at room temperature for 23 hours (stoppered), then evaporated to dryness *in vacuo*. The residue was dissolved in 15 ml. of water, neutralized by stirring with 50 mg. of Amberlite IRC-50(H),[24] filtered, and evaporated to dryness *in vacuo*. The residual crude XVIII was a colorless oil which, after solution in absolute ethanol and re-evaporation (finally at 35° and 0.9 mm.), weighed 528 mg. (102%) and showed no carbonyl absorption in the infrared. On periodate oxidation, this oil consumed only 0.042 ± 0.005 equivalent of oxidant. The remaining 499 mg. was dissolved in 50 ml. of 0.01 *M* aqueous acetic acid, and the *p*H (measured potentiometrically) was adjusted to that of 0.01 *M* aqueous acetic acid by the addition of 0.3–0.4 ml. of 2 *M* aqueous acetic acid. The resulting solution was diluted with an additional 12.5 ml. of 0.01 *M* aqueous acetic acid, then refluxed under nitrogen for 1.75 hours.[14] The cooled, colorless solution, which gave a positive Benedict test, was evaporated to dryness *in vacuo* (bath 40°). The residual crude XIX, after solution in methanol and re-evaporation *in vacuo*, was converted to its anilide (402 mg.), m.p. 150–153° dec., by the method of Hardegger, *et al.*[15] Recrystallization from methanol gave 304 mg. (41%) of white crystals, m.p. 173–175° dec. A mixture with authentic anilide[34] gave no depression in m.p.; the infrared spectra of the two samples were identical. The product of a preliminary run had m.p. 175–177° dec. and $[\alpha]^{27}D$ +175° (1% in pyridine); reported[15] m.p. 172–173° dec. and $[\alpha]D$ +171° (1% in pyridine).

Acknowledgments.—We wish to thank Miss C. M. Brown and staff for the microanalyses, Dr. Peter Lim for interpretation of the infrared data, and Dr. L. K. Moss for the periodate oxidation data and optical rotations.

(34) Obtained from the California Foundation for Biochemical Research, Los Angeles 63, California.

MENLO PARK, CALIF.

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION, CORNELL UNIVERSITY MEDICAL COLLEGE]

Sedimentation Studies of Fractions of Deoxyribonucleic Acid[1]

BY HERBERT S. ROSENKRANZ[2] AND AARON BENDICH

RECEIVED JULY 2, 1958

Eight fractions of calf thymus DNA obtained by chromatography on a column of the anion-exchanger ECTEOLA have been examined in the analytical ultracentrifuge in dilute solution (0.003%). These fractions, amounting to 56.5% of the DNA recovered from the column with eluents of increasing ionic strength and then increases in *p*H, showed progressive increases in $s_{50\%}$ from 10.9 to 24.1 *S* (compared with 15.8 *S* for the original). The original DNA showed the usual wide spread in sedimentation coefficients. The spread was very narrow for the early fractions but broadened considerably for the later ones. Evidence is presented that the fractionation procedure discriminates among DNA's, in part, on the basis of properties related to their sedimentation coefficients and that the method does not lead to measurable physical alterations.

It has been shown[3–6] that the deoxyribonucleic acid (DNA) of various sources could be separated into different fractions by means of chromatography on the substituted cellulose anion exchanger ECTEOLA.[7] These fractions were found to differ in their biological activity and base composition.[4,6,8,9] From a study of the behavior of various nucleotides and DNA specimens on columns of ECTEOLA, it was inferred that the chromatography could discriminate among DNA molecules on the basis of size.[5,6,10] The present report deals with a study of the sedimentation behavior of such chromatographic fractions in the analytical ultracentrifuge.[11]

Earlier studies have indicated that DNA was heterodisperse with respect to sedimentation coefficient.[12,13] In order to assess conclusions drawn from physico-chemical measurements on such inhomogeneous material, the validity of which might be influenced by the polydispersity,[14,5] a number of approaches have been made to obtain more homogeneous specimens. Schumaker and Schachman[13] obtained a fraction of DNA with a narrower sedimentation distribution than the original by applying the technique of zone centrifugation. Shooter and Butler[15,16] (*cf.* also[17]), using moving boundary centrifugation, obtained a fraction of DNA with a lower average sedimentation coefficient than the original.

Experimental

Nucleic Acid.—The DNA was prepared from fresh calf thymus glands by the Schwander and Signer method.[18] Several of the properties of this preparation (designated as "S-II" in a previous publication[19] have been reported.[6,19]

Chromatography.—An adaptation of the previously described[3,6] chromatographic method was used to fractionate 78.5 mg. of the DNA (1 mg. per ml. of 0.001 *M* NaCl) adsorbed on a column (7.4 × 2.8 cm.) of ECTEOLA-SF-1[6] in the cold room. The column was washed with 425 ml. of tris buffer (tris-(hydroxymethyl)-aminomethane), 0.01 *M*, *p*H 7. A gradient elution system with two mixing chambers was attached to the column. The description and mathematical treatment of this arrangement have been discussed previously.[6] The stock solution, saturated with chloroform and consisting of 0.5 *M* NaCl (in 0.01 *M* tris), was allowed to drip into the first mixing chamber containing 250 ml. of

(1) This investigation was supported by funds from the American Cancer Society, National Cancer Institute, National Institutes of Health, Public Health Service (grant #CY-3190), and from the Atomic Energy Commission (Contract #AT(30-1), 910).

(2) Pre-doctoral Fellow of the Alfred P. Sloan Foundation.

(3) A. Bendich, J. R. Fresco, H. S. Rosenkranz and S. M. Beiser, THIS JOURNAL, **77**, 3671 (1955).

(4) A. Bendich, H. B. Pahl and S. M. Beiser, *Cold Spr. Harbor Symp. Quant. Biol.*, **21**, 31 (1956).

(5) A. Bendich, H. B. Pahl, H. S. Rosenkranz and M. Rosoff, *Soc. Exp. Biol. Symp.*, **12**, 31 (1958).

(6) A. Bendich, H. B. Pahl, G. C. Korngold, H. S. Rosenkranz and J. R. Fresco, THIS JOURNAL, **80**, 3949 (1958).

(7) E. A. Peterson and H. A. Sober, *ibid.*, **78**, 751 (1956).

(8) H. B. Pahl, S. M. Beiser and A. Bendich, *Federation Proc.*, **16**, 230 (1957).

(9) S. M. Beiser, H. B. Pahl, H. S. Rosenkranz and A. Bendich, in preparation.

(10) M. Rosoff, G. di Mayorca and A. Bendich, *Nature*, **180**, 1355 (1957).

(11) A preliminary report on these studies is given in H. S. Rosenkranz and A. Bendich, *Federation Proc.*, **17**, 299 (1958).

(12) K. V. Shooter and J. A. V. Butler, *Nature*, **175**, 500 (1955); *Trans. Faraday Soc.*, **52**, 734 (1956).

(13) V. N. Schumaker and H. K. Schachman, *Biochim. et Biophys. Acta*, **23**, 628 (1957).

(14) P. Doty, *J. Cell Comp. Physiol.*, **49**, suppl. 1, 27 (1957).

(15) K. V. Shooter and J. A. V. Butler, *Nature*, **177**, 1033 (1956).

(16) K. V. Shooter and J. A. V. Butler, *J. Polymer. Sci.*, **23**, 705 (1957).

(17) P. Doty, in "The Chemical Basis of Heredity," Ed. McElroy and Glass, Johns Hopkins Press, Baltimore, Md., 1957, p. 550.

(18) H. Schwander and R. Signer, *Helv. Chim. Acta*, **23**, 1521 (1950).

(19) L. F. Cavalieri, M. Rosoff and B. H. Rosenberg, THIS JOURNAL, **78**, 5239 (1956).

0.01 *M* tris, the contents of which were led into a second mixing chamber which also contained 250 ml. of this diluent. All collections were made with a photoelectric-activated constant volume collector (25 ml.) in test-tubes at approximately 2-hour intervals. When the limit concentration of 0.5 *M* NaCl was reached, about 25 ml. of 0.5 *M* NaCl in 0.01 *M* tris was left on top of the column and elution with 2.0 *M* NaCl and 0.1 *M* NH_3 was begun. When no more DNA was eluted, a gradient system with two mixing chambers was again established, each of which contained 250 ml. of 0.1 *M* NH_3 in 2.0 *M* NaCl. The stock solution consisted of 1 *M* NH_3 in 2 *M* NaCl and elution was continued until a base line optical density of 0.014 at 260 mμ was reached. The column then was washed with 0.5 *M* NaOH and the residual DNA (less than 1% of the original) was eluted. The optical density of each collection at 240, 250, 260 and 280 mμ was determined in the Beckman Model DU quartz spectrophotometer. The chromatographic profile of the DNA based upon the 260 mμ readings is given by the heavy outline in Fig. 1. In this plot the area under the heavy outline is directly proportional to DNA concentration. The recovery, based on the 260 mμ absorbancies, was 98% of the DNA applied to the column.

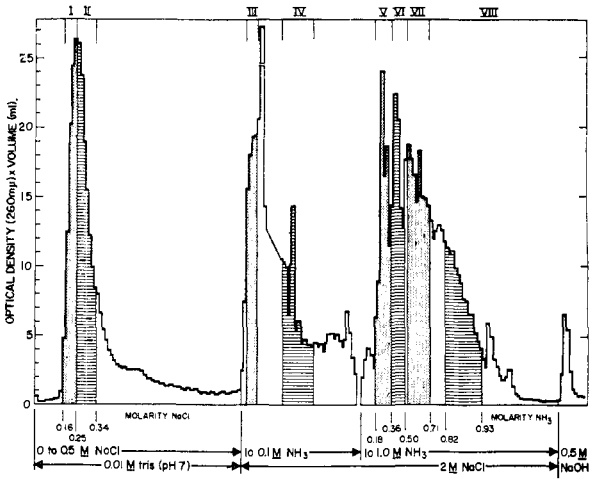

Fig. 1.—Gradient elution profile obtained by chromatography of 78.5 mg. of calf thymus DNA on a 10-g. column of ECTEOLA-SF-1 (7.4 × 2.8 cm.; 0.21 meq./g.). The area under the heavy outline is directly proportional to the quantity of DNA; recovery 98%. The eight fractions selected for further studies are indicated by the shaded areas and the Roman numerals above them.

Pooling of Tubes.—For further studies, the contents of selected collection tubes were pooled to give fractions I through VIII (Fig. 1). These fractions, described in Table I, were dialyzed (0 to 3°) against several changes of 0.01 *M* NaCl and concentrated about fivefold *in vacuo* at 10 to 20° in a rotating evaporator. These solutions were then equilibrated by dialysis in the cold room against several changes of 0.20 *M* NaCl and subsequently examined in the analytical ultracentrifuge (see below). The solutions, saturated throughout this procedure with chloroform, were at all times stored at 0 to 3° until analyzed.

TABLE I

AVERAGE SEDIMENTATION COEFFICIENTS ($S_{50\%}$) OF DNA FRACTIONS

Fraction	% of original	Molarity of effluent NaCl	Molarity of effluent NH_3	$s_{50\%}$
Original	100	.....	.....	15.8
I	6.2	0.16–0.24	.....	10.9
II	8.1	0.25–0.34	.....	11.6
III	6.6	~2	~0.1	12.8
IV	6.0	2.0	0.1	14.4
V	6.1	2.0	0.18–0.36	14.4
VI	6.1	2.0	.36– .50	19.2
VII	8.2	2.0	.50– .71	19.2
VIII	8.2	2.0	.82– .93	24.1
	56.5			

Ultracentrifugal Analysis.—Solutions containing 0.003% DNA in 0.20 *M* NaCl were analyzed at 59,780 r.p.m. (259,700 *g*) in 12 mm. cells in a Spinco Model E ultracentrifuge equipped with ultraviolet optics.[12,13] The integral distributions of sedimentation coefficients, corrected to 20° and water, were calculated by an adaptation of the method of Schumaker and Schachman[13] and are reproduced in Fig. 2.

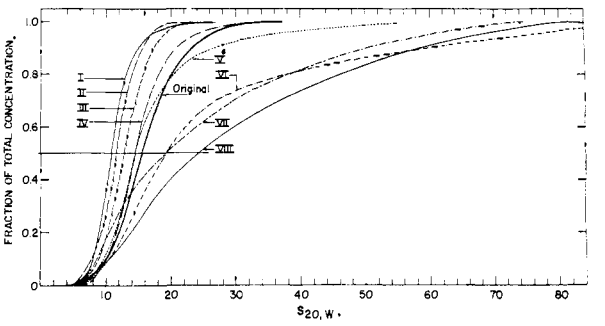

Fig. 2.— Integral sedimentation coefficient distribution curves of calf thymus DNA and 8 fractions thereof obtained by chromatographic fractionation on ECTEOLA.

Effect of Exposure of DNA to Ammonia.—Aliquots (5 ml.) of 0.003% solutions of the original DNA and DNA fraction II in 0.20 *M* NaCl were dialyzed for five days in a cold room (0 to 3°) against 1 liter of 1 *M* NH_3 in 2 *M* NaCl. They were then equilibrated by dialysis as above against 0.20 *M* NaCl and analyzed in the ultracentrifuge. No significant ultraviolet absorbing (260 mμ) material was lost throughout this procedure.

Reconstructed Sedimentation Distribution Curve.—From the data in Fig. 2, the proportion of each fraction which sedimented with a given sedimentation coefficient (at 5 Svedberg unit intervals) was determined from the ordinate readings. Each of these proportions was then weighted according to the occurrence of each fraction in the original DNA (given as "per cent. of original" in Table I), and the per cent. values, corrected in this fashion, were summed and then divided by 56.5% to correct for the total DNA which was analyzed. The calculated distribution is given in Fig. 3 together with that of the original for comparison.

Average Sedimentation Coefficients.—The average sedimentation coefficients ($s_{50\%}$) were estimated from the 0.5 intercepts of the sedimentation coefficient distribution curves (Fig. 2) and are given in Table I. The error in determination of $s_{50\%}$ from repeat ultracentrifugal analyses is less than 0.3*S*.

Results and Discussion

The curve for the original DNA (heavy line, Fig. 2) shows the usual wide spread in sedimentation coefficients seen previously.[18,19,20] It can be seen from Fig. 2 and Table I that there is a progression in average sedimentation coefficients ($s_{50\%}$) of the fractions which correlates with the increases in ionic strength and then *p*H of the solutions required for their elution. It should also be observed that some of the early fractions (fractions I through IV) show sedimentation coefficient distributions which are narrower than the original. Fraction V has a distribution which is very nearly the same as the original and the remaining fractions (VI through VIII) are fractions in which high sedimentation coefficients preponderate. Thus, in the case of fraction VIII, (which represents about 8% of the

(20) J. A. V. Butler, D. M. Phillips and K. V. Shooter, *Arch. Biochem. Biophys.*, **71**, 423 (1957).

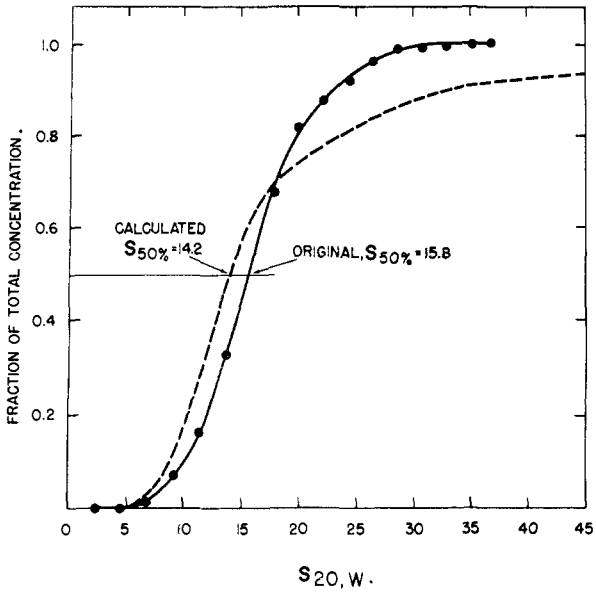

Fig. 3.—Comparison of sedimentation coefficient distribution curves of calf thymus DNA with curve calculated by combining the sedimentation data of eight individual fractions.

original DNA) 50% of the material has sedimentation coefficients larger than 24 *S*.

To determine whether the sedimentation behavior of fractions VI through VIII (*i.e.*, those with high $s_{50\%}$ and wide distribution of sedimentation coefficients) might have resulted from exposure to ammonia and 2 *M* NaCl, the effect of such conditions was studied. It was found that a five-day exposure of unfractionated DNA and also of an early DNA fraction (fraction II) to 1 *M* NH_3 and 2 *M* NaCl did not affect significantly the sedimentation distribution curves of these materials; these conditions were more severe than any to which the DNA was subjected during the course of the chromatography.

It would appear from these data that the DNA is a mixture of molecules which vary in sedimentation coefficient (see also ref. 12, 13, 20) and that the chromatography discriminates among these molecules on the basis of properties related to their sedimentation coefficients. The chromatographic procedures can thus be used to prepare fractions of DNA of graded sedimentation coefficients.

There are some serious questions which arise in connection with the chromatography, the most important being whether these fractions were present as such in the original unfractionated DNA or whether the chromatography had led to their formation (*i.e.*, are the fractions artifacts?). Evidence already has been obtained[8,9] that no significant alteration of the DNA had been produced by the chromatography when this procedure was applied to DNA active in pneumococcal transformation. All of the transforming activity which had been applied to ECTEOLA columns could be recovered in the chromatographic fractions.

The present data can be used to decide whether any alterations (due to the chromatography) had taken place as measured by the ultracentrifugal technique. This was done by calculating the sedimentation distribution curve that would have been obtained if the fractions examined were mixed together in the proportion of their occurrence in the original DNA and by comparing such a reconstructed curve with that of the unfractionated DNA. (The validity of such a calculation has been demonstrated experimentally.[21]) Such a comparison is given in Fig. 3. The average sedimentation coefficients ($s_{50\%}$) agree quite closely (14.2 *S* *vs.* 15.8 *S*). An examination of these curves shows that the calculated curve is consistent with the original within the experimental error. For technical reasons the eight fractions selected account for only 56.5% of the total material recovered and therefore do not constitute a random sample in the strict sense. The non-randomness of the sampling has resulted in a selection of fractions with sedimentation coefficients which are either considerably higher or considerably lower than the original. The largest errors in determining the distribution curves are in estimating the terminal (or "tail") regions and therefore errors in these regions would be magnified when the data for the eight curves are combined.

It would appear, at first glance, that some of the fractions (VI through VIII) contain material in the high sedimentation coefficient range (>40 *S*) not present in the original. The total amount of DNA with values of *S* in that range is so small that it could not have been detected in the original, unfractionated DNA.

It is concluded that the chromatographic fractions preexisted in the original DNA. In addition to the evidence mentioned above, further support for this conclusion comes from the behavior which the fractions show on alkalization to *p*H 13.[22] The hyperchromic effect at 260 mμ produced by this treatment was that expected of undenatured DNA (unpublished results).

The fractions studied here have been found to differ among themselves in their ability to inhibit the transforming activity of pneumococcal DNA.[9] They also differ[23] in relative abilities to serve as primers in the enzymatic synthesis of DNA.[24]

Similar fractions separated by this technique from pneumococcal DNA showed qualitative as well as quantitative differences in their transforming activity.[4,8,9] These different biological activities are thus presumably associated with molecules of different physical and chemical properties.

The authors take pleasure in acknowledging the interest and guidance of Drs. Marion Barclay, Morton Rosoff, Howard K. Schachman and G. B. Brown.

NEW YORK 21, NEW YORK

(21) H. S. Rosenkranz and A. Bendich, THIS JOURNAL, in press.

(22) R. D. Hotchkiss, in "Methods in Enzymology," Vol. III, S. P. Colowick and N. O. Kaplan, editors, Academic Press, Inc., New York, N. Y., 1957, p. 708.

(23) Unpublished results with I. R. Lehman and A. Kornberg.

(24) J. Adler, M. J. Bessman, I. R. Lehman, H. K. Schachman, E. S. Simms and A. Kornberg, *Federation Proc.*, **17**, 178 (1958).