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

Fractionation of Deoxyribonucleic Acids on Columns of Anion Exchangers; Methodology[1]

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A number of anion exchangers have been studied for their suitability in the chromatographic fractionation of DNA. The substituted cellulose ECTEOLA was found to be the most useful and a reproducible column procedure has been developed which effects extensive fractionation of the DNA from a number of diverse sources. The procedure affords a quantitative recovery of the DNA in the form of numerous fractions. The fractions have been found to differ in base composition. Other aspects of the basis of the fractionation are discussed. A series of preparations of ECTEOLA have been studied and these have been found to differ not only with respect to adsorptive capacity for DNA and smaller anions but also in the chromatographic profiles obtained with DNA. A measure of the resolving power of the exchanger is seen in the separation of DNA from polyadenylic acid and in the fractionation of DNA containing 5-bromouracil from that which does not.

A number of techniques have been developed which effect partial separation of the polynucleotide components of the deoxyribonucleic acids (DNA) isolated from single sources. The techniques employed have included differential centrifugation (or solubility),[4-6] fractional dissociation of nucleoprotein,[7-9] differential solution in alkali[10] and chromatography on columns of histone,[11,12] anion exchangers[13,14] and calcium phosphate gels[15]; see also ref. 16.

Since these various methods (with the possible exception of the alkali treatment)[10] are not expected to lead to the cleavage of covalent linkages in DNA, it has been concluded that the total DNA of single sources is actually a mixture of different molecular species. Discussions of the heterogeneous nature of DNA and its biological significance have appeared.[17-20]

DNA carries a net negative charge at a wide range of *p*H. This circumstance, coupled with its macromolecular and heterogeneous character, suggested that DNA might be treated as a mixture of polyanions. Accordingly, the suitability of anion-exchange chromatography was investigated as an approach to the separation of the component polynucleotides.

Several anion exchangers were studied. The strong-base resin Dowex-1,[21] which has proved to be so useful in the fractionation of simpler nucleotides,[22-24] showed a low capacity for DNA. Furthermore, that small portion which did adsorb to the resin was found to be too tightly bound for useful chromatography. This also has been the experience of other investigators,[25] although Kawade and Watanabe have reported partial success with this exchanger.[26] More promising results were obtained with the weak-base resin Amberlite IR-4B.[27] By eluting with salt solutions of increasing ionic strength, much of the adsorbed DNA could be recovered from this exchanger as numerous (16 to 200) "peaks."[28] Further use of the Amberlite was abandoned, however, because of the lack of reproducibility and the observed shedding into the eluates of large amounts of ultraviolet-absorbing resin impurities.

The most encouraging results were obtained with certain nitrogen-containing cellulose derivatives developed by Peterson and Sober for protein and peptide separations.[29-32] One of these exchangers, DEAE-cellulose, prepared[30] by reaction of alkaline cellulose with β-chloroethyldiethylamine to yield diethylaminoethyl-cellulose has proved valuable for the fractionation of proteins and peptides (*loc. cit.*) but not for nucleic acids. Although not

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particularly useful thus far for proteins, another exchanger, ECTEOLA-cellulose (prepared by the interaction of alkaline cellulose with epichlorohydrin and triethanolamine),[30,33] has shown considerable promise in the chromatography of DNA,[14,19,20,34,35] ribonucleic acids (RNA),[14,36] tobacco mosaic virus[37] and in the purification of bacteriophages.[38,39] Aside from the fact that the two exchangers differ in basic strength (pK_a) in 0.5 *M* NaCl DEAE = 9.5; ECTEOLA = 7.5),[30] ECTEOLA probably contains cross-linkages involving nitrogen whereas DEAE undoubtedly does not. These factors probably contribute to the difference in the relative utility of these materials in the chromatography of proteins and nucleic acids.

This report deals with the suitability of ECTEOLA for the chromatography of DNA, details of the experimental techniques which have been developed and a discussion of some of the factors which appear to be related to the basis of the separations achieved. Other papers concerning the application of this procedure to various problems of biological interest appear elsewhere.[20,40]

Experimental and Results

Nucleic Acids.—The calf thymus DNA (sodium salt) used in many of these experiments was prepared from fresh glands by the Schwander and Signer procedure.[41] Some of the properties of this DNA (designated as "S-II" in a previous publication[42]) have been reported.[42] The preparation had a weight average sedimentation coefficient ($S_{20,w}$) of 15.8 when determined as a 0.003% solution in 0.2 *M* NaCl.[43,44] Other nucleic acids or nucleotides used in these studies are described below.

Capacity and Affinity Studies. (a) **Ion Exchangers.**—The preparation of the substituted cellulose ion exchangers employed in these studies has been described in detail by Peterson and Sober.[30,45]

Among the materials studied were the cation-exchanger CM (prepared[30] from alkaline cellulose and monochloroacetic acid, hence containing *C*arboxy *M*ethyl groups) and a wood cellulose, Solka-Floc[46] (270 to 325 mesh). These cellulose preparations were characterized by their capacity for small ions as determined by potentiometric titration with standard hydrochloric acid at room temperature in 0.5 *M* NaCl. This property is expressed (Table I) in terms of milliequivalents of ionizing groups per g. of exchanger (*cf.* ref. 30). In many instances, the nitrogen contents also were determined. These exchangers could be reproduced with reasonably similar properties provided the published synthetic sequence[30] was closely adhered to (compare, for example, ECTEOLA SF-1 and -4), although variations in the time of contact or in the proportions of the reactants resulted in preparations with appreciably different characteristics.

TABLE I

PROPERTIES OF CELLULOSE ION-EXCHANGERS

Exchanger[a]	Nitrogen, %	Ionizing groups, meq./g.	Adsorption capacity, mg. DNA/g.
DEAE-PC-1[b]	0.14	0.1	2.0
DEAE-PC-2[b]	1.09	.8	11 to 13
DEAE-W[b]	..	.27	3.2
ECTEOLA-W-1[b]	0.29	.17	3.0
ECTEOLA-W-2	..	.3	7.2
ECTEOLA-SF-1	.51	.20	7.2
ECTEOLA-SF-2	.75	.27	10.0
ECTEOLA-SF-3	.26	.04	1.6
ECTEOLA-SF-4	.48	.21	8.4
ECTEOLA-SF-5	.47	.24	10.1
CM-W[b]	..	.5	0
(CELLULOSE-SF	..	..	0)

[a] PC and SF are wood celluloses; W is a cotton cellulose [See E. A. Peterson and H. A. Sober, THIS JOURNAL, **78** 751 (1956)]. [b] Specimens of these exchangers were kindly furnished by E. A. Peterson and H. A. Sober.

ECTEOLA-SF-1, -4 and -5 were made according to the 75–80° heating procedure[30]; the SF-5 differed only in that it was a larger batch (200 g.) than the first two (60, 100 g.). ECTEOLA-SF-2 was prepared without the initial heating, as described.[30] For ECTEOLA-SF-3, a solution of 3.5 ml. of triethanolamine and 60 ml. of epichlorohydrin in 30 ml. of dioxane was stirred into 60 g. of Solka-Floc which had been blended with a solution of 60 g. of NaOH in 150 ml. of water.[45] The heating and isolation procedure[30] was then followed closely.

b. Adsorption Capacities for DNA.—To determine the capacity of each adsorbent for DNA, both a column and a batch procedure were used. In the column procedure, a slurry of the cellulose (0.5 g.) in 0.01 *M* phosphate buffer, (*p*H 7.0) was poured onto a coarse porosity glass filter disc supported in a column (0.8 cm. inner diam.) and washed by gravity drip with 150 to 250 ml. of 0.5 *N* NaOH followed by about 300 ml. of the phosphate buffer. An excess of a solution of calf thymus DNA (usually 10.0 mg. dissolved in 200 ml. of the buffer) was passed through the column at room temperature at a rate of 3 ml. per hour. Hourly collections were made, and the column was considered to be saturated when the optical density of the effluent at 260 mμ (Beckman model DU spectrophotometer) approached very closely that of the original solution. The adsorptive capacity of the exchanger for DNA (mg. DNA/g. exchanger) was calculated from that amount of nucleic acid removed from solution under these circumstances.

Neither cellulose itself nor the cation-exchanger CM exhibited any measurable affinity for DNA (Table I). On the other hand, the anion-exchangers DEAE and ECTEOLA (see ref. 33) showed adsorption capacities from approximately 1 to 13 mg. of DNA per g. These capacities varied with the nitrogen content and with the number of acid-titratable groups (Table I), although no simple relationship was discerned. The biological source of the cellulose [cotton (W) or wood (SF)] also influenced the capacity of these exchangers for DNA (as well as for proteins[30]).

For the determination of adsorption capacity by the batch procedure, the DNA (*ca.* 2 mg. in 40 ml.) was stirred continuously for 0.5 hr. with 100 mg. of the exchanger. The exchanger was removed by centrifugation and the amount of DNA left in the supernatant then determined by ultraviolet spectrophotometry. The adsorption capacities determined by the two procedures agreed to within 5 to 15%. A longer contact time in the batch procedure (one to seven days of constant mixing) resulted in about a 15% increase in adsorption. The capacity of an exchanger was only slightly affected by temperature. At 3°, 9% less DNA was adsorbed than at 28°, but when the suspension

(33) For brevity, this anion exchanger will be referred to here simply as ECTEOLA. This designation is derived[30] from a contraction of the words *e*pichlorohydrin and *t*ri*e*thano*l*a*m*ine.

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(44) K. V. Shooter and J. A. V. Butler, *Trans. Faraday Soc.*, **52**, 734 (1956).

(45) We are indebted to Drs. Peterson and Sober, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, for generous gifts of many of their exchangers, for instructions for their preparation and for valuable discussions.

(46) We are grateful to the Brown Company, Berlin, New Hampshire, for a gift of this cellulose (SF).

of ECTEOLA (SF-1) which had been saturated with DNA at 3° was warmed to 28°, the additional 9% was readsorbed. However, the exchanger, saturated at 28°, did not release any DNA when the temperature was lowered to 3°.

No detectable amounts of DNA were adsorbed from solution (room temperature) by ECTEOLA-SF-1 in the presence of 0.5 *M* NaCl (buffered in 0.01 *M* phosphate at *p*H 5.2, 6.2, 7.0 or 8.1) even after five days of contact. When, after equilibration of the ECTEOLA with 0.5 *M* NaCl, the saline was replaced with 0.01 *M* phosphate buffer, *p*H 7.0, the exchanger again exhibited its usual affinity for DNA.

The capacity of these exchangers for DNA was found to be considerably lower than that reported for a number of proteins. A preparation of DEAE which contained about 0.8 meq. of basic groups per gram was found to adsorb, by weight, approximately 30 times more crystalline bovine plasma albumin[30] than it did DNA. However, the specific adsorption of the protein by a preparation of ECTEOLA (*ca.* 0.3 meq./g.) was only about five times larger than for DNA. The very large differences in the molecular weights between this protein and DNA may serve to explain, in part, these differences in their relative adsorption to the two exchangers. When calf thymus DNA[41] (1 mg./ml.) was heated at 100° for 1 hour in water and the solution adjusted to 0.01 *M* phosphate, *p*H 7.0, the capacity of ECTEOLA SF-1 for the modified DNA was increased by about 100%. Such heat treatment is known to decrease the molecular weight of DNA considerably.[4]

Since the preparation of ECTEOLA involves the interaction of cellulose with the bifunctional epichlorohydrin and the polyfunctional triethanolamine, its preparation undoubtedly results in the formation of cross-linkages which are not possible in DEAE. A decrease in the degree of cross-linking of anion-exchangers results in a decrease in ion selectivity,[47] and, in accord with this observation, we have found that ECTEOLA shows a much higher resolving ability than does DEAE for the component polynucleotides of DNA. This resolving power was found to vary according to the properties (Table I) of the given preparation of ECTEOLA. Those with a low adsorption capacity for DNA (for example, ECTEOLA-SF-3) resolved the DNA polynucleotides less than did ECTEOLA-SF-1 and -4. Of the exchangers studied, ECTEOLA-SF-1 (capacity 7.2 mg. DNA/g.) appeared to possess the most desirable properties in that a greater difference in chromatographic profiles could be obtained with the different specimens of DNA examined (see below).

c. **Affinity of ECTEOLA for Monodeoxyribonucleotides.**—A solution of 0.50 mg. of ammonium deoxyadenylate, 0.45 mg. of calcium thymidylate, 0.48 mg. of deoxycytidylic acid and 0.67 mg. of magnesium deoxyguanylate[48] in 100 ml. of distilled water was passed through a column containing 0.5 g. of ECTEOLA-SF-1 (5 × 0.8 cm.) at a rate of approximately 2 ml. per hour. The optical density (at 260 mμ) of the effluents, collected at two-hour intervals, remained at about one-sixth the value of the original mixture until it all had passed through the column. The column was then washed with 0.01 *M* phosphate (*p*H 7.0; rate, 5 ml. per hour) and the remaining nucleotides were found to be quantitatively removed after 40 ml. of solution had been collected. This result indicates that ECTEOLA possesses a low affinity for monodeoxyribonucleotides and that the bonds between them and the exchanger can be completely broken by 0.01 *M* phosphate, *p*H 7.0.[49]

d. **Affinity of ECTEOLA for Adenosine Triphosphate (ATP).**[50]—A freshly prepared solution of crystalline disodium adenosine triphosphate[51] (3.0 mg. in 3.0 ml. of water) was passed slowly through a column (5.9 × 0.8 cm.) containing 0.5 g. of ECTEOLA-SF-1. The column was washed with 0.01 *M* phosphate buffer, *p*H 7.0, at a rate of about 1 to 4 ml. per hour. ATP first appeared in the effluent after 34 ml. had been collected. The peak concentration was not obtained until a further 24 ml. had passed through. Quantitative recovery of the ATP required the passage of a total of 130 ml. of the buffer.

e. **Affinity of ECTEOLA for Oligonucleotides.**—The calf thymus DNA was digested[52,53] with crystalline pancreatic deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.). A portion of the digest (corresponding to 3.6 mg. of the original DNA) was passed through a column of ECTEOLA-SF-1 (0.5 g., 4.5 × 0.8 cm.; rate, 3 ml. per hour) with quantitative retention of ultraviolet absorbing (260 mμ) material. The column was washed with 0.01 *M* phosphate buffer, *p*H 7.0, at a rate of 3 to 7 ml. per hour. Two-hour collections were examined in the Beckman Spectrophotometer at selected wave lengths in the ultraviolet. The elution pattern has been reproduced.[20] A sharp peak was obtained at a retardation volume of about 43 ml. About 25% of the original ultraviolet-absorbing material was recovered in the phosphate wash. A sodium chloride gradient was established using a two-mixing chamber system (see below) and the digest remaining on the column was eluted when a concentration of 0.19 to 0.20 *M* NaCl was reached. There was a 97% recovery, the main peak occurring in the region of 0.11 *M* NaCl.

Enzymatic digests of this type have been found[54] to consist of 17% of mono- and dinucleotides with appreciable amounts of oligonucleotides of the order of hepta- or octadeoxyribonucleotides. Although the nucleotides which were obtained in the two main fractions were not identified, it may be inferred from the previous experiments described above that the 0.01 *M* phosphate fractions consisted of material smaller in size than octanucleotides.

The experiment was repeated using ECTEOLA-SF-2 which has about a 30% greater affinity for DNA than does ECTEOLA-SF-1 (Table I). The elution diagram is given in Fig. 1. A peak which contained about 32% of the ultraviolet-absorbing (260 mμ) material present in the original digest was obtained after 83 ml. of the phosphate solution had passed through. This was followed by an additional 21% in a second peak which required 340 ml. more of the wash fluid. With the exception of perhaps 2%, the remaining digest was eluted from the column after a NaCl concentration of 0.19 *M* had been attained by a two-mixing chamber gradient system. Although the resolving power of ECTEOLA-SF-2 for DNA was less than that of the SF-1 preparation, the exchanger with the higher capacity appeared to be more useful for the resolution of the smaller-size nucleotides present in the digest.

Column Chromatographic Studies. a. Preparation of Columns of ECTEOLA.—A coarse-porosity sintered Pyrex funnel (0.8 cm. i.d. × 12 cm. height) was sealed to the bottom end of a 2 × 10 cm. tube fitted with a standard taper female joint at the upper end. A slurry of 0.5 g. of ECTEOLA in about 20 ml. of 0.01 *M* phosphate, *p*H 7.0, was poured into the column and allowed to settle under gravity. A glass tip was attached to the bottom of the column by means of a piece of washed gum rubber tubing and a thumb-

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(48) These nucleotides were obtained from the California Foundation for Biochemical Research, Los Angeles, Calif. The first three nucleotides were found to be homogeneous by paper chromatography using solvent systems containing either sodium dihydrogen phosphate or sodium citrate in aqueous isoamyl alcohol [C. E. Carter, THIS JOURNAL, **72**, 1466 (1950)]. The magnesium deoxyguanylate chromatograms, however, showed an ultraviolet-absorbing "spot" in addition to the one expected.

(49) When a solution of mixed monodeoxyribonucleotides or the *dialyzable* fraction of a deoxyribonuclease digest of DNA is kept in contact with a *suspension* of ECTEOLA-SF-1 for a period of 2 to 7 days under sterile conditions, about 20 to 60% of the nucleotide material is adsorbed by the exchanger. The character of the material adsorbed to the exchanger has been so altered that strong solutions of NaCl are required for partial desorption. (Some of the adsorbed material is so firmly held that 0.5 *N* NaOH is required for quantitative elution). It has been found that 10 to 60% of the desorbed material, depending on the experiment, was non-dialyzable against dilute solutions of NaCl. In view of the non-dialyzability and the fact that "polymeric" material formed by this treatment was rendered dialyzable by digestion with deoxyribonuclease, a polymerization apparently had occurred. This was not found to occur when a digest was applied to a *column* of ECTEOLA-SF-1 and chromatographed in the usual manner (H. S. Rosenkranz and A. Bendich, unpublished experiments).

(50) This experiment was performed by Miss Barbara Huberman.

(51) This preparation, obtained from Schwarz Laboratories, Inc., Mt. Vernon, N. Y., was found to be homogeneous by paper chromatography in ammonium sulfate–isopropyl alcohol and 1-propanol systems (circular OR-10, Pabst Laboratories, Division of Pabst Brewing Co., Milwaukee, Wis., Jan., 1956).

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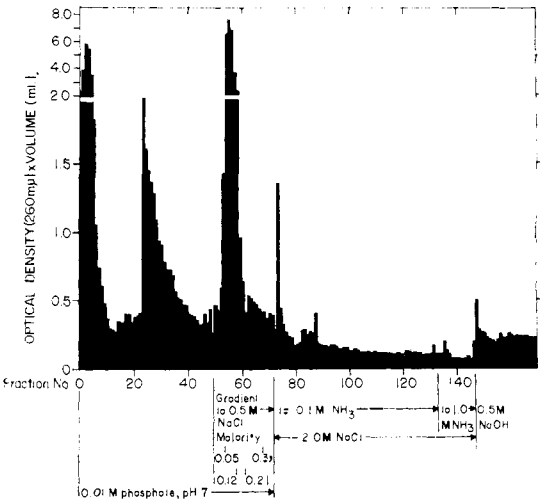

Fig. 1.—Chromatography of an oligonucleotide mixture from a deoxyribonuclease digest of 3.0 mg. of calf thymus DNA on a column of ECTEOLA-SF-2 (4.2 × 0.8 cm.; 0.5 g.; flow rate, about 6 ml./hr.) using a gradient elution system with two mixing chambers. Collections were made at two-hour intervals. For properties of ECTEOLA-SF-2, see Table I.

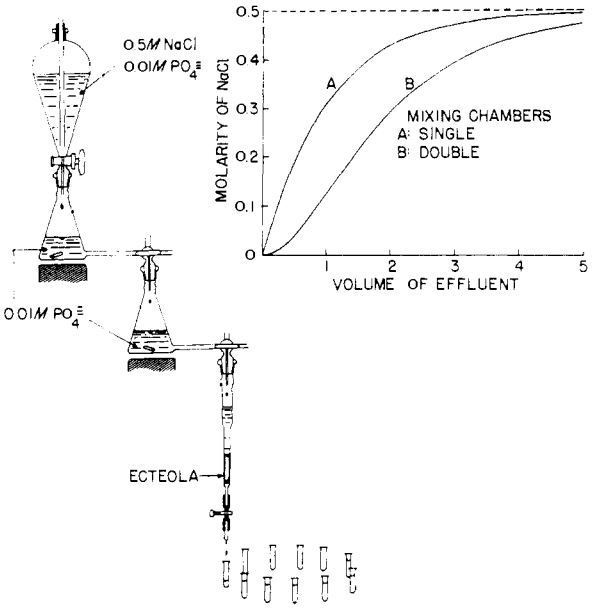

Fig. 2.—Diagram of the apparatus used for gradient elution with two mixing chambers. The inset shows the change of salt concentration as a function of the volume of effluent.

screw-clamp was used to permit adjustment of flow rate. A standard taper dropping-funnel (one liter) containing 0.5 *N* NaOH was connected to the top of the column and the exchanger washed (about 5 to 10 ml. per hour) until the effluent showed a negligible optical density (*less than 0.015* at 260 mμ). Excessive washing did not appear to be injurious to the exchanger, and it was found desirable to continue the alkali washing for 3 to 4 days. The excess supernatant wash was followed by washing with 0.01 *M* phosphate, *p*H 7.0, at a similar rate for 3 to 4 days. The *p*H and optical density (base-line usually below 0.015 at 260 mμ) of the effluents were checked. About 5 ml. of the buffer was left on top of the exchanger. With the exception of the attachment of the drip-tip, all connections were glass-to-glass. A diagram of the apparatus is shown in Fig. 2.

In a typical experiment, 1 to 3 ml. of a solution of DNA (1 mg. per ml. in either 0.01 *M* phosphate buffer, *p*H 7.0, or in 0.001 *M* NaCl) was pipetted slowly into the fluid above the freshly-washed exchanger. The solution was allowed to percolate by gravity through the exchanger at a rate not exceeding 2 to 4 ml. per hour. The effluent was collected in test-tubes on a fraction collector. When all but about 0.5 ml. had passed through the column, the walls were washed down with a few ml. of buffer and the wash fluid permitted to pass through until about 0.5 ml. remained. A dropping funnel containing several hundred ml. of the phosphate buffer was then attached and the ECTEOLA-DNA washed (3 ml./hr.) until the base-line optical density was reached (usually overnight). Excessive washing (up to one week) failed to dislodge detectable amounts of DNA. The column was then ready for elution of the DNA.

With the exception of the chromatography of pneumococcal transforming DNA which was carried out at 0 to 3°, all other experiments were performed at room temperature. To prevent microbial growth during the experiments, all neutral and ammoniacal solutions were saturated with reagent-grade chloroform. All eluents excepting 0.5 *N* NaOH contained 0.01 *M* phosphate.

b. **Discontinuous Elution.**—Solutions of graded concentrations of NaCl were passed through (*ca.* 5 to 6 ml./hr.) until a base-line or a low constant optical density (260 mμ) was reached. The NaCl concentration was increased stepwise from 0.05, to 0.1, 0.2, etc., until 2.0 *M* was reached. The ultraviolet absorption at 240, 250, 260, 280 and 290 mμ of each column collection was determined. With calf thymus DNA[41] and ECTEOLA-W-2, increases up to 0.6 *M* NaCl were required to elute 65% of the DNA[14] but an increase to 2.0 *M* NaCl did not improve the recovery of DNA. It was necessary to adjust the *p*H of solutions of 0.5 to 2.0 *M* NaCl to above 8 before additional DNA was removed; a further 25% required a *p*H of 8.9. The remaining 15% was eluted with 0.5 *M* NaOH. A chromatographic profile, previously described,[14] showed two large fractions between 0.2 and 0.4 *M* NaCl which amounted to 50% of the applied DNA. These two fractions were not resolved, however, when a single-mixing chamber gradient elution system was employed.

c. **Gradient Elution: Single Mixing Chamber.**—A gradient elution system was established by having a stock of 0.5 *M* NaCl–phosphate solution (contained in a standard-taper dropping funnel reservoir) drip into a 150-ml. erlenmeyer mixing chamber containing either 100 or 125 ml. of 0.01 *M* phosphate, *p*H 7.0, and a Teflon-sealed stirring bar-magnet which was continuously rotated by an external magnetic stirrer. The mixing chamber was connected to the column *via* a standard-taper joint. The NaCl concentration gradient follows the exponential course shown in Fig. 2 (Curve A) in which the units of volume plotted on the abscissa are equal to the volume in the mixing chamber. The chromatographic pattern obtained with 1.0 mg. of calf thymus DNA revealed only a single asymmetric peak in the 0 to 0.5 *M* NaCl region which corresponded to about 40% of the applied DNA whereas several peaks were obtained in this region with the discontinuous elution technique described in the previous section. (Those peaks were also not resolved when a linear salt gradient was employed.)

d. **Gradient Elution: Two Mixing Chambers.**—Since the use of salt gradients which increased exponentially or linearly failed to give the separate peaks in the 0.2 to 0.4 *M* salt range which were obtained with the discontinuous system,[14] a gradient was established in which this range of concentration was achieved more gradually. This was arranged by interposing a second mixing chamber containing a volume of diluent phosphate equal to that in the first. A diagram of the apparatus is given in Fig. 2. Curve B shows the gradient which is achieved with this system when the NaCl concentration of the stock solution in the dropping funnel is 0.5 *M*. Thus, when a volume of eluting fluid equal to that (for example, 100 ml.) in each mixing chamber has passed through the apparatus, a concentration of NaCl of 0.13 *M* will have been reached, and after a second volume the concentration attained is 0.30 *M*, etc. (With a single mixing chamber, the NaCl concentration is already 0.32 *M* after the passage of one volume.) For any small increment of eluent, after the passage of a measured volume of effluent, the NaCl molarity can be read either directly from the s-shaped curve or calculated from the following relationship which describes the s-shaped curve

$$[NaCl]_{effluent} = [NaCl]_{stock} - [NaCl]_{stock}\left(\frac{V+1}{e^V}\right)$$

where

$$V = \frac{\text{cumulative vol. passed through}}{\text{vol. in mixing chamber}}$$

The validity of these methods for determining salt concentration has been confirmed by direct analysis of chloride content in the effluent by Mohr titration.[55] The salt concentration–volume relationship is strictly valid only if the original volumes in the two mixing chambers remain constant throughout the procedure. Volume changes can be avoided by the use of sufficiently long (about 4 to 5 cm.) heavy wall glass capillary tubing (2 mm. i.d.) both in the drip tips leading from the stopcock connections between the mixing chambers and in the dropping funnel.[56]

Ordinarily, 125 ml. of 0.01 *M* phosphate was placed in each of the two 150-ml. mixing chambers and about 700 ml. of the phosphate–0.5 *M* NaCl in the upper funnel. A flow rate of between 2 and 5 ml. per hour was established by adjusting the screw clamp. On occasion, a heavy-wall tube of 2 to 3 mm. i.d. and 2 feet in height was placed between the lower mixing chamber and the column if additional hydrostatic pressure was required in order to maintain a desired flow rate. To maintain a constant hydrostatic head, a Mariotte tube was inserted into the dropping funnel (see Fig. 2) through a tight-fitting rubber stopper. The flow rate was checked frequently and adjusted as needed.

Collections were made at two-hour intervals in test-tubes of equal diameter in an automatic fraction collector[57] capable of holding fifty tubes in each of four concentric rows so that four separate columns could be accommodated concurrently. Occasionally, a photoelectric-activated constant volume (8 ml.) collector[58] also was used. To expedite the transfer and optical density reading of the tube contents in the Beckman spectrophotometer, a Gilson-Transferator[58] was employed. Optical densities were measured at 240, 250, 260, 280 and 290 mμ. Effluent volumes were determined by visual comparison with a calibrated tube of diameter equal to that of the collection tubes. The flow rates were reasonably constant except in the region 0.18 to 0.28 *M* NaCl when a considerable slowing-down occurred due to expansion of the column matrix and adjustment with the screw-clamp was found to be necessary.

When the limit concentration of 0.5 *M* NaCl was nearly attained (0.48 *M*), the eluting solution was changed. It had been found, with ECTEOLA-SF-1 and *calf thymus DNA*, that very little additional DNA could be eluted if the salt concentration were raised from 0.5 to 2.0 *M* NaCl or if only 0.1 *M* NH_3 or 1.0 *M* NH_3 were used after a NaCl concentration of 0.5 *M* had been reached. However, a large fraction of the calf thymus DNA was obtained if *both* the salt concentration and the *p*H were raised simultaneously. An analogous situation was described in the chromatography of cytochrome *c* on the cation exchanger IRC-50 in that the elution of this protein depended upon changes in the ionic strength as well as the *p*H.[59]

Accordingly, the apparatus was dismantled with about 10 ml. of fluid left on top of the exchanger in the column. A dropping funnel which contained 2.0 *M* NaCl/0.1 *M* NH_3 was attached, and the elution continued until a base-line optical density at 260 mμ (not greater than 0.025) was reached. A continuous gradient elution schedule was again set up with 2.0 *M* NaCl/1.0 *M* NH_3 in the dropping funnel and 125 ml. of 2.0 *M* NaCl/0.1 *M* NH_3 in each of the two mixing chambers, with the flow rate the same as before. Aqueous ammonia was chosen to increase *p*H (to about 11.0) since it could be removed easily from the fractions by evacuation if required. In addition to the usual optical density and volume measurements, the *p*H also was determined. A base-line optical density (at 260 mμ) of 0.015 to 0.020 was usually attained after the passage of about 800 ml. The column was then washed with 0.5 *N* NaOH. In all instances in which ECTEOLA-SF-1 was employed, quantitative recoveries (±5%) of nucleic acid were obtained. After base-line was reached and the regenerated exchanger re-equilibrated with phosphate, it was stored in the refrigerator and could be reused several times.

Alterations in the elution schedule described above were occasionally introduced for the study of nucleic acids from other sources. Because the presence of phosphate buffer precluded the direct estimation of DNA-phosphorus, other buffer systems were employed both in this (see below) and another study.[60]

The two-mixing chamber gradient system was adopted as a routine procedure for the comparison of the chromatographic profiles of small samples of DNA from different sources[20] and in studies concerned with the effect on DNA of a particular procedure (*i.e.*, partial hepatectomy, irradiation)[20] or inhibitor (for example, 5-bromouracil).[61] Other investigators also have found gradient elution (in contrast to discontinuous elution) to be particularly suitable for the analysis of small quantities of material.[62]

e. Character and Reproducibility of Chromatographic Patterns.—The chromatographic profile of Schwander–Signer calf thymus DNA using the two mixing chambers and ECTEOLA-W-2 has been presented in a preliminary communication[14]; its profile on a column of ECTEOLA-SF-1 is reproduced in Fig. 3. Other profiles with ECTEOLA-SF-1 have been presented elsewhere.[20]

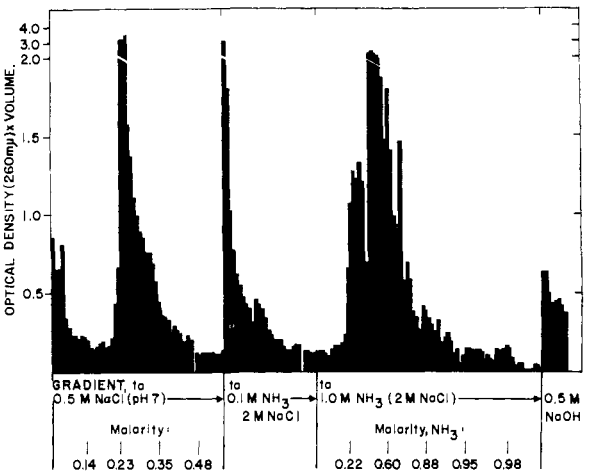

Fig. 3.—Chromatography of 3 mg. of calf thymus DNA on a column of ECTEOLA-SF-1 (6.7 × 0.8 cm.; 0.5 g.; flow rate, about 6 ml./hr.) using a gradient elution system with two mixing chambers. Collections were made at two-hour intervals.

As another example, the chromatographic pattern of the DNA from the white cells of an untreated patient with chronic granulocytic leukemia is shown in Fig. 4.[63] The DNA was prepared by the Dounce detergent procedure[64] modified in that salt-free solvents were avoided. The highly polymeric preparation showed $\epsilon(P)^{18} = 6{,}500$ and contained H_2O = 19.9, N = 12.2, P = 7.10 and Na = 5.69% (atomic N/P = 3.79). Paper chromatographic

(55) I. M. Kolthoff and E. B. Sandell, "Textbook of Quantitative Inorganic Analysis," The Macmillan Co., New York, N. Y., 1943, p. 509.

(56) An apparatus properly designed for this purpose is available from Kopp Laboratory Supplies, Inc., 1680 Second Ave., New York, N. Y.

(57) A Technicon time-operated collection, manufactured by the Technicon Chromatography Co., Chauncey, N. Y., was modified by removal of the automatic stops.

(58) Manufactured by Gilson Medical Electronics, Madison, Wisconsin.

(59) N. K. Boardman and S. M. Partridge, *Nature*, **171**, 208 (1953).

(60) L. Astrachan, E. Volkin and M. H. Jones, THIS JOURNAL, **79**, 130 (1957).

(61) A. Bendich, H. B. Pahl and G. B. Brown, in "The Chemical Basis of Heredity," McCollum–Pratt Institute Symposium (W. D. McElroy and B. Glass, eds.), Johns Hopkins Press, Baltimore, Md., 1957, p. 378.

(62) R. S. Alm, R. S. P. Williams and A. Tiselius, *Acta Chem. Scand.*, **6**, 826 (1952).

(63) We are indebted to Dr. G. di Mayorca for a gift of this specimen and for carrying out the chromatographic analysis.

(64) E. R. M. Kay, N. S. Simmons and A. L. Dounce, THIS JOURNAL, **74**, 1724 (1952).

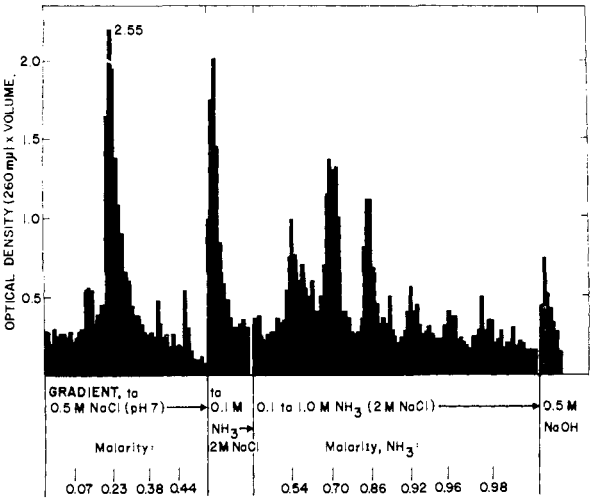

Fig. 4.—Chromatography on a column of ECTEOLA-SF-1 (6.2 × 0.8 cm.; 0.5 g.; flow rate about 5 ml./hr.) of 3 mg. of DNA isolated from human leucocytes. A gradient elution with two mixing chambers was used. Collections were made every 2 hours.

analysis[65] of base composition on formic acid hydrolysates gave adenine = 29.9, thymine = 28.7, guanine = 20.4 and cytosine = 21.0 moles per 100 moles of base; recovery = 94% based on phosphorus. A neutral solution ($O.D._{260}$ = 0.6) in 10^{-2} *M* NaCl showed a 38% increase in O.D. at 260 mμ on acidification to *p*H 3 and a 31% increase on alkalinization to *p*H 13 (see ref. 42 and 66). The weight average sedimentation coefficient ($S_{20,w}$)[43,44] of a 0.003 mg./ml. solution in 0.2 *M* NaCl was 19.3.

The extent to which the chromatographic profiles of DNA on columns of ECTEOLA can be reproduced is illustrated in Fig. 5 in which are presented independent chromatograms using DNA isolated[64] from the nuclei of rat liver

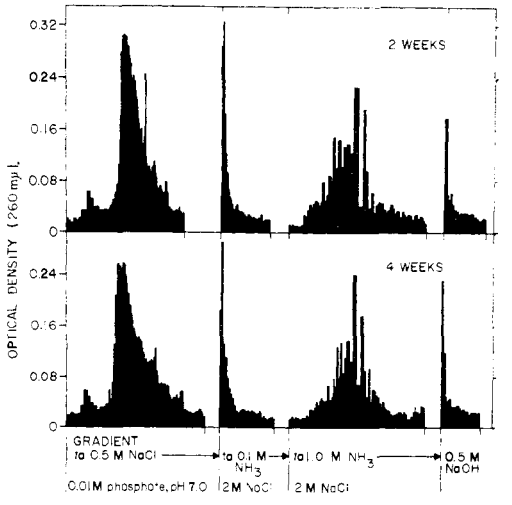

Fig. 5.—Chromatography of regenerating rat liver DNA, two and four weeks after partial hepatectomy. Each column contained 0.5 g. of ECTEOLA-SF-1 and 3 mg. of DNA. Both columns were run simultaneously. The flow rate was carefully maintained at 4 ml./hr., with collections made at two-hour intervals. A gradient elution system with two mixing chambers was used.

(65) A. Bendich, in "Methods in Enzymology," Vol. III, (S. P. Colowick and N. O. Kaplan, editors), Academic Press, New York, N. Y., 1957, p. 715.

(66) R. D. Hotchkiss, in ref. 65, p. 708.

2 and 4 weeks following partial hepatectomy.[67] In fact, most of these peaks are essentially superimposable. Such closely spaced, but separate, peaks are obtained provided that an elution rate of about 3 to 5 ml. per hour is not exceeded. At this rate, a complete chromatogram requires a total time of approximately 3 weeks. An increase in elution rate causes the individual peaks to smear with a concomitant decrease in resolving power. Since the method is capable of considerable reproducibility (for other examples of reproducibility, see Figs. 3 and 4 of ref. 20), the large differences seen in the profiles of DNA from different sources (such as, for example, from rat kidney and brain, or T6r and T6r+ bacteriophage)[20] are believed to reflect intrinsic differences in the DNA specimens and in the distribution of the component polynucleotides therein.

f. **Resolving Power.**—Experiment 1—A study was carried out on the ability of the chromatographic procedure to distinguish between normal *E. coli* DNA and that which contains 5-bromouracil in place of a portion of the thymine. A culture of a thymine-requiring mutant of *E. coli*, Strain I,[68] was grown in the presence of 5-bromouracil-2-C^{14} in a medium supplemented with thymine.[69] Simultaneously, a separate culture was grown under similar conditions except for the omission of 5-bromouracil. The harvested cells were mixed in the proportion of two parts of normal bacteria to one part of bromouracil-grown cells. The DNA was isolated[61] from the mixed cells by a modification[58] of a detergent method.[64] Three and nine-tenths mg. of the mixed DNA (containing a total of 252,000 c.p.m.) was applied to a 0.5 g. column of ECTEOLA-SF-1 (5.0 × 1.2 cm.) and the two-mixing chamber chromatographic technique employed. The chromatographic profile which was obtained is shown in Fig. 6 (stripped area) and the specific

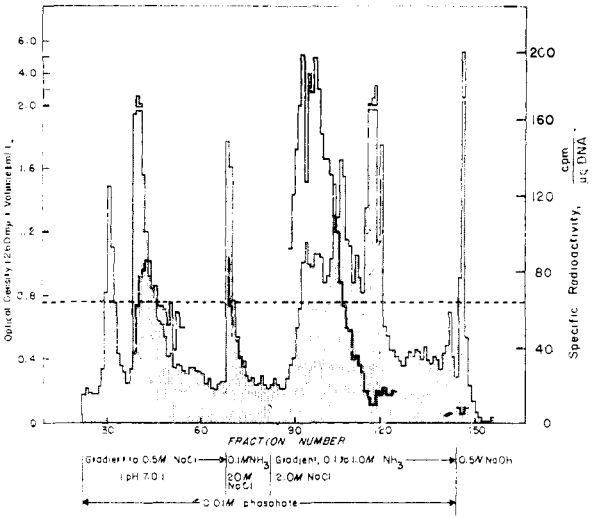

Fig. 6.—Chromatography of ECTEOLA-SF-1 (5.0 × 1.2 cm.; 0.5 g.; flow rate, about 7 ml./hr.) of 3.9 mg. of DNA isolated from an *E. coli* mixture composed of two parts of cells grown in the absence of 5-bromouracil-2-C^{14} and one part of cells grown in the presence of this pyrimidine. The stippled area represents the DNA content of a fraction (left-hand ordinate). The horizontal dashed line shows the specific radioactivity of the starting, unfractionated DNA. A gradient elution with two mixing chambers was used. Collections were made at two-hour intervals. See ref. 61.

(67) The effect of partial hepatectomy on the chromatographic patterns of the DNA of the regenerating liver is discussed elsewhere.[40]

(68) We are indebted to Dr. S. Zamenhof for this strain and for instructions for the isolation of DNA: see S. Zamenhof, B. Reiner, R. De Giovanni and K. Rich, *J. Biol. Chem.*, **219**, 165 (1956).

(69) The synthesis of the labeled bromouracil from uracil-2-C^{14} is from H. B. Pahl, M. Gordon and R. Ellison, submitted to *Arch. Bioch. Biophys.* Details of the growth of the bacteria and the isolation of the DNA therefrom have been given.[61]

TABLE II

BASE COMPOSITION OF COLUMN FRACTIONS OF CALF THYMUS DNA

Fraction no.	Fraction as % of original[a]	Composition of eluent, M NaCl	Composition of eluent, M NH_3	Molar base composition (Adenine = 1.00[b]) Thymine	Guanine	Cytosine	AD/THY	GU/CY	(AD + GU)/(THY + CY)	Recovery,[c] %
Original	100			0.97	0.77	0.77	1.03	1.00	1.02	95
50-1, 53-4	3.5	0.20–0.29		.91	.61	.81	1.10	0.75	0.94	91
55-7	3.4	.29– .31		.88	.64	.84	1.14	.76	.95	89
59-64	2.6	.34– .39		.94	.71	.82	1.06	.87	.97	90
146-161	3.2	2.0	(TRIS, *p*H 9.5)	.97	.73	.83	1.03	.89	.96	92
197	3.3	2.0	0.1	.88	.67	.74	1.14	.91	1.03	89
199-200	3.6	2.0	.1	.95	.64	.74	1.05	.87	0.96	98
206-211	3.1	2.0	.1	1.01	.75	.79	0.99	.95	.97	95
236-7	3.3	2.0	.25	0.98	.63	.74	1.02	.85	.95	100
239	5.4	2.0	.35	.95	.68	.72	1.05	.95	1.01	95
242	6.9	2.0	.45	.98	.78	.78	1.02	1.00	1.01	99
244	3.1	2.0	.53	.94	.86	.90	1.06	0.96	1.01	104

[a] These values represent the amount of DNA in each fraction as a percentage of the original, unfractionated DNA (65 mg.) which was applied to the column. The total fractions analyzed amounted to 41% of the original. [b] These values are the average of six determinations, except for the guanine of fraction number 146–161 which is the average of two analyses. Average deviation is less than 2%. [c] $\frac{\text{Total } \mu M \text{ of bases recovered}}{\text{Total } \mu M \text{ of P in hydrolysate}} \times 100$.

radioactivity (c.p.m. per μg. DNA) of many of the individual collections (after dialysis to remove salt) is shown by the heavy black line (right-hand ordinate, Fig. 6).[70] Had the chromatographic procedure failed to separate the DNA isolated from the mixed cultures, the specific radioactivity values would have fallen on the horizontal dashed line (*i.e.*, c.p.m. per μg. = 68, since this line represents the specific radioactivity of the original, unfractionated mixed DNA). Fifty-eight % of the individual collections were assayed for C^{14} content, and these accounted for 67% of the total radioactivity.

Some of the fractions (numbers 93 and 97) were three times as active as the unfractionated DNA; these fractions were undoubtedly derived exclusively from the DNA of those bacteria grown in the presence of the radioactive pyrimidine since their specific radioactivities were diluted threefold by the 2:1 admixture of bacterial cells. Other fractions (such as numbers 118 and 147) were almost devoid of radioactive 5-bromouracil and were probably derived from the DNA of the normal bacteria. The large peak encompassing fractions 118 to 124 was largely missing when the DNA from only bromouracil-containing cells was chromatographed in a separate experiment (*cf.* ref. 61). The results thus indicate that the chromatographic procedure is capable of resolving these related specimens of DNA.

Experiment 2.—A solution containing 2.0 mg. of calf thymus DNA and 0.5 mg. of polyadenylic acid[71,72] in 0.01 *M* phosphate buffer, *p*H 7.0, was applied to a column (5.2 × 0.8 cm.) of 0.5 g. of ECTEOLA-SF-1 and a chromatogram developed using the two-mixing chamber technique. The chromatographic profile in the 0 to 0.43 *M* NaCl range is shown in Fig. 7. The heavy black line represents the total optical density at 260 mμ. Each of the column collections was assayed for DNA by the Ceriotti method[73] and for $(AMP)_n$ by the orcinol method.[74] The first peak (maximum at 0.23 *M* NaCl) was composed almost exclusively of DNA and the second peak (maximum at 0.34 *M* NaCl) was largely $(AMP)_n$. In this experiment about 25% of the total DNA and over 80% of the total $(AMP)_n$ were recovered in the neutral NaCl region. The remaining 20% of the $(AMP)_n$ was spread out in such small quantities in the later chromatographic regions that accurate quantitative assessment of these quantities in the presence of the DNA fractions was difficult. The chromatography procedure thus permitted a relatively sharp separation of the bulk of the $(AMP)_n$ from the first DNA peak. The presence of the $(AMP)_n$ did not significantly affect the position or the contour of this DNA peak (compare with Fig. 3).

The chromatographic procedure has been found to have sufficient resolution to separate pneumococcal DNA and RNA into several fractions.[20] Additional facets of the resolving power of ECTEOLA columns are seen in the separation of pneumococcal transforming DNA (streptomycin resistance) into fractions possessing widely differing specific activities, the partial separation of streptomycin, penicillin and mannitol "markers"[75] of the transforming DNA of *pneumococcus*[20,76] and the differences in base composition of chromatographic fractions from calf thymus DNA (Table II).

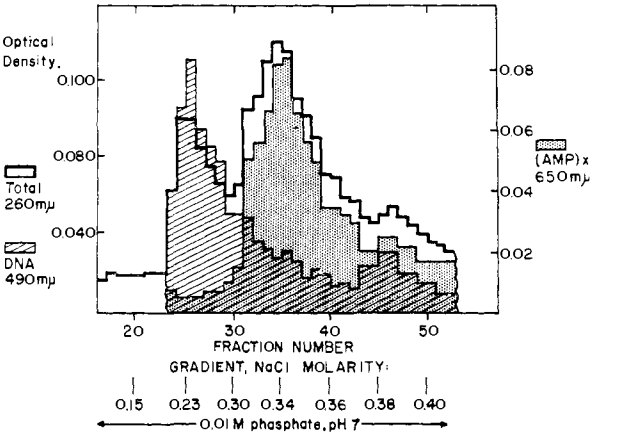

Fig. 7.—Chromatographic separation of ECTEOLA-SF-1 (5.2 × 0.8 cm.; 0.5 g.; flow rate, about 5 ml./hr.) of a solution containing 2 mg. of calf thymus DNA and 0.5 mg. of polyadenylic acid. Collections were made every two hours. A gradient elution system with two mixing chambers was used.

Properties of Column Fractions. a. Base Composition.—A column (7.4 × 2.8 cm.) of ECTEOLA-SF-1 was prepared in the usual manner, except that 0.01 *M* Tris [tris-(hydroxymethyl)-aminomethane] buffer, *p*H 6.8, was used throughout in place of the phosphate buffer since the phosphorus content of the fractions was to be determined. A solution of 65 mg. of calf thymus DNA in 70 ml. of the Tris buffer

(70) An internal Geiger–Müller flow counter (Radiation Counter Laboratories Mark 12, Model 1, helium-isobutane gas) was used.

(71) We are indebted to Prof. S. Ochoa for a gift of the enzymatically synthesized polyadenylic acid, $(AMP)_n$.

(72) This material had a molecular weight of from 80,000 to 140,000, R. C. Warner, in ref. 61, p. 562.

(73) G. Ceriotti, *J. Biol. Chem.*, **214**, 59 (1955).

(74) W. Mejbaum, *Z. physiol. Chem.*, **258**, 117 (1939).

(75) R. D. Hotchkiss and J. Marmur, *Proc. Nat. Acad. Sci.*, **40**, 55 (1954).

(76) H. B. Pahl, S. M. Beiser and A. Bendich, *Federation Proc.*, **16**, 130 (1957).

was placed on the column and a chromatogram developed using the double mixing chamber technique. Several individual (each 25 ml.) or pooled collections, each containing from 3 to 7 mg. of DNA which together amounted to 41% of the original sample, were selected for base analysis. The calculated composition of the solutions used to elute these fractions is given in Table II. The fractions were dialyzed (with less than 10% loss) against distilled water to remove salts and concentrated to small volume (*ca.* 2 ml.) *in vacuo* in a rotating evaporator. The chilled concentrates were adjusted to about 0.1 *M* NaCl by addition of solid NaCl and two volumes of ethanol added to precipitate the DNA. The fibrous precipitates were collected by centrifugation, washed with ethanol and ether and dried. They were hydrolyzed with 88% formic acid for base composition by paper chromatographic analysis[65] and phosphorus estimation.[77] The base analyses are listed in Table II. The average molar recovery based upon phosphorus was 95% (see ref. 18).

The molar base ratios (with adenine taken as 1.00), which are the average of six determinations, show a deviation of less than 2% from the average, and, taken together with the recovery of 95%, the variations in base composition are considered to be experimentally significant. It would appear that the column procedure has effected a separation, from the total sample, of fractions of DNA of varying composition. When the adenine to thymine and guanine to cytosine ratios are computed, significant departures from the widely-accepted (see ref. 18) value of unity are noted (Table II). This procedure would therefore appear to have a basis of separation that is different from those of other published methods[7-9,11,12] all of which yield fractions of progressively changing base composition which, however, still show very little departure from the unity ratio for the base pairs listed above.

(77) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

To ascertain whether the dialysis of the DNA fractions had altered their base compositions, the original DNA was exhaustively dialyzed against distilled water, precipitated and analyzed as above. No significant change was seen in the base composition.

A discussion of the possible significance of these data in terms of the Watson–Crick double helix formulation of DNA[78,79] has appeared.[20] A noteworthy feature of these data is the average ratio *total* purines to *total* pyrimidines, which, for these fractions, is 0.98 ± 0.03.

b. Other **Properties.**—Fractions obtained with eluents of increasing ionic strength and increasing *p*H have been found to show increasing sedimentation coefficients when examined (*cf.* ref. 44, 43) in the analytical ultracentrifuge equipped with ultraviolet optics. Upon alkalinization to *p*H *ca.* 13, such fractions have shown the hyperchromic effects at 260 mμ expected of "undenatured" DNA.[66] The results of these various studies as well as those dealing with the problem of rechromatography will be presented elsewhere. Preliminary accounts of these studies have appeared.[20,40,80,81]

Acknowledgments.—The authors take pleasure in acknowledging the advice and valuable help of Dr. Sam M. Beiser, Dr. George B. Brown, Dr. Giampiero di Mayorca and Dr. C. P. Rhoads.

(78) J. D. Watson and F. H. C. Crick, *Nature*, **171**, 737 (1953); *Cold Spring Harbor Symposia Quant. Biol.*, **18**, 123 (1953).

(79) F. H. C. Crick, in ref. 61, p. 532.

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

(81) H. S. Rosenkranz and A. Bendich, *Federation Proc.*, **17**, 299 (1958).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, THE UNIVERSITY OF CALIFORNIA, BERKELEY]

Inositol Phosphates: Pinitol 4-Phosphate and (−)-Inositol 3-Phosphate

BY GORDON L. KILGOUR AND CLINTON E. BALLOU

RECEIVED JANUARY 14, 1958

The synthesis of 3-O-methyl-(+)-inositol 4-phosphate and (−)-inositol 3-phosphate are described. These substances were prepared as model compounds for the study of the properties of inositol phosphates in general.

myo-Inositol occurs in nature both free and in conjugated forms. Of the latter, which may represent up to 90% of the total, the phosphate esters are a major component.[1] Prominent among these phosphate compounds are the hexaphosphoric acid ester (phytic acid) and the widely-distributed inositol-containing phospholipids, from which inositol mono- or diphosphates are obtained on hydrolysis.

myo-Inositol mono- or diphosphates have been isolated (in varying degrees of purity) from inositol phospholipids of brain, heart, liver, wheat germ and soya beans.[2] Others have been obtained from chemical and enzymatic hydrolysis of phytic acid.[3] One compound, believed to be *myo*-inositol 2-phosphate, has been synthesized by the phosphorylation of 1,3,4,5,6-penta-O-acetyl-*myo*-inositol.[4] It has since been found that this pentaacetate undergoes a base-catalyzed acetyl migration from the equatorial 1- or 3-position to the axial 2-position during methylation with methyl iodide and silver oxide.[5] In fact, Angyal, *et al.*, have made use of a similar migration to prepare 1,3-di-O-methyl-*myo*-inositol from the (±)-1,4,5,6-tetraacetate.[6] Since the phosphorylation step in the above synthesis[4] required a high temperature and was carried out in pyridine, there is a possibility that the product is actually the racemic (±)-1-phosphate.[7]

The migration of phosphate groups during the isolation of naturally-occurring inositol phosphates is very likely. The possibility of migration was mentioned by Malkin and Poole[8a] while Hawthorne and Chargaff[8b] discounted the possibility of migra-

(1) P. Fleury and P. Balatre, "Les Inositols," Masson et Cie., Paris, 1947.

(2) See review by J. Folch and L. N. LeBaron, *Can. J. Biochem. and Physiol.*, **34**, 305 (1956).

(3) J. Courtois, *Bull. soc. chim. biol.*, **33**, 1061 (1951); A. Desjobert, *ibid.*, **36**, 1293 (1954); M. H. McCormick and H. E. Carter, *Biochem. Preps.*, **2**, 65 (1952).

(4) B. M. Iselin, THIS JOURNAL, **71**, 3822 (1949).

(5) L. Anderson and A. M. Landel, *ibid.*, **76**, 6130 (1954).

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