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# Sedimentation Studies on the Interaction of Proflavine with Deoxyribonucleic Acid

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A method is described of using photography to measure the concentrations of a small ligand (proflavine) above and below the boundary of a macromolecule (DNA, both native and denatured) sedimenting in the ultracentrifuge. The measurements are used to determine the extent of the binding of proflavine to DNA, and the results compared with those obtained by a spectrophotometric method. The results obtained by the two methods agree within 10%, thus validating the spectrophotometric technique under these conditions. The variation of the sedimentation coefficient with the extent of binding of proflavine was also studied. The results are discussed in relation to previously observed changes in the viscosity of the solutions.

Since 1960 the ultracentrifuge has been used increasingly for the study of interactions between large and small molecules, particularly by Schachman and co-workers (Schachman, Gropper, Hanlon & Putney, 1962; Schachman, 1963; Steinberg & Schachman, 1966). In these studies photoelectric scanning devices have been used for following the changes in light-absorption that occur when the interacting system of macromolecule and ligand is subjected to a high centrifugal field. Such scanning devices are expensive and not yet available in many Laboratories, so one of the main objects of this study was to assess the feasibility of using the commoner photographic method of recording light-absorption to obtain quantitative information about such interacting systems, since these are likely to be of increasing interest in biochemistry. The system chosen for examination was proflavine and DNA, since this has been much studied by other methods (Bank & Bungenburg de Jong, 1939; Albert, 1951; Michaelis & Granick, 1945; Michaelis, 1947; Peacocke & Skerrett, 1956).

Both equilibrium dialysis and a spectrophotometric method were used by Peacocke & Skerrett (1956) to measure the binding of proflavine to DNA and their results indicated two stages of binding: an initial strong binding with a limit of 1 dye molecule bound/4 or 5 nucleotide residues followed by a weak binding with a limit of 1 dye molecule bound/nucleotide residue. Lerman (1961) proposed a model for the DNA–acridine complex associated with strong binding, according to which the planar acridine molecules intercalated between adjacent base pairs of the DNA double helix, with a consequent extension, and unwinding, of the polynucleotide chains. This model has been supported by other studies (Luzzati, Masson & Lerman, 1961; Cairns, 1962), but it is difficult to reconcile with the observations (Lawley, 1956; Drummond, Simpson-Gildemeister & Peacocke, 1965; Liersch & Hartmann, 1965) that the extent of binding of proflavine to denatured DNA is at least as great as that to the completely helical DNA.

Comparisons (Lawley, 1956; Drummond *et al.* 1965; Liersch & Hartmann, 1965) of the binding of proflavine by native and denatured DNA have usually made use of the displacement of the spectrum of proflavine that occurs when it is bound to the DNA. Although the spectral shifts induced by native and denatured DNA are of the same type, and exhibit the requisite isosbestic point, there is a possibility that the similarity of proflavine binding so determined may be the fortuitous result of a balance between opposing shifts in the spectrum of proflavine. For these spectra are composite and involve at least two distinct transitions (Blake & Peacocke, 1967), which may be affected differently by (i) whether the binding is strong or weak and (ii) whether the DNA is native or denatured. A method of comparing the binding of native and denatured DNA that did not exploit the spectral shift caused by binding was desirable and provided an additional incentive in applying sedimentation methods.

Hence another object of this study was to compare the extent of binding of proflavine to native and denatured DNA, by means of the ultracentrifuge. A quantitative study has also been made of the decrease in the sedimentation coefficient of DNA that occurs when it binds proflavine

(Lerman, 1961). By relating the observed changes in sedimentation coefficient to previous observations (Drummond, Pritchard, Simpson-Gildemeister & Peacocke, 1966) on the viscosities of these complexes, further insight should be obtained into their shape and hydrodynamic behaviour.

## EXPERIMENTAL

### *Materials*

Calf thymus DNA was prepared by a modification of the method of Kay, Simmons & Dounce (1952). The RNA content of the preparation was found to be 0·5% by the method of Webb (1956), and the protein content was found to be 1% by a modification of the method of Lowry, Rosebrough, Farr & Randall (1951). The concentrations of DNA solutions were determined by phosphorus analysis (Jones, Lee & Peacocke, 1951). The calf thymus DNA was native by the criterion of the increase in ultraviolet absorption caused by heating. Proflavine was obtained from British Drug Houses Ltd. (Poole, Dorset) as the hemisulphate and was dried to constant weight and stored in a vacuum desiccator (for other details see Drummond *et al.* 1965, 1966).

### *Determination of binding by 'sedimentation dialysis'*

*Principles.* If a solution containing a small ligand molecule and a macromolecule is subjected to a high centrifugal field, the concentration of free ligand remains constant throughout the cell, since it is far too small to sediment even in these high fields. But the macromolecule sediments and its concentration, after a slight correction for the radial dilution effect, remains constant in the plateau region. The sedimentation process may be followed by absorption optics at a wavelength at which the ligand absorbs, but the macromolecule does not, and a photograph should yield a microdensitometer trace similar to Fig. 1. Such traces show the movement of ligand bound to the sedimenting macromolecules. The absorption of light in the region $A$ of the cell is due only to free ligand, whereas the absorption in region $B$ is due to both bound and free ligand. From the absorption of the incident light by these regions, as measured photographically, it should be possible to determine a binding curve for the ligand–macromolecule system. The method corresponds to the equilibrium-dialysis method in that the sedimenting boundary replaces the dialysis membrane. If the wavelength used in the 'sedimentation-dialysis' method is the isosbestic wavelength of the ligand–macromolecule system, the ligand molecules have the same extinction coefficients whether bound or free and, of particular relevance to the proflavine–DNA system, no assumptions about the spectral shift need to be made in order to calculate the amounts of free and of bound proflavine.

An equation relating the concentration of ligand in region $A$ in the cell (see Fig. 1) to that of free ligand in region $B$ has been derived by Schachman (1959). The equation involves the sedimentation coefficients of the macromolecule, the ligand and the complex, and also the molar concentrations of the constituents and the number of binding sites on each macromolecule. In the present case the molar concentration of DNA is so low, and the number of binding sites is so high, that the concentration of free ligand in region $B$ can differ from that in region $A$ by only about one part in a thousand. Thus the proflavine concentration above the boundary is a good measure of the concentration of free proflavine below the boundary.

*Equipment.* All studies were made at 20° in a Beckman–Spinco model E ultracentrifuge fitted with an absorption optical system and a Beckman monochromator for selecting wavelengths in the visible and ultraviolet range.

By substituting a high-pressure xenon lamp for the ordinary mercury-vapour source supplied by the manufacturer and incorporating a more efficient condensing system it was possible to obtain light of sufficient intensity for the photographic work described below with band widths usually of about 2m$\mu$, or at the most 6m$\mu$ in the early stages of the work. With the low-intensity source, as supplied, the band width needed to obtain sufficient intensity was too large to apply the principle of analysis already outlined.

The photographic film employed was Kodak CF8 and it was developed with Kodak D19b developer under carefully standardized conditions. Film densities were measured by means of a Joyce–Loebl microdensitometer.

*Development.* Proflavine was known to adsorb heavily on a variety of materials and a preliminary investigation of the amount adsorbed by ultracentrifuge centrepieces made of various materials was necessary. Aluminium centrepieces adsorbed proflavine strongly and were therefore unsuitable. Kel F centrepieces adsorbed much less than aluminium-filled Epon centrepieces on first use, but it was discovered that an aluminium-filled Epon centrepiece which had been pretreated with proflavine solution subsequently adsorbed even less proflavine than Kel F centrepieces and this Epon centrepiece was therefore used in these studies.

The main practical problem is to relate the blackening of the film (film density) caused by the light passing through a proflavine solution to the proflavine concentration. A plot of film density against the concentration of proflavine solutions in an ultracentrifuge cell could not be obtained with sufficient accuracy and reproducibility and this approach had to be abandoned.

The theory of the photographic process suggested another possibility. If a photographic film is exposed to a constant light intensity, $I$, for a time, $t$, the film density, $d$, measured as a deflexion of the microdensitometer, is a function of the logarithm of the product of the light-intensity and the time, i.e.:

$$d = \mathrm{f}(\log I . t) = \mathrm{f}(\log I + \log t)$$

This function may be evaluated either by plotting $d$ versus $\log I$ at constant $t$, or by plotting $d$ versus $\log t$ at constant $I$ (Fig. 2). The latter method is very much easier in practice, and if the units of $I$ are suitably chosen the two methods will yield exactly the same curve. (Other units of $I$ would simply move the curve parallel to the $\log I$ axis, without altering its shape.) Thus if the film-response curve is evaluated by exposing a film to constant light-intensity for various times and plotting $d$ versus $\log t$, the $\log t$ scale may be used as a $\log I$ scale for extinction measurements, since the units in which $I$ is measured are not important in evaluating extinctions. Thus if an ultracentrifuge cell contains an absorbing solution and also a region of 'zero' absorption (e.g. the air space or a reference hole), the extinction of the solution, relative to that region, may be evaluated from the densitometer deflexions in these two

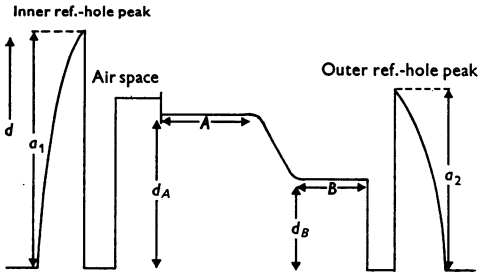


Fig. 1. Idealized microdensitometer trace illustrating the principle of the sedimentation-dialysis method and the measurements made. *d* is the extinction of the film measured by the microdensitometer.

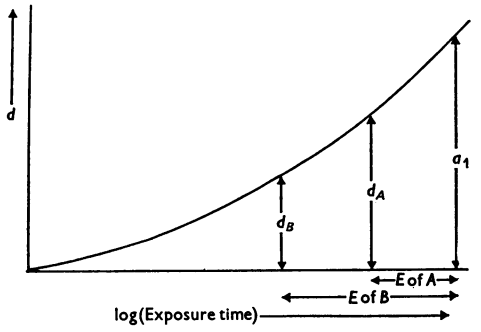


Fig. 2. Film-response curve determined in the sedimentation-dialysis method, illustrating the method of calculating extinctions from the microdensitometer traces. *E*, extinction.

regions of the photography, by using the film-response curve to obtain the corresponding values of $\log I$ ($=\log t$). The difference in the two $\log I$ values so obtained is the extinction of the solution relative to this 'internal standard'. This is illustrated in Figs. 1 and 2 for a case where the inner reference hole has been used as the internal standard. The apparent extinction of water (or the solvent), relative to the same internal standard, must be determined by the same procedure and subtracted from the apparent extinction of the solution to obtain the extinction of the solution relative to water.

The advantage of this approach to the problem is that the film-response curve may be evaluated afresh during each experiment simply by taking a series of photographs of the cell with different exposures and using all of them to plot the film-response curve and any one of them to deduce the extinction of the solution. In this way variations of film response or developing conditions are largely eliminated. In theory the most suitable region to take as the internal standard is the air space above the solution column, since this will approach closely to the true zero level. However, in these experiments the absorption of the air space was found to be poorly reproducible (possibly as a result of adsorption of proflavine on the quartz windows). It was therefore necessary to use one of the two reference holes as the internal standard.

*Experimental procedure.* Extinction measurements with the absorption optical system of the ultracentrifuge were made with cells of path length 3 mm. and 12 mm. Mixtures of DNA and proflavine were made up as follows: 5 ml. of proflavine solution (approx. 50 $\mu$M) in 0·1 M-NaCl and 5 ml. of 0·1 M-NaCl were pipetted into a series of test tubes and various volumes of DNA solution added. The mixtures were made up to 12 ml. by the addition of 0·1 M-NaCl solution. A preliminary run in the ultracentrifuge with the cell containing water was carried out at low speed and photographs were taken with various exposures to determine the apparent 'extinction' of water. After deceleration the rotor was removed and the cell refilled with the solution. The cell was accelerated to about 40 000 rev./min. and after about 25 min. a series of photographs of different exposure times were taken with light of wavelength 454 m$\mu$, the isosbestic point of the DNA–proflavine system. The slit width on the ultracentrifuge monochromator was chosen so that the film density produced by a 60 sec. exposure approached the maximum that could be recorded by the microdensitometer. From each microdensitometer trace the distances $a_1$, $a_2$, $d_A$ and $d_B$ (Fig. 1) were measured. ($a_1$ is greater than $a_2$ because the two reference holes are the same size, and so the inner hole subtends a greater angle at the centre of the rotor than does the outer hole.) The distances $a_1$ and $a_2$ were plotted against the logarithm of the exposure time, *t*, for the corresponding photograph, to give film-response curves (such as Fig. 2). The $\log t$ values corresponding to the distances $d_A$ and $d_B$, both measured at the same time of exposure as the particular $a_1$ used for comparison, were read off from the response curves. Subtraction of these values from the $\log t$ values corresponding to the peak heights (e.g. $a_1$ in Fig. 2, which is plotted for the inner-reference-hole peak) from the same trace gave values for the apparent 'extinctions' of proflavine in regions *A* and *B* relative to the internal standard (the reference-hole peak). Values for these apparent extinctions were calculated relative to each of the reference holes as internal standard, and the average was taken. Similar treatment for the microdensitometer traces of the exposures taken with the cell containing water gave values for the 'extinction' of water also relative to the internal standards. The true extinctions (relative to water) of proflavine in any level of the cell were then obtained by subtraction of the apparent 'extinction' of water from that of proflavine at the level in question. Hence proflavine concentrations in regions *A* and *B* could be obtained by use of the extinction coefficient of proflavine at the isosbestic wavelength. The molar concentration, *c*, of free proflavine is that in region *A* above the DNA boundary and the concentration of bound proflavine, $c_b$, is the molar concentration of proflavine in region *B* below the boundary minus *c*. Then the number, *r*, of molecules of proflavine bound/DNA nucleotide residue is:

$$r = c_b/P$$

where *P* is the molar concentration of DNA phosphorus in the solution. It was therefore possible to obtain plots of *r* against *c*, binding curves, from these sedimentation measurements.

### *Determination of binding by spectrophotometry*

These determinations were made at 440 m$\mu$ for comparison with binding curves determined from sedimentation and

followed the procedures fully described elsewhere (Peacocke & Skerrett, 1956; Drummond *et al.* 1965).

### *Sedimentation velocity*

It was desired to determine the variation with $r$ of the sedimentation coefficient, $S^0$, of the DNA–proflavine complex at infinite dilution, so that, although the DNA concentration is varied, $r$ must be kept constant. Various amounts of DNA were pipetted into test tubes so as to give calculated final concentrations of 1–8mg./100ml., and proflavine solution was then added to each tube. The volumes of proflavine were calculated as follows. For a given value of $r$, the molar concentration, $c$, of free proflavine has a definite value, so that $T_L$ ($=rP+c$), the total concentration of proflavine in the final volume, is also determined once $P$ is fixed. The mixtures were diluted with 0·2M-NaCl solution and water to give a solution of ionic strength 0·1. A set of solutions of various concentrations, but with $r$ constant, was prepared in this way on the basis of the previously determined relation between $r$ and $c$ at ionic strength 0·1.

A wavelength of 460m$\mu$ was used to follow the sedimenting boundary in the ultracentrifuge in order to maximize the optical absorption of the bound proflavine. The exposure time and the slit width on the monochromator were chosen so that the film density corresponding to the plateau region ($B$ of Fig.1) was on the linear part of the film-response curve. Rotor speeds were about 35000rev./min. Sedimentation coefficients were determined for the point corresponding to an extinction at 460m$\mu$ which was the arithmetical average of the extinctions in the plateau and DNA-free regions.

## RESULTS AND DISCUSSION

*Binding curves.* The binding curves of proflavine on native and denatured DNA as determined by sedimentation dialysis are given in Fig. 3 and compared with the parallel observations by the spectrophotometric method in Fig. 4.

*Sedimentation-dialysis method.* The analysis of the microdensitometer traces revealed certain sources of error.

(i) The internal reference peaks were not always of identical shape and a summation of errors occurred when the actual extinction of the proflavine solution relative to water was calculated.

(ii) Contamination of the condensing lens by oil from the drive mechanism of the ultracentrifuge produced a considerable and variable background in the microdensitometer traces. This 'noise' was worse with visible light than with ultraviolet light.

(iii) In some experiments the apparent 'extinction' of the solution determined from the film density varied across the cell for unknown reasons. The most likely explanation is the presence of an unequal adsorption of proflavine on the quartz windows of the cell.

The binding curves measured by sedimentation dialysis agreed satisfactorily with the binding curves

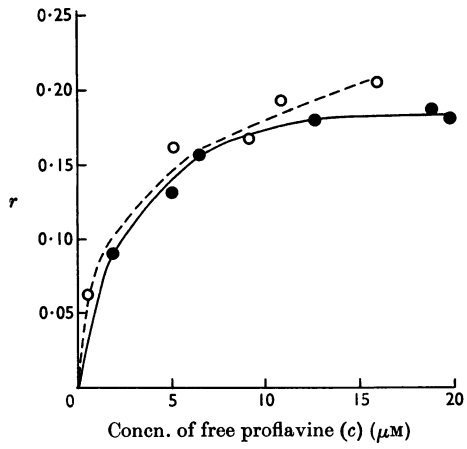


Fig. 3. Binding curves determined by the sedimentation-dialysis method. ●, Native DNA; ○, denatured DNA.

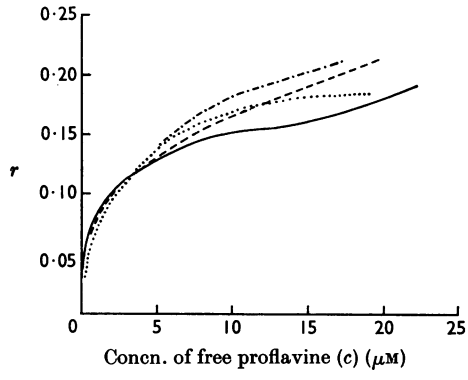


Fig. 4. Binding curves for the interaction of proflavine with DNA. ——, Native DNA (spectrophotometric method); ·····, denatured DNA (spectrophotometric method); – – – –, native DNA (sedimentation-dialysis method); –·–·–, denatured DNA (sedimentation-dialysis method).

determined spectrophotometrically (Fig. 4) up to $r=0·15$, but diverged beyond this point, though probably the divergence was no greater than the probable error in the spectrophotometric method in this range when binding by the weak process is beginning to be significant in amount. The broad agreement ratifies both methods, within the limits expressed in Fig. 4, and in particular validates (within these limits) the basic assumption of the spectrometric method that the extinction coefficient of bound proflavine at 440m$\mu$ is independent of $r$ in this range.

The accuracy of the sedimentation-dialysis method as developed here is limited by the use of

photographic techniques and the difficulties in finding a suitable internal standard. Electronic scanning devices to measure the extinction across the ultracentrifuge cell during sedimentation seem able to remove most of the sources of error arising from the use of photography, although errors due to adsorption of the small ligand molecule on the centrepiece and windows will still arise. However, even if only photographic techniques are available, estimates of binding to within 10% seem to be attainable if suitable care is taken in their calibration.

*Binding of proflavine to native and denatured DNA.* The binding of proflavine to denatured DNA is greater (Fig. 3) than the binding to native DNA when $r > 0{\cdot}16$, which occurs at free proflavine concentrations greater than $80\,\mu$M when the ionic strength is 0·1. The difference in binding at $r > 0{\cdot}16$ may be attributed to the greater tendency of proflavine to bind to denatured DNA by the weak binding process, since at this ionic strength the inflexion point beyond which weak binding predominates occurs in the range $r = 0{\cdot}12$–$0{\cdot}17$. At $r < 0{\cdot}12$, when the strong binding process predominates, native and denatured DNA bind to the same extent, within the limits of error (10%) of both the sedimentation-dialysis and spectrophotometric methods. This confirms the reality of the problem posed for the intercalation model, if this is conceived as identifying strong binding with intercalation between base pairs into a double helix (Lerman, 1961), and suggests that this model needs to be modified to explain strong binding to unwound polynucleotide chains, perhaps along the lines already suggested (Pritchard, Blake & Peacocke, 1966).

*Sedimentation velocity.* Sedimentation coefficients were determined for a series of mixtures with various concentrations of native DNA, but with a concentration of proflavine such that the extent of binding, $r$, was constant within each series. The results are expressed in terms of $1/S$ versus DNA concentration (g./dl.) in Fig. 5. (The additional subscript $r$ refers to measurements at a particular extent of binding $r$.) Extrapolation of the least-squares lines to infinite dilution afforded values of $S^0_{20,r}$. The values of $S^0_{20,r}$ are plotted against $r$ in Fig. 6, in which the vertical lines represent the maximum error determined by the least-squares method.

The decrease in the sedimentation coefficient with increasing $r$ (Fig. 6) is in agreement with previous observations (Lerman, 1961; N. F. Ellerton & D. U. Jordon, unpublished work), and implies an increase in frictional coefficient of DNA when proflavine is bound. This change in $S$ must be combined with the known changes in viscosity

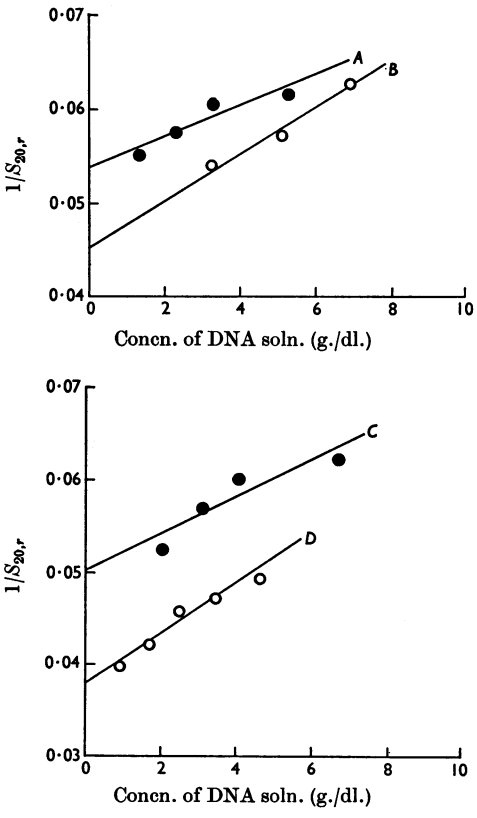


Fig. 5. Set of extrapolated curves for the DNA–proflavine complex at various values of $r$, to obtain $S^0_{20}$. *A*, $r = 0{\cdot}16$; *B*, $r = 0{\cdot}07$; *C*, $r = 0{\cdot}12$; *D*, $r = 0$.

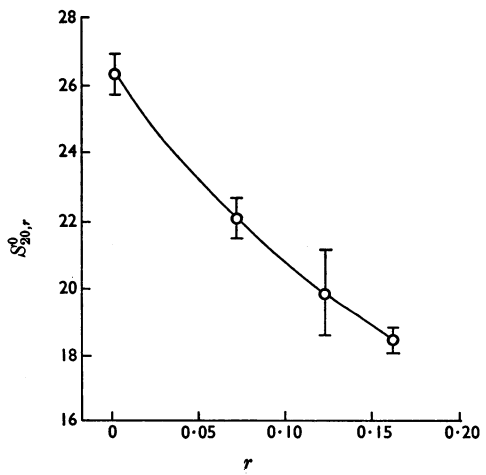


Fig. 6. Dependence of $S^0_{20}$ on $r$ (number of proflavine molecules bound/nucleotide residue) (ionic strength 0·1).

by the usual equations (Scheraga, 1961) to enable conclusions to be drawn about the nature of the change occurring, although it is clear that there must be an extension of the molecule, since the changes in molecular weight on binding are relatively small. The equation relating $S$ and intrinsic viscosity, $[\eta]$, is (Scheraga, 1961):

$$\beta = \frac{NS^0_{20}[\eta]^{1/3}\eta_0}{M^{2/3}(1-\bar{v}\rho)}$$

where $N$ is Avogadro's number, $S^0_{20}$ is the sedimentation coefficient at infinite dilution measured at 20°, $[\eta]$ is the intrinsic viscosity at the same temperature, $\eta_0$ and $\rho$ are the specific viscosity and density respectively of the solvent at 20°, $M$ is the molecular weight of the macromolecule and $\bar{v}$ is its partial specific volume. $\beta$ is a constant whose significance for DNA has been closely examined (Eigner & Doty, 1965). If $\beta_r$ and $\beta_0$ are the values of $\beta$ for DNA, with and without bound proflavine, and these subscripts have the same meaning for other quantities, then:

$$\beta_r/\beta_0 = \frac{(S^0_{20,r}/S^0_{20,0})([\eta]_r/[\eta]_0)^{1/3}}{(M_r/M_0)^{2/3}}$$

since $\bar{v}$ of DNA should change very little with proflavine binding. The variation in molecular weight with the degree of binding may be calculated from $r$ (since $M_r = M_0[1 + r.M_{\text{proflavine}}/M_{\text{nucleotide}}]$). The relative variation of $S^0_{20,r}$ with $r$, observed in the present study, has been combined with the results of an earlier study (Drummond *et al.* 1966) on the dependence of $[\eta]$ on $r$ to calculate the variation in $\beta_r/\beta_0$ with $r$. A decrease of about 20% in $\beta_r/\beta_0$ at $r = 0{\cdot}16$ was inferred (Fig. 7). As derived by Mandelkern & Flory (1952), $\beta$ is dependent only on macromolecular shape and has a value of $2{\cdot}4 \times 10^6$ for flexible coils. The best authenticated values for DNA are between $2{\cdot}4 \times 10^6$ and $2{\cdot}6 \times 10^6$, the limiting value being $2{\cdot}4 \times 10^6$ at very high molecular weights (Eigner & Doty, 1965). A change in $\beta$ of DNA of about 20% on the binding of proflavine could only be explained if parameters of shape or flexibility altered. We have attempted to interpret this change in terms of the worm-like-chain model for DNA, as developed by Eizner & Ptitsyn (1962). According to the relationships presented by Eizner & Ptitsyn (1962, Fig. 4) a decrease in $\beta$ (their $\phi^{1/3}P^{-1}$) of 20% could only be explained if the parameter $\lambda$, which is 32 for DNA, decreases to about 10 when proflavine is bound. The parameter $\lambda$ is, in their treatment, the ratio of the persistence length, $a$, of the worm-like chain of DNA to the length, $b$, of the hydrodynamically independent segments that constitute the chain. Given this change in $\lambda$ from 32 to 10, on the basis of the decrease in $\beta$, the concomitant changes on binding of proflavine in $a$ and $b$ can be deduced from the equation (Eizner & Ptitsyn, 1962, eqn. 11) that relates these quantities to the observed intrinsic viscosities. The value of the radius of the segments was kept constant at 10 Å, previously deduced as the best value for DNA (Drummond *et al.* 1966). The value of $b$ then changed from 22 Å to 27·5 Å on binding of proflavine to the extent $r = 0{\cdot}16$, and $a$ changed from 700 Å to 300 Å. Such an increase in $b$ implies a proportionate increase in contour length, which explains the increase in viscosity and decrease in sedimentation coefficient. However, along with this change there is apparently a decrease in persistence length, which would normally be taken to indicate an increase in flexibility. This latter inference seems to be required by the observation that $\beta$ (and so $\lambda$) decreases when proflavine is bound. In other words the decline in sedimentation coefficient on binding of proflavine is steeper than one would expect, if the flexibility was unaltered, from the increase in contour length implied by the concomitant increase in viscosity.

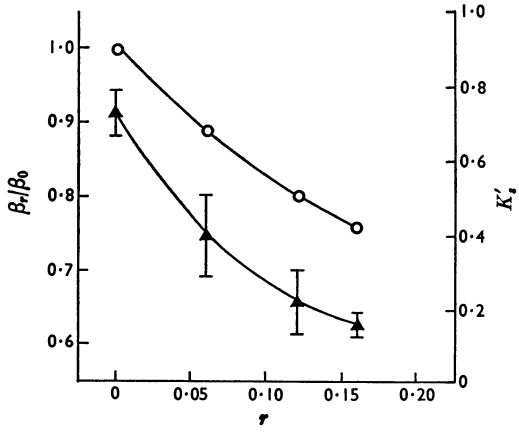


Fig. 7. Dependence of $\beta_r/\beta_0$ (○) and $K'_s$ (▲) on $r$ (ionic strength 0·1).

The equation:

$$1/S = (1/S^0)(1 + K'_s[\eta]c)$$

(where $c$ is the concentration of DNA and the superscript zero represents infinite dilution, as above) has been postulated as representing the empirical dependence of sedimentation coefficient on concentration. Eigner & Doty (1965) concluded that, over a molecular-weight range $0{\cdot}3 \times 10^6$–$30 \times 10^6$ $K'_s$ has a constant value for DNA of $0{\cdot}8 \pm 0{\cdot}1$. The values of $S$ and $[\eta]$, as functions of $r$, which were used to determine $\beta_r/\beta_0$ may also be used to calculate the variation of $K'_s$ with $r$ and this is shown in Fig. 7. The coefficient $K'_s$ decreases markedly from a value of 0·72 for the original DNA to 0·15 at $r = 0{\cdot}16$, as a result of the binding of

proflavine. Creeth & Knight (1965) have suggested that values of $K'_s \leqslant 1{\cdot}6$ can be interpreted for globular proteins in terms of particle shape, and that, like $\beta$, $K'_s$ is dependent primarily on shape, but is more sensitive than $\beta$. From the empirical equation quoted by Creeth & Knight (1965) the axial ratio of the equivalent prolate ellipsoid may be calculated to increase from about 35 for native DNA to about 260 when proflavine is bound to an extent of $r = 0{\cdot}16$. No great significance can be attached to these exact values, since their treatment is confined to neutral macromolecules, but the results do indicate a considerable extension of the molecule when proflavine binds to DNA.

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