delay.

Deproteinization. The isolation procedures discussed above are, of course, deproteinizations; however, all samples at this stage were still contaminated with protein, even though in many cases they had the appearance and the N/P ratio of pure DNA. Aggregation was indicated by the relatively high molecular weights and low radii of gyration. The presence of protein was apparent from the results of proteolytic and surface denaturation treatments. Chymotrypsin (one part Worthington crystalline enzyme to 50 parts DNA, by weight, in 0.1 bicarbonate buffer, pH 7.4, at 37° for 4 hours) was effective in reducing the aggregation of *E. coli* B DNA (10), whereas trypsin and carboxypeptidase were not. Shaking with chloroform-normal octanol (9:1 by volume: Sevag procedure) had the same effect as chymotrypsin. Since the enzyme treatment proved to be touchy, with the enzyme itself often mediating aggregation, the chloroform-octanol (hereafter denoted by C-0) treatment was adopted routinely. The DNA solution was shaken at room temperature with an equal volume of fresh C-0 for 20 minutes, and the procedure repeated until no more denatured protein appeared as a thin film at the interface. Subsequent C-0 treatments (see below), when necessary, were generally repeated three times, even though little or no material was visible at the interface. Shaking was generally accomplished with a Burrell wrist-action shaker at 250 linear (10 cm) strokes per minute, using a stoppered glass centrifuge bottle. The important factor seems to be the complete emulsification of the two layers, rather than the exact rate of shaking. The maximum DNA concentration that could be efficiently disaggregated in this manner was 0.3 mg/ml. Volumes as high as 100 ml have been successfully treated in this manner. The various aqueous solvents used are indicated in Table I and discussed below.

After deproteinization, the sedimentation constant distributions became narrower and

shifted as a whole to lower values. This showed that the bulk of the DNA rather than just a small fraction had been disaggregated.

Quality of the DNA. Four criteria were used before a sample of DNA was considered to be of good quality. (1) Hyperchromic effect: 32 to 35 per cent with alkali or 40 per cent with acid, at 260 mμ. If the DNA has not been completely deproteinized, the hyperchromic effect with acid may appear high because of a slight turbidity due to the precipitation of nucleoprotein. This was routinely checked at 320 mμ before assuming hyperchromicity to be a valid indication of the undenatured state. (2) Helix-coil transition: the melting point curves showed one sharp transition in a range of about 10°; the hyperchromicity was 35 to 40 per cent. (3) Sedimentation: only one boundary was observed in the ultracentrifuge. The width of the sedimentation-constant distribution was reasonably constant and narrow for all DNA samples: about 85 per cent of the material fell within a range of 10 svedberg units. No slowly sedimenting material was present, except in the case of the S-180 DNA, which exhibited a small shoulder at the slow end of the boundary. (4) Length-to-mass ratio: light scattering showed this to increase during disaggregation, finally attaining a high and constant value for any given sample.

Heat Denaturation. Heat denaturation of DNA was carried out at a concentration of 0.1 mg/ml or less, in 0.2 M NaOAc or Na_2HPO_4 between pH 8-8.5, by heating for 10 minutes in boiling water. No hydrolysis occurs in this pH range, but if the pH is lower extensive degradation can result. If such denatured DNA is allowed to stand for several hours, aggregation begins and proceeds indefinitely. It was found that the original molecular weight could often be restored by a single chloroform-octanol treatment, using an appropriate aqueous solvent. Sometimes it was necessary to raise the pH to 12 in order to effect complete disaggregation. If divalent metal ions, which cause aggregation, were present, the solvent was made 0.02 M in EDTA or the solution was passed through a sephadex (14) column to remove the ions before C-0 treatment.

HINTS IN HANDLING DNA

In this section we will enumerate some points, to be discussed later, which can lead to difficulties in the interpretation of molecular weights. (1) Incomplete deproteinization. Traces of protein are often responsible for residual aggregation in DNA samples. Such aggregation, apparently lateral, decreases the length-to-mass ratio calculated from light-scattering data. This ratio can thus be used as an index during deproteinization. It is probable that no single method of treatment will remove all the protein. (2) Cymotrypsin and other enzymes. Removal of residual proteins from DNA with proteolytic enzymes is difficult because the enzymes themselves interact with the DNA, causing aggregation. (3) Formation of pellets. The formation of DNA pellets from solutions in the preparative ultracentrifuge produces aggregation of the DNA. For example, a sample of pneumococcus DNA had a molecular weight of 1.7 million before ultracentrifugation. After forming a pellet and redissolving it overnight in 0.01 M Tris, pH 9.5, the molecular weight was found to be fifty million. (This DNA could be returned to its original molecular weight by shaking with C-O; see Results.) Thus, isolation procedures should not include pelleting unless aggregation is not relevant to the ensuing measurements. (4) Denatured DNA. Addition of divalent metal ions such as Mg^{++} causes immediate and

extensive aggregation of denatured DNA, as shown by light scattering. The presence of such ions as contaminants in other reagents should be kept in mind. Dialysis of denatured DNA in Visking casing also leads to extensive aggregation, presumably because of traces of ions such as Cu^{++} and Zn^{++}, which diffuse out of the substance of the casing. Even when the concentration of divalent ions is extremely low, denatured DNA aggregates slowly. This process can be reversed by shaking with C-O, as described in the experimental section. (5) Fractionation. Shaking with C-O often results in the appearance of both DNA and protein at the interface, especially when the treatment is carried out at high ionic strengths. The higher molecular-weight DNA is preferentially precipitated in this way, leaving relatively low molecular-weight DNA in the supernatant, as will be shown in the following section. Thus, what is actually a fractionation process can give the appearance of degradation. This misapprehension is doubtless responsible for the widely felt distrust of deproteinization by shaking with C-O. It will be shown below that conditions can be adjusted, *e.g.* by lowering the salt concentration and raising the pH, so that DNA is not lost from the solution to the interface. (6) Dialysis. We have found that Visking cellophane casing, at least in the presence of EDTA, releases an apparently non-dialysable material whose turbidity interferes with light-scattering experiments by indicating a high molecular-weight component. This material is completely extracted by C-O. (7) Precipitation of DNA. Alcohol precipitation of DNA may cause aggregation, necessitating C-O treatment to regain the original molecular weight. Furthermore, storage of DNA in the form of fibers sometimes results in property changes. Such samples are sometimes very difficult to dissolve, even with prolonged stirring at low ionic strength (0.002 M sodium chloride), and those that do dissolve are found to be aggregated. To avoid this possibility, we store our samples as concentrated solutions at 4°. (8) Aging. Deproteinized DNA solutions which have remained at 4° for several months frequently show signs of aggregation. On the other hand, solutions grossly contaminated with protein are sometimes slowly degraded. The aggregation, probably a very slow interaction between DNA and minute amounts of protein and divalent metal ions, is extremely difficult and sometimes impossible to reverse. Kirby (15) has demonstrated that such a slowly occurring reaction is possible. We have therefore deproteinized our DNA samples as soon as possible after isolation and utilized them without delay.

RESULTS AND DISCUSSION

A. Disaggregation. Table I contains molecular weights and radii of gyration of the various DNA samples studied. All samples except 20 to 23 and 25 and 26 were isolated using the duponol method. The column headed "Treatment" shows the subsequent deproteinization method. Since shaking with C-O was found to yield a low molecular weight, presumably because of efficient deproteinization, it was of prime importance to establish the validity of this method. That is, it had to

TABLE I

MOLECULAR WEIGHTS OF DNA FROM VARIOUS SOURCES

Sample*	Sample code	Source	Treatment‡	$M_W \times 10^6$ §	$(\overline{\rho^2})^{\frac{1}{2}}$ ‖
1	TS-388	C.T. (whole tissue)¶	None	3.3	1500 Å
2	TS-487	C.T. (whole tissue)	C-O, 0.2 M NaCl, pH 6	1.9	1280
3	JS-288	C.T. (nuclei-sucrose)	None	2.8	1130
4	JD-SN-221	C.T. (nuclei-sucrose)	None	3.4	1080
5	JD-SN-236	C.T. (nuclei-sucrose)	C-O, 0.2 M NaCl, pH 9.4	1.8	1490
6	JD-CN-117	C.T. (nuclei-citric)	None	4.8	1160
7	JD-CN-141	C.T. (nuclei-citric)	C-O, 0.2 M NaCl, 0.1 M EDTA, pH 8.5	1.7	1320
8	JD-SSN-52	S-180 (nuclei-sucrose)¶	None	14.0	1140
9	JD-SSN-55	S-180 (nuclei-sucrose)	C-O, 0.2 M NaCl. pH 6	1.4	1300
10	LC-SI-422	S-180 (nuclei-sucrose)	C-O, 0.2 M NaOAc, pH 7.8 or 9.5	1.3	1160
11	LC-SI-430	S-180 (nuclei-sucrose)	C-O, 0.2 M NaOAc, pH 12	1.2	935
12	JD-SCN-36(38)	S-180 (nuclei-citric)	None	2.6	1540
13	JD-SCN-50	S-180 (nuclei-citric)	C-O, 0.2 M NaCl, pH 6	1.6	1080
14	I	*E. coli* B	None	11.0	1600
15	I	*E. coli* B	Chymotrypsin	2.6	1300
16	Ib-49	*E. coli* B	C-O, 0.2 M NaCl, pH 6	2.4	1620
17	WO-241	*E. coli* 15_{T-}	C-O, 2 M NaCl; then 0.2 M NaCl, 0.1 M EDTA, pH 8.5	1.2	1120

TABLE I *concluded*

18	JD–II	Pneumococcus	None	5.0	1060
19	JD–II–380	Pneumococcus	C–O, 0.2 M NaCl, pH 6	2.3	1250
20	LC–I–458	Pneumococcus (NaOAc)	None	5.0	900
21	LC–I–450	Pneumococcus (NaOAc)	C–O, 0.2 M NaOAc, pH 7.8	1.5	1300
22	LC–II–540	Pneumococcus (NaOAc)	C–O, 0.2 M NaOAc, pH 7.8	1.6	1100
23	Lem	Pneumococcus (NaBr)	None	2.1	1140
24	E2E3I–380	Pneumococcus	C–O, 0.1 M NaCl, pH 9.3	1.7	1000
25	KS–370	Salmon sperm**	None	8.0	1400
26	KS–455	Salmon sperm**	C–O, 0.2 M NaCl, pH 12	2.0	1480
27	TS–492	Sea urchin sperm	None	12.5	1900
28	TS–497	Sea urchin sperm	C–O, p.2 M NaCl, pH 6	2.2	1440
29	TS–499	Sea urchin sperm	C–O, 0.2 M NaCl, pH 9.4	2.2	1490

* Samples 1 to 19, 24, and 27 to 29 were isolated by the duponol method, which is a modification (5) of the Kay, Simmons, and Dounce method (7). Sample 23 was isolated by the Emanuel and Chaikoff NaBr method (8). Samples 20 to 22 were isolated by a modification of the Meyers and Spizizen NaOAc method (9).

‡ The treatment indicated was applied to the DNA after the initial isolation procedure. Shaking with chloroform-octanol (C–O) is described in the Experimental section; the aqueous solvent used for this is indicated in the table. The pH of samples 5, 10, 11, 24, 26, and 29 was adjusted with sodium hydroxide. The trisodium salt of EDTA was used.

§ Weight-average molecular weight from light scattering.

‖ Z-average radius of gyration, from light scattering.

¶ C.T., calf thymus; S–180, mouse sarcoma–180.

** This sample of DNA was prepared by Dr. K. A. Stacey, by extraction of the sperm with 1 *M* NaCl.

be proven that the observed decreases in molecular weight were indeed due to disaggregation rather than to degradation of the DNA molecules by shear.

Several experiments were carried out to establish this point. *E. coli* B DNA (sample 14, Table I), which had a molecular weight of 11 million after duponol isolation, was shaken with C-O; the weight decreased to 2.4 million. When a different aliquot of the 11 million sample was treated with chymotrypsin, directly in the light-scattering cell, the molecular weight decreased to nearly the same value, 2.6 million. Since the latter treatment involved no shaking or shear at all, not even pouring or pipetting, we conclude that shaking with C-O produces deproteinization but no shear degradation. That deproteinization did in fact occur was obvious from the film of denatured protein found at the interface, while all the DNA remained in the aqueous phase.

The C-O treatment was used on DNA samples from a number of different sources. In each case the molecular weight dropped to a characteristic value, which could not be lowered by further shaking with C-O. Nearly the same characteristic value was attained by different aliquots of the same sample and by different preparations from the same source (even when isolated by a variety of methods). The results shown in Table I are representative of a much larger number of similar observations: for example, twelve separately isolated samples of *E. coli* 15_{T-}, treated with C-O (see sample 17, Table I), all had a molecular weight of 1.2 ± 0.1 million. The fact that the characteristic molecular weight values for DNA from different sources are not the same, although all were achieved by shaking with C-O, demonstrates again that these low molecular weights are not shear-produced artifacts.

The C-O treatment is also capable of returning artifically aggregated DNA samples to their original molecular weights. For example, a solution of pneumococcus DNA of molecular weight 1.7 million was centrifuged in the preparative ultracentrifuge until a DNA pellet formed. When redissolved, its molecular weight was 50 million. Shaking with C-O at pH 9.5 lowered the molecular weight to 1.7 million again. The process was repeated five times, with the same results each time. There is no doubt that the effect of C-O shaking, here, is simply disaggregation. The same is true in the case of denatured DNA, the aggregation of which is reversed by shaking with C-O.

The argument may be raised that degradation had already occurred during the original isolation of DNA, since the duponol method, for example, involves foam and mechanical shear. DNA was therefore isolated from pneumococcus by a modification of the sodium acetate method of Myers and Spizizen (9) which involves no foam, shaking, or stirring, and compared to a sample isolated by the duponol method (Table I, samples 20 and 18, respectively). The molecular weights were identical (5.0 million) before any further deproteinization. After shaking with C-O they both decreased in molecular weight to similar values (1.5 and 2.3 million, samples 21 and 19 respectively). That the reduced molecular weights are not iden-

tical probably means that traces of protein are still present in sample 19; several other duponol-C-O-treated samples (*e.g.* sample 24, 1.7 million) had essentially the same molecular weight as the sodium acetate-C-O-treated sample. In any case the molecular weight of the duponol-isolated DNA was at no time lower than that of the sodium acetate-isolated DNA, and we therefore deduce that the foam and stirring in the duponol procedure do not degrade this material. These results were corroborated by isolating pneumococcus DNA by the saturated sodium bromide method of Emanuel and Chaikoff (8), which involves no foam and only very slight mechanical shear (slow filtration through celite). The molecular weight of this DNA (sample 23), 2.1 million, was *lower* than that of the duponol-isolated sample (5 million), and in fact was about the same as that of the samples prepared by duponol plus C-O treatment (2.3 to 1.7 million). Thus, since treatment of pneumococcus DNA by the duponol and C-O procedures yields the same result as treatment by the sodium acetate and C-O procedures or by the sodium bromide procedure alone, shear can be eliminated as a factor in producing the low molecular weights observed.

The C-O procedure was next subjected to a direct test. Incompletely deproteinized calf thymus DNA (Table I, sample 4) in 2 M NaCl was shaken with chloroform-octanol. Under these conditions a considerable amount of DNA is removed from solution and appears in the interface phase. The molecular weight of the 40 per cent of the DNA which remained in the supernatant solution was directly determined by light scattering. The DNA at the interface was extracted with 0.002 M sodium chloride and made 0.2 M for light scattering; this constituted 59 per cent of the starting material. Ninety-nine per cent of the DNA was recovered. The weight-average of the molecular weights determined for the supernatant (2.0 million) and interface (4.2 million) DNA fractions was 3.3 million ($[4.2 \times 10^6] \times 0.59 + [2.0 \times 10^6] \times 0.40$)—essentially the same as the original molecular weight of 3.4 million. We can conclude that no degradation occurred as a result of the shaking procedure, for the calculated weight would otherwise have been lower than the original. However, on shaking the same calf thymus DNA in 0.2 M sodium chloride, pH 9.4, with C-O, there is no loss of DNA at the interface (*i.e.*, 100 per cent of the DNA remains in the supernatant solution), and the molecular weight decreases to 1.8 million (sample 5). This effect must be considered a dissociation, involving bonds (probably electrostatic, because of the effect of pH) weaker than covalent ones, since shaking produced no cleavage of DNA molecules in the first case.

The conditions under which C-O disaggregation is most effective vary with the source of the DNA, depending no doubt on the nature and amount of the contaminating protein. The procedures listed in Table I gave reproducible results and the disaggregated samples possess the minimum molecular weights so far obtainable. The conditions employed were adopted only after others had been eliminated; in this connection, we wish to describe some of the general experiences which led to

successful disaggregation procedures. It is clear that there are no general requirements for disaggregation. DNA from some sources can be disaggregated merely by shaking (with C-O) in 0.1 M salt, whose pH is about 6. For other samples 2 M salt is more effective, although still other samples are fractionated thereby. We ordinarily use 0.2 M sodium chloride or sodium acetate. The addition of 0.01 to 0.1 M EDTA is often helpful, and a pH between 9 and 10 is essential in some cases.

After any manipulation of a DNA sample, such as precipitation and dissolving, dialysis, or even merely storage for several weeks, we have found it advisable to shake the solution again with C-O. Samples which can be disaggregated only at high pH often reaggregate if the pH is lowered. Any experiments which depend upon molecular weight must be performed immediately and with a minimum of handling of the disaggregated DNA, if reaggregation is to be avoided.

The behavior of denatured DNA is more extreme than that of undenatured DNA. It is much more sensitive to the presence of divalent metal ions; for example, a denatured DNA sample of molecular weight 2 million aggregated immediately to about 10 million upon addition of magnesium chloride (final concentration, 0.001 M). Heating briefly to 60° in the presence of EDTA or removal of ions on a sephadex column is frequently required before disaggregation of denatured DNA can be effected. It is sometimes necessary to raise the pH to 12 during C-O treatment. Even on standing, denatured DNA aggregated much faster and to a much greater extent than undenatured DNA. This difference is probably due to the availability of the bases of denatured DNA for intermolecular bonding.

Our experience, and that of others (1, 15), together with the relatively high and inconstant molecular weights reported in the literature, indicate that DNA prepared by most, if not all, procedures contains small amounts of protein and divalent metal ions, one or both of which link the DNA molecules together to form complex aggregates. The behavior of the system during the disaggregation treatments may be interpreted in the following way. Dissociation (16) of the aggregates is brought about by the low shearing forces which must be generated in the formation of surfaces between chloroform-octanol and the aqueous phase with which it is shaken. Since the shearing force is not great, the extent of dissociation is determined by the pH (16) and the nature of the contaminating proteins. For example, the higher the pH the fewer the charged protein amino groups available for binding to DNA, and therefore the weaker the complex. The protein may be denatured at the C-O surface, and if it is then insoluble in the aqueous phase it precipitates and is found at the interface. EDTA chelates any divalent ions that may be present, making them unavailable for complex formation with DNA and (probably nonbasic) protein. Heating to 60° may accelerate either chelation by EDTA or dissociation of the aggregates.

An appropriate combination of these conditions appears to be capable of disaggregating any sample of DNA, but nonetheless traces of soluble protein and

divalent ions must remain in the solution, since reaggregation can occur. Possibly the protein is not permanently denatured; at any rate, the protein-metal ion links between DNA molecules apparently re-form very slowly on standing. The reaggregation is slow probably because of specific configurational requirements and the repulsion between the negatively charged DNA molecules. It is accelerated by anything that increases contact between molecules; *e.g.,* precipitation with alcohol or formation of a pellet in the ultracentrifuge. (Note also that C-O disaggregation is unsuccessful at DNA concentrations above 0.3 mg/ml.) Likewise, increasing the concentration of protein or metal ions, *e.g.* by adding proteolytic enzyme, by changing the pH so that more charged groups appear on the protein already present, or by dialyzing in casing contaminated with divalent metal ions, can also produce rapid aggregation. C-O treatment, under appropriate conditions, can often (temporarily) dissociate the aggregates again, even though no protein is removed from the solution by such treatment.

In support of this interpretation, Kirby (15) has found that, in the phenol extraction procedure, rat liver DNA can be released from protein only in the presence of salts which interact with the protein and chelate divalent metal ions. Some salts are more effective than others; those that are successful for rat liver DNA are less so for rat tumor or wheat germ DNA, indicating the source specificity and difference in properties of the proteins involved. EDTA, which does not interact with protein, is ineffective at pH 6.5 but successful at pH 10 in extracting DNA from rat liver. Thus it appears that the protein is less strongly bound at the higher pH. DNA prepared by most of the extraction procedures tested contained measurable quantities of protein with a relatively high proportion of acidic amino acids. Calf thymus DNA prepared by the detergent method contained about 1 per cent of such protein. The evidence suggests that aggregation involves divalent metal ions linking protein carboxyl groups and DNA phosphates. Accordingly, Kirby found that DNA is adsorbed by collagen when divalent (but not monovalent) ions are present. This reaction takes place *very slowly,* less than 50 per cent of the DNA being adsorbed in 14 days.

Schumaker and Marano (17) have also presented evidence that a reversible change, possibly an aggregation-disaggregation reaction, occurs in very dilute solutions of DNA above pH 9. We have confirmed their observation of the stepwise sedimentation behavior of DNA under those conditions, using DNA from calf thymus, *E. coli,* and pneumococcus, after both partial and complete disaggregation. This is in accord with the observed effects of pH and concentration on disaggregation by C-O treatment.

B. Light Scattering. Light-scattering theory states that extrapolation of the angular envelope to 0° yields the reciprocal of the weight-average molecular weight, $1/M_w$. However, the theoretical particle-scattering factor curves show that, when the radius of gyration is greater than about 2500 A, extrapolation to obtain

$1/M_w$ may be inaccurate because of possible curvature of the angular envelope at angles too low for measurement. The radii of gyration recorded in Table I are in the range 1000-1600. The question has nevertheless been asked (18, 19) whether, even for such samples, there is any slight downward curvature of the angular envelope below the smallest angle of observation (25°-30°) which, when overlooked, results in speciously low molecular weights. The question has been answered empirically before (2, 2*a*), and the same treatment applies to the present samples. Briefly, one assumes that downward curvature does occur and, therefore, that the extrapolated value of $1/M_w$ is too high; one then assigns other, lower values to $1/M_w$. With these assigned values one then calculates theoretical particle-scattering curves. These are compared to those for chains of varying degrees of stiffness (20). Although stiffness and polydispersity parameters cannot be solved for explicitly, one can consider the extreme cases; it was thereby found that, for all the samples in this study, an M_w about 5 to 7 per cent higher than that recorded in Table I is the maximum value possible. For higher assumed M_w's, the particle-scattering curves are kinked and fall below the theoretical curves for any degree of stiffness or polydispersity. We deduce, therefore, that the light-scattering molecular weights for these samples are the weight-average molecular weights.

In addition to this reasoning there are two experimental results which lead to the same conclusion. The first concerns the molecular weight of denatured DNA. When DNA is denatured by brief exposure to heat, acid, or alkali, under conditions which minimize hydrolysis and reaggregation, the molecular weight remains the same as that of the undenatured material (21). However, because of the collapse of the molecular structure, the angular dependence of scattered light is markedly decreased and extrapolation to the ordinate ($1/M_w$) is unequivocal. The question of reaggregation was eliminated by denaturing several samples of DNA directly in the light-scattering cell. Heat-denatured samples were cooled immediately, and light-scattering measurements were made within 10 minutes of denaturation. Samples allowed to stand in the light-scattering cell began to aggregate after several hours. All measurements performed on denatured samples were made before this happened. Also, the hyperchromic rise was checked with alkali after light scattering and was found in all cases to have the low value characteristic of denatured DNA.

The second experimental result involves equilibrium sedimentation in a cesium chloride gradient. Meselson and Stahl (22) have found that both native and heat-denatured salmon sperm DNA form bands of the same width when centrifuged in a density gradient, indicating that heat denaturation causes no molecular weight change. In a parallel experiment we have found that light scattering also shows no molecular weight change (23). The same set of results has been obtained in this laboratory with calf thymus DNA; other mutually consistent light-scattering and gradient sedimentation experiments have also been performed on bacterial DNA samples (23).

On the basis of the calculations and experimental results noted above we conclude that light scattering provides the weight-average molecular weight for the DNA samples of this study.

C. Interpretation of Molecular Weight Data. We have presented evidence which shows that the low molecular weights in Table I were not caused by shear degradation during isolation or deproteinization. One question remains: the possibility of autolysis before the degradative enzymes had been denatured or inhibited. Since the enzyme contents of whole cells and of nuclei isolated by two different procedures undoubtedly differ, the molecular weights of DNA samples prepared therefrom might be expected to reflect such differences, *if* any enzyme action occurs. Calf thymus DNA samples isolated by the duponol method did show some differences: the molecular weights were 3.3 million (sample 1) from whole tissue, 2.8 and 3.4 million (samples 3 and 4) from nuclei isolated by the sucrose method, and 4.8 million (sample 6) from nuclei isolated by the citric acid method. However, C-O treatment reduced all these molecular weights to essentially the same low value: 1.9, 1.8, and 1.7 million, respectively. Mouse sarcoma-180 DNA from sucrose- and citric acid-isolated nuclei (samples 8 and 12) showed an even greater difference initially (14 and 2.6 million), but after C-O treatment the molecular weights were close to the same: 1.4 and 1.6 million (samples 9 and 13; see also 10 and 11). The large variation in molecular weight observed for these DNA samples before complete deproteinization is of no significance, in view of the fact that the differences could subsequently be abolished without degrading the DNA. The duponol-isolated samples apparently contained traces of different proteins, and were therefore aggregated to different degrees. Such discrepancies probably account for much of the disagreement in the literature with respect to the molecular weight of DNA, and illustrate the fallacy of assuming that larger weights are more "native."

Further evidence against autolysis is provided by the pneumococcus DNA samples in Table I. As a precaution, in several preparations the living cells were centrifuged rapidly (10 minutes), suspended in 0.1 M EDTA—0.1 M sodium chloride at pH 7.5, and immediately heated to 60° to denature intracellular enzymes. After 5 minutes the cells were lysed and the sodium acetate isolation procedure was carried out, while maintaining the 60° temperature. Thus the probability of enzymatic attack on the DNA was diminished. The final molecular weight of this DNA (samples 21 and 22) was the same as that of DNA prepared after lysing the cells at room temperature (sample 24). Furthermore, a variety of different media and lytic agents have been employed in this laboratory in the preparation of *E. coli* 15_{T-} DNA, with no effect on the final molecular weight.

It is doubtlessly significant that seven diverse sources, possessing different types and amounts of enzymes and other proteins, all have yielded DNA of relatively low molecular weight (1.2 to 2.4 million). These are among the lowest molecular weights reported in the literature, for undegraded DNA, and have been attained only

after testing many different methods and conditions of preparation. No further treatment has yet been found which will reduce the weights to lower values. The small scatter in the final molecular weights from some sources indicates that the larger samples still contain some aggregated material. It is possible, of course, that all the samples are still somewhat aggregated, although the fact that the same minimum weight is always attained regardless of the cause or extent of previous aggregation speaks against this.

Two criteria—minimal molecular weight and maximal length-to-mass ratio—were found useful in recognizing disaggregated DNA. The ratio of radius of gyration to molecular weight provides a convenient index of the latter. Of course the Z-average radius of gyration, obtained from light scattering, refers to the configuration of the molecules in solution, rather than directly to the contour length, so the index is only approximate. Despite large decreases in molecular weight, the radius of gyration remains very nearly the same; see, for example, samples 8 and 9, 14 and 16, and 25 and 26 in Table I. The same is also true of artificial aggregates, and for this reason we have been able to obtain meaningful measurements of molecular weights as high as 50 million. It appears, therefore, that the aggregation of DNA molecules is primarily lateral rather than head-to-tail.

SUMMARY

The purpose of this investigation was to define aggregation operationally and to try to arrive at a meaningful molecular weight for DNA. Toward this end, a number of isolation procedures were employed to prepare DNA from a total of seven sources. Molecular weights were determined by light scattering, which was shown to yield weight-average molecular weights for the samples studied. A variety of treatments subsequent to isolation have been investigated, and a list of pitfalls which may bring about aggregation during the handling of DNA has been compiled. Certain conclusions concerning disaggregation have been reached. First, we investigated the effects of shear and enzymatic attack during the isolation of the DNA samples examined, and found them to be undetectable. In this connection, it was demonstrated that shaking with a chloroform-octanol mixture causes no degradation of DNA. Degradation can therefore be eliminated as a factor in producing the low molecular weights observed. Second, the ratio of radius of gyration to mass indicates that aggregation is primarily of the lateral type, rather than end-to-end; the length of the aggregates appears to remain substantially unaltered as disaggregation proceeds. We have found this ratio to be a useful index to the extent of aggregation. Third, a variety of molecular weights can be obtained for DNA prepared by different methods from a single source, but all these converge to a narrow range as a result of the disaggregation procedure. The molecular weights found for disaggregated DNA from all seven sources are low, and the radii of gyration are high. Finally, a hypothesis explaining the observations regarding aggregation has

been presented. It appears that the various high molecular weights reported in the literature result from aggregation due to protein and/or divalent metal ion contamination.

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