semi-solid which on crystallization from pentane yielded 29 mg. (47%) of material with m.p. 145–150°. Two recrystallizations of this material from benzene–petroleum ether gave 7 mg. of fine needles, m.p. 158–159°, undepressed by admixture with an authentic specimen[3] of A-homo-3,4-seco-cholestane-3,4-diol (Xb).

CAMBRIDGE 39, MASS.

[CONTRIBUTION FROM THE BIOLOGICAL AND MEDICAL SCIENCES DIVISION, U. S. NAVAL RADIOLOGICAL DEFENSE LABORATORY]

A Modified Calcium Phosphate for Column Chromatography of Polynucleotides and Proteins[1]

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Specimens of calcium phosphate, prepared at different *p*H values and subsequently modified by heat or alkali, have been investigated as possible absorbents for the column chromatography of polynucleotides and proteins. One such specimen, precipitated at *p*H 6.7 and boiled with saturated calcium hydroxide has the desired characteristics of ease and reproducibility of preparation, high flow rate, stability, capacity and degree of resolution. It has been used to fractionate deoxyribonucleic acid and polyadenylic acid, to separate the latter from the former, and to separate albumin from the nucleic acid of a 10 to 1 mixture of the two by gradient elution with neutral phosphate buffers.

It is the purpose of this communication to describe the development, preparation, certain chemical and physical properties and chromatographic characteristics of a new calcium phosphate preparation suitable for column chromatographic separation of DNA[2] from certain other macromolecules such as proteins and lower molecular weight polynucleotides. Notable progress recently has been made by a number of workers in the development of adsorbents for column chromatography of proteins,[3–5] of DNA[6–12] and of RNA.[13] For the most part these methods were applied to the resolution of, and demonstration of heterogeneity in, a single previously purified chemical species, *i.e.*, protein, DNA or RNA. Previous reports from this Laboratory[14,15] indicated that DNA could be separated, in mixtures, from the products of minimal enzymatic digestion of DNA (polynucleotides) by elution with a phosphate buffer gradient from calcium phosphate columns, and that by a similar method it could be demonstrated that certain polynucleotides from DNA were liberated in mouse spleen tissue following whole body X-irradiation.[16]

In the present report a systematic study has been made of the effect of *p*H of precipitants, and of heating with alkalies, in the preparation of calcium phosphate for column chromatography. Variations in the resulting calcium phosphate preparations were followed by the criteria of (a) flow rates through standard packed columns, (b) X-ray diffraction powder patterns, (c) chemical analyses (molar calcium/phosphorus ratios) and (d) chromatographic behavior with DNA. These studies have led to a calcium phosphate preparation suitable for the column chromatography of DNA, bovine plasma albumin, polyadenylic acid, RNA,[17] and certain mixtures thereof. In addition, a simplified method of preparing a calcium phosphate with properties similar to those of the semi-hydroxylapatite of Tiselius and co-workers[3] will be described.

Experimental

Calcium Phosphate Preparations.—A series of 0.5 *M* sodium phosphate buffers ranging in *p*H from 6.8 to 7.8 by intervals of 0.2 unit was prepared. Calcium phosphate was precipitated by admixing 100 ml. of 0.5 *M* calcium chloride solution (1 drop per second) with 120 ml. of each buffer under mechanical stirring at room temperature. In this manner phosphate ions were continuously in over-all excess during the entire precipitation process. Each of the resulting suspensions was stirred for one additional hour and then allowed to settle. The *p*H of each supernatant solution was

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(2) The following abbreviations have been used: DNA, deoxyribonucleic acids; RNA, ribonucleic acids; RNAse, ribonuclease. For purposes of this report, calcium phosphate is abbreviated, CP. CP, followed by arabic numerals represents calcium phosphate precipitated by the manner described in the text from sodium phosphate buffer initially of *p*H indicated by the arabic numerals. Thus, CP 6.7 represents a calcium phosphate specimen precipitated from sodium phosphate buffer initially at *p*H 6.7. CPA represents calcium phosphate prepared as described by boiling CP 6.7 in a solution of ammonium hydroxide. CPM represents calcium phosphate prepared by boiling CP 6.7 with calcium hydroxide solution. CPS represents a prepared standard of pure anhydrous secondary calcium orthophosphate while CPHA represents a prepared standard of pure hydroxylapatite. CPT represents a sample of calcium phosphate prepared according to the method of Tiselius.[3]

(3) A. Tiselius, S. Hjerten and O. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

(4) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(5) J. Porath, *Arkiv Kemi*, **11**, 97 (1957).

(6) G. L. Brown and M. Watson, *Nature*, **172**, 339 (1953).

(7) G. L. Brown and A. V. Martin, *ibid.*, **176**, 971 (1955).

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

(9) A. Bendich, H. B. and S. M. Beiser, *Cold Spring Harbor Symposia Quant. Biol.*, **21**, 31 (1956).

(10) H. Ephrussi-Taylor, "Proc. 3rd International Congress of Biochemistry," Brussels, 333, 1956, Academic Press, Inc., New York, N. Y., 1956.

(11) G. Semenza, *Arkiv. Kemi*, **11**, 89 (1957).

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

(13) D. F. Bradley and A. Rich, *ibid.*, **78**, 5898 (1956).

(14) R. K. Main and L. J. Cole, U. S. Naval Radiological Defense Laboratory Technical Report, USNRDL-TR-88, 22 May 1956.

(15) R. K. Main and L. J. Cole, *Arch. Biochem. Biophys.*, **68**, 186 (1957).

(16) R. K. Main, L. J. Cole and M. E. Ellis, *Nature*, **180**, 1285 (1957).

(17) R. K. Main, M. J. Wilkins and L. J. Cole, *Science*, **129**, 331 (1959).

determined with a glass electrode. Each precipitate was washed by decantation 7 times with 600 ml. of 0.005 *M* phosphate buffer, *p*H 6.7, and stored in sufficient buffer to make a total volume of 50 ml. For purposes of identification these will be referred to as CP 6.8, CP 7.0, CP 7.2, CP 7.4, CP 7.6 and CP 7.8, respectively. Specimen CP 8.8 was precipitated by the dropwise addition of 0.5 *M* $CaCl_2$ solution to 0.5 *M* Na_2HPO_4 solution (*p*H 8.8) as outlined above.

Specimens CPM and CPA were each precipitated by the dropwise addition of 0.5 *M* $CaCl_2$ solution to 0.5 *M* phosphate buffer solution *p*H 6.7 as described, for example, for the preparation of CP 6.8. Immediately following the initial 1 hour stirring period, each precipitate was washed by decantation once with 600 ml. of distilled water. With the aid of an additional 600 ml. of distilled water, each of these precipitates was transferred to a separate 4 liter erlenmeyer flask. To the first (CPM), 750 ml. of a filtered solution of calcium hydroxide (saturated at room temperature) and 5 drops of 0.2% solution of phenolphthalein were added. The resulting suspension was boiled vigorously for 30 minutes.[18] A preliminary experiment indicated that this amount of $Ca(OH)_2$ solution was just sufficient to attain and to maintain a pink color with phenolphthalein (*p*H 8.5) in the suspension throughout the boiling period. When the boiling was stopped, the granular precipitate settled rapidly and was decanted free of the supernate while still hot. The precipitate was washed by decantation with 7, 600-ml. portions of 0.005 *M* phosphate buffer, *p*H 6.7, and stored in a final volume of 50 ml. with this buffer. Specimen CPA differed in preparation from CPM only in that NH_4OH, instead of $Ca(OH)_2$, was used as the alkalizing agent. Sufficient concd. NH_4OH solution was added to attain and, at intermittent intervals during the boiling period, to maintain a pink color with phenolphthalein.

Specimen CPT was prepared according to the method of Tiselius, *et al.*[3]

Determination of Flow Rates.—The eluent flow rate through a column of each of the above calcium phosphate specimens was measured under certain arbitrary standard conditions. Columns were uniformly packed in glass chromatography tubes of the type described by Tompkins, *et al.*[19] All tubes were alike; measuring 10 mm. inside diameter, 150 mm. in length, and 2 mm. inside diameter for the capillary bore tubing sections. A pledget of glass wool covered by a filter paper disc, supported each column. A slurry of calcium phosphate in 0.005 *M* phosphate buffer was added to a tube in four portions of 4 ml. each. The first portion was settled by gravity. Subsequent portions were added and packed under air pressure of 3 p.s.i. (0.21 kg. per sq. cm.) until settling ceased. A layer of fluid continuously was maintained above the column surface. Final additions (or withdrawals) brought the height of each column to exactly 7.5 cm. The eluent flow rate through each column, measured under a pressure head of 10 inches of 0.005 *M* phosphate buffer, was expressed in terms of ml. per hour. For a given calcium phosphate preparation the flow rate, measured under these conditions in each of 4 such chromatographic tubes, agreed in value to within ± 3%.

Chromatographic Procedures.—In initial experiments the chromatographic behavior of columns of each specimen toward DNA[20] was determined in the following manner: DNA (2 mg. in 4 ml. of 0.005 *M* buffer) was applied during a period of 45 minutes and chromatographed on each column by the method of discontinuous gradient elution with sodium phosphate buffers at *p*H 6.7, of increasing molarity according to the elution schedules shown in the accompanying illustrations. Eluent flow rates of 4.8 ml. per hour were maintained automatically by a constant rate flow device described elsewhere.[21] Fractions (4.8 ml.) were collected hourly by an automatic fraction collector. Chromatography was carried out at a temperature of 6°. The optical absorbance of each fraction was measured in a Beckman Model DU spectrophotometer at wave lengths 260 mμ (A_{260}) and 280 mμ (A_{280}). In later experiments, the same methods and elution schedules were applied to the chromatography of polyadenylic acid,[22] bovine plasma albumin[23] and to certain mixtures of these with DNA on columns of CPM.

X-Ray Diffraction Analysis of Calcium Phosphate Specimens.—X-Ray diffraction powder patterns[24] were determined on significant specimens by means of a G. E. Model XRD3 diffraction unit equipped with a diffractometer. A copper target and nickel filter were used. Pure reference specimens were prepared; anhydrous secondary calcium orthophosphate ($CaHPO_4$) by the method of Perloff and Posner,[25] designated CPS, and hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$) by Wallaeys' procedure,[26] designated CPHA.

Chemical Analyses.—The molar calcium to phosphorus ratio of calcium phosphate specimens was determined in duplicate.[27] Calcium was determined by titration of the oxalate with standard permanganate. Phosphorus was estimated gravimetrically as the magnesium pyrophosphate.

Total apparent recoveries of DNA from the column were determined by two independent methods. By the first, the sum of the optical absorbance (A_{260})X volume units of each of the fractions eluted was compared with the $A_{260}X$ volume units given by 2.00 mg. of DNA dissolved in 40 ml. of 0.01 *M* phosphate buffer *p*H 6.7. By the second method, the total amount of DNA placed on the column were each determined by the DNA-indole method of Keck.[28] This method is based on the deoxyribose moiety of DNA. In the hands of the authors, the most sensitive form of this method permitted the reliable determination of 1.0 microgram of DNA per ml.

To detect possible depolymerization of DNA during or immediately following passage through the column, the criteria of (a), rechromatography and (b) a test of the "hyperchromic effect" of Kunitz[29] were applied to chromatographed fractions. Selected eluate fractions were rechromatographed after pervaporation to small volumes while enclosed in dialysis bags suspended in a stream of dry air at 6°, followed by dialysis against 0.005 *M* phosphate buffer at *p*H 6.7. Results of this test are presented in Table II. Selected fractions of DNA eluted from each of several columns, as well as freshly prepared solutions of unchromatographed DNA were immediately treated with DNAse according to the method of Kunitz[29] in order to compare the "hyperchromic effect" of each. Results of these experiments are presented in the text, see Results—Elution Profiles—DNA.

In experiments concerning the chromatography of DNA–albumin mixtures, albumin in the DNA fractions was determined by the method of Lowry, *et al.*[30] As used by the present authors this method permitted the determination of 2.5 micrograms of albumin per 0.5 ml. DNA in the albumin fractions, as determined by the method mentioned above.[28] Results of these experiments are presented in the text, see Results—Elution Profiles—Protein and Protein-DNA Mixture.

(18) Within the limits of experimental error, no additional changes were produced in CPM by extending this boiling period from 30 minutes to 3 hours, as measured by X-ray diffraction powder patterns, molar Ca/P ratios or by eluent flow rates through standard columns.

(19) E. R. Tompkins, J. X. Khym and W. E. Cohn, THIS JOURNAL, **69**, 2769 (1947).

(20) Calf thymus DNA, Lot No. 569, was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. A 0.1% solution of Worthington calf thymus DNA has a relative viscosity of 5–30 and exhibits birefringence of flow, according to the manufacturer. Solutions of this DNA act as a satisfactory biological "primer" for the enzymatic synthesis of DNA, *cf.*, F. J. Bollum and V. R. Potter, *J. Biol. Chem.*, **233**, 478 (1958). Specimens of this DNA (see under Results—Elution Profiles, DNA) give a "hyperchromic effect" of 33% when treated with DNAse.

(21) R. K. Main, L. J. Cole, L. M. Bryant and S. K. Morris, *Anal. Chem.*, **29**, 1558 (1957); R. K. Main, *ibid.*, **30**, 531 (1958).

(22) The authors are indebted to Dr. R. F. Beers, Jr., for the gift of polymerized adenylic acid (Poly-A No. 63); mean molecular weight 1.8×10^6.

(23) Crystallized bovine plasma albumin, Lot No. P67704, was a product of Armour Laboratories, Chicago.

(24) Acknowledgment is made to Mr. G. M. Gordon, Division of Mineral Technology, University of California at Berkeley and to Mr. Jack T. Quan and Mr. Joseph D. O'Connor, U. S. Naval Radiological Defense Laboratory for the X-ray diffraction powder analyses.

(25) A. Perloff and A. S. Posner, *Science*, **124**, 583 (1956).

(26) R. Wallaeys, Thesis, Univ. of Paris, 1952, as reported by A. S. Posner and A. Perloff, *J. Research Natl. Bur. Standards*, **58**, 279 (1957).

(27) We are indebted to Mr. Clarence Callahan for performing the calcium and phosphorus analyses.

(28) K. Keck, *Arch. Biochem. Biophys.*, **63**, 446 (1956).

(29) M. Kunitz, *J. Gen. Physiol*, **33**, 349 (1950).

(30) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Results

As shown in Table I, a series of calcium phosphate specimens CP 6.8–7.8 was prepared, packed in standard columns and tested as to their eluent flow rate characteristics by the methods outlined under Experimental. A relationship was noted between the initial *p*H of each sodium phosphate reagent used in the precipitation and these flow rate values. For this series of calcium phosphate preparations, the reagent with the lowest initial *p*H favors production of a calcium phosphate possessing the fastest eluent flow rate characteristics. This relationship is illustrated in columns 2 and 4 of Table I.

TABLE I

INFLUENCE OF *p*H OF PHOSPHATE REAGENT ON CALCIUM PHOSPHATE PRECIPITATED THEREFROM: ELUENT FLOW RATES AND LIMITED CHROMATOGRAPHIC BEHAVIOR

Calcium phosphate specimen	Initial *p*H phosphate reagent[a]	Final *p*H supernate[b]	Eluent flow rate[c]	% DNA eluted[d]
CP 6.7	..	..	28.5	64
CP 6.8	6.8	3.8	28.5	65
CP 7.0	7.0	4.2	25.0	71
CP 7.2	7.2	4.5	25.0	63
CP 7.4	7.4	5.4	20.0	66
CP 7.6	7.6	6.2	20.0	39
CP 7.8	7.8	6.5	5.5	0
CP 8.8	8.8[e]	6.7	7.5	0

[a] 120 ml. of 0.5 *M* sodium phosphate buffers at *p*Hs indicated to which 100 ml. of 0.5 *M* $CaCl_2$ solution (*p*H 6.35) was added. [b] Following completion of precipitation of calcium phosphate and one additional hour of stirring. [c] Flow rate of 0.005 *M* sodium phosphate buffer, *p*H 6.7, through standard columns of each calcium phosphate specimen, expressed as ml. per hour. Experimental conditions as in text. [d] Per cent. of DNA (2.0 mg. in 4.0 ml. of 0.005 *M* phosphate buffer placed on column) recovered in 50 ml. of 0.005 *M*, *p*H 6.7, phosphate buffer eluent. [e] The sodium phosphate reagent in this singie instance was 0.5 *M* Na_2HPO_4 solution.

In exploratory trials of these specimens as column material for the chromatography of DNA, certain of these were eliminated from further consideration. Two mg. of DNA was placed on columns of each calcium phosphate and eluted with 50 ml. of 0.005 *M* phosphate buffer *p*H 6.7. Results of these experiments are shown in column 5 of Table I. It is evident that of these specimens, only CP 7.8 and CP 8.8 retained sufficient DNA during the elution with 50 ml. of 0.005 *M* phosphate eluent to be of value for the chromatography of DNA.

In order to take advantage of the comparatively larger crystal size (as reflected in the fast eluent flow rate) of CP 6.8 and CP 6.7, these preformed crystals were boiled with dilute alkaline solutions of $Ca(OH)_2$, NH_4OH and NaOH. Treatment of specimen CP 6.8 with $Ca(OH)_2$ according to the method used in producing CPM brought about an increase in relative eluent flow rate in standard columns of the latter from 28.5 ml. per hour to 50 ml. per hour. Treatment of CP 6.8 with NH_4OH to produce CPA caused scarcely any change in eluent flow rate. Treatment of CP 6.8 with NaOH resulted in a material causing unsuitably slow eluent flow rates.

Physical and Chemical Properties of Calcium Phosphate Preparations.—X-Ray diffraction powder patterns and chemical analyses (molar calcium to phosphorus ratios) helped to identify the nature of these specimens. As determined, CP 8.8 has the crystal structure[31] and approximate molar Ca/P ratio, 1.07, (theoretical, 1.00) of brushite ($CaHPO_4 \cdot 2H_2O$). CP 6.7 has the crystal structure[31] and approximate molar Ca/P ratio, 0.98 (theoretical 1.00) of anhydrous secondary calcium orthophosphate ($CaHPO_4$). These results were verified by comparison with the X-ray pattern and Ca/P ratio of a standard $CaHPO_4$ preparation (CPS). Since the X-ray diffraction patterns of CPM appeared somewhat similar to those of $CaHPO_4$, one might infer from these data alone that preparation CPM is $CaHPO_4$. However, both the molar Ca/P ratio, 1.20, and the chromatographic characteristics of CPM (as shown in a later section) are incompatible with such a conclusion. Both preparation CPT, a sample of the semi-hydroxyl apatite prepared according to the method of Tiselius and co-workers,[3] and preparation CPA, exhibited an X-ray pattern similar to that of a pure specimen of hydroxyl apatite ($Ca_{10}(PO_4)_6(OH)_2$). The molar Ca/P ratio of the latter (designated CPHA) was 1.67 (theoretical 1.67), while that of each of the former was 1.53. Chromatogaaphy of DNA on columns of either CPT or CPA gave identical chromatograms. Eluent flow rates through columns of CPT or CPA were of the same order of magnitude: 22 ml. per hour and 25 ml. per hour, respectively. The above data indicate that CPT and CPA are alike, if not identical. The eluent flow rate through a standard column of CPHA was too slow (0.5 ml. per hour) to warrant further consideration of this sample for purposes of column chromatography.

Stability of Calcium Phosphate Preparations.—Crystalline specimen CP 8.8 originally gave an X-ray diffraction powder pattern identical with that derived from $CaHPO \cdot 2H_2O$. After storage in a tightly capped bottle at room temperature for six months, an aliquot from this bottle gave an X-ray diffraction powder pattern closely similar to that shown by CPS ($CaHPO_4$). This result indicates that the dihydrate is unstable, and with time, even at room temperature, changes into the more stable anhydrous form. These findings are in agreement with those of Hill and Hendricks[32] and Whittaker, *et al.*[33] Anhydrous dicalcium phosphate does not readily become hydrated when placed in contact with solutions with which the dihydrate is the stable solid phase.[34]

Appreciable changes were difficult to detect in a specimen of CPM during storage as a suspension in 0.005 *M* phosphate buffer, *p*H 6.7, at room temperature for 7 months. The criteria of stability were X-ray diffraction powder patterns, eluent flow rates through standard packed columns, and elution profiles of DNA. Nevertheless, as a precau-

(31) Alphabetical and Numerical Indexes of X-ray Diffraction Patterns, American Society for Testing Materials, Philadelphia, pp. 37 and 241 (1954).

(32) W. L. Hill and S. B. Hendricks, *Ind. Eng. Chem.*, **28**, 440 (1936).

(33) C. W. Whittaker, F. O. Lundstrom and J. H. Shimp, *ibid.*, **26**, 1307 (1934).

(34) H. Bassett, Jr., *Z. anorg. Chem.*, **59**, 1 (1908).

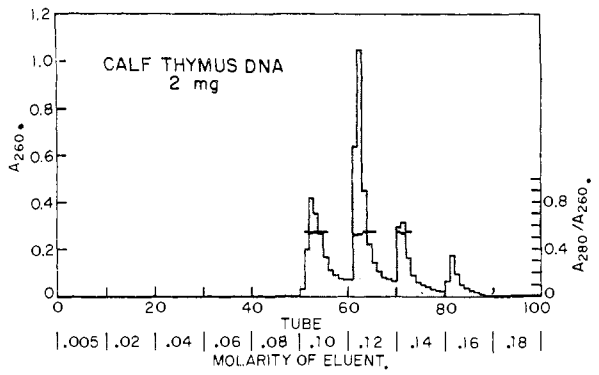

Fig. 1.—Chromatography of calf thymus DNA (2.0 mg. dissolved in 4.0 ml. of 0.005 *M* phosphate buffer *p*H 6.7 placed on column at rate of 4.0 ml. per 45 min.) on calcium phosphate, CPM, column (10 × 75 mm.); temperature 6°. Elution by discontinuous gradient elution. Eluent, sodium phosphate buffers, *p*H 6.7, at molarities indicated. Eluent flow rate maintained at 4.8 ml. per hour. Optical absorbance of eluates (A_{260}) plotted according to scale at left. Values of bars (A_{280}/A_{260}) measured on scale at right. The ratio A_{280}/A_{260} for fractions of DNA eluted at molar eluent concentrations of 0.10, 0.12, 0.14 and 0.16 was 0.54 ± 0.01 as compared with a corresponding value of 0.54 for the original DNA solution before chromatography.

tionary measure the authors recommend that CPM, be stored in the cold.

Elution Profiles.—The chromatographic behavior of calf thymus DNA, bovine plasma albumin, polyadenylic acid and mixtures of certain of these is illustrated by Figs. 1–3. Columns of CPM were used for this purpose in each instance.

DNA.—Figure 1 is a typical chromatogram of calf thymus DNA. Fractions of DNA were eluted at molar eluent concentrations of 0.10, 0.12, 0.14 and 0.16. The total apparent recovery of DNA from the column was 82.6% as determined by optical absorbance at 260 mμ and 83.2% by the method of Keck.[28] It is therefore evident that under the rather mild experimental conditions of elution used, approximately 17% of the DNA remained on the column.

TABLE II

RECHROMATOGRAPHY OF DNA FRACTIONS

Eluent buffer concn. at which fraction was originally eluted, *M*	Eluent buffer concn. at which rechromatographed fraction was eluted			
	0.10 *M*	0.12 *M*	0.14 *M*	0.16 *M*
	% eluted fraction			
0.10	10	90	0	
	10	90	0	
0.12	0	60	40	0
	0	73	27	0
0.14		0	90	10
		0	71	29

The elution data of the rechromatographed fractions from two separate experiments are shown in Table II. For fractions originally eluted by 0.12 and 0.14 *M* buffers, respectively, it is evident that on rechromatography of each of these, a major portion of DNA was eluted at the original concentration of eluent which initially caused that fraction to be eluted from the column. On rechromatography of the fraction originally eluted by 0.10 *M* buffer, this fraction, for the most part, was eluted by 0.12 *M* buffer. This latter result is reminiscent

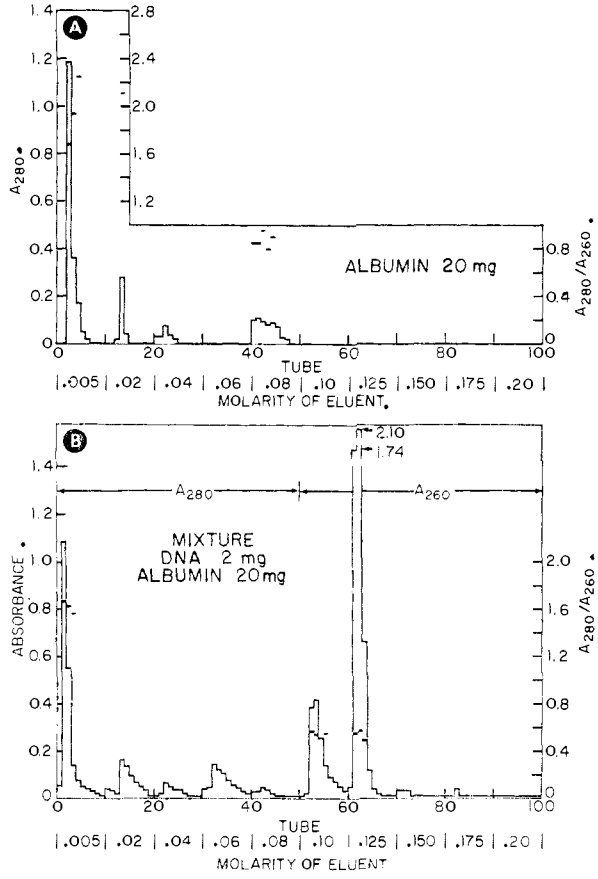

Fig. 2.—(A) chromatography of bovine plasma albumin (20.0 mg. dissolved in 4.0 ml. of 0.005 *M* phosphate buffer *p*H 6.7) placed on column, 10 × 75 mm., of calcium phosphate CPM at rate of 4.0 ml. per 45 min. Elution by discontinuous elution. Eluents, sodium phosphate buffers, *p*H 6.7, at molarities indicated; temperature 6°. Eluent flow rate maintained at 4.8 ml. per hour. Optical absorbance of eluates (A_{260}) plotted according to scale at left. Values of bars (A_{280}/A_{260}) measured on scale at right. All significant values of A_{280}/A_{260} for albumin fractions 1 (*i.e.*, tube numbers 3–8) and 2 (*i.e.*, 13–15) were within the limits 2.0 ± 0.3. Corresponding values for fraction 4 (tube numbers 40–47) were within the limits 0.88 ± 0.07. A_{280}/A_{260} of the original albumin solution placed on the column was 1.75. (B) chromatography of mixture of bovine plasma albumin (20.0 mg.) with calf thymus DNA (2.0 mg.) dissolved in 4.0 ml. of 0.005 *M* phosphate buffer *p*H 6.7 and placed on column, 10 × 75 mm., of calcium phosphate CPM at rate of 4.0 ml. per 45 min. Conditions of elution same as in A. Optical absorbance of eluates in tubes 1–50 inclusive, measured at 280 mμ (A_{280}) and of eluates in tubes 51–100 measured at 260 mμ (A_{260}), plotted as ordinates on the left. The A_{280}/A_{260} values of portions of fractions 1, 2 and 4 (tube numbers 1–10, 11–20 and 31–40) are, respectively, 1.61 ± 0.03, 1.41 and 1.12. These compare favorably with such values for corresponding albumin fractions as shown in Fig. 2A. The A_{280}/A_{260} values of fractions 6 and 7 (tubes 51–60 and 61–70, respectively) are within the limits 0.53 ± 0.02. The value of this ratio for solutions of unchromatographed DNA is 0.54 and for DNA eluates, as shown in Fig. 1, is 0.54 ± 0.01.

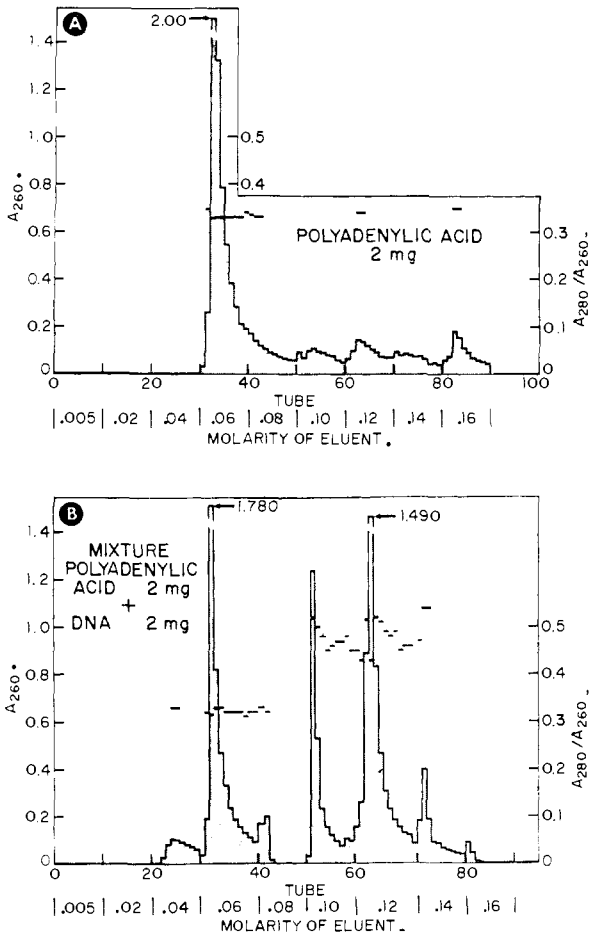

Fig. 3.—(A) chromatography of polyadenylic acid (2.0 mg. dissolved in 4.0 ml. of 0.005 *M* phosphate buffer *p*H 6.7 placed on column at rate of 4.0 ml. per 45 min.) on calcium phosphate, CPM, column (10 × 75 mm.); temperature 6°. Elution by discontinuous gradient elution. Eluent, sodium phosphate buffers, *p*H 6.7, at molarities indicated. Eluent flow rate maintained at 4.8 ml. per hour. Optical absorbance of eluates (A_{260}) plotted according to scale at left. Values of bars (A_{280}/A_{260}) measured on scale at right. (B) chromatography of mixture of polyadenylic acid (2.0 mg.) with calf thymus DNA (2.0 mg.) on calcium phosphate, CPM, column (10 × 75 mm.). Conditions of placement of mixture (solvent volume 4.0 ml.) on column and elution from column, same as in A. Optical absorbancy data plotted as in A.

of the results obtained by Semenza[11] concerning rechromatography of the first DNA fraction eluted from a hydroxylapatite column. These results (by the present authors) cannot be ascribed to the pervaporation and dialysis processes *per se*, since a sample of DNA previously pervaporated and dialyzed gave the same chromatogram as that derived from an equal amount of DNA not previously pervaporated and dialyzed.

The above rechromatography data, as well as those derived from experiments based on the well known[29] hyperchromic effect produced by the action of DNAse on "undegraded" DNA, discount the possibility of depolymerization of DNA during its passage through the column. The maximum increases in A_{260} of DNAse-treated DNA specimens (whether they were chromatographed solutions) over those of corresponding untreated (with DNAse) controls, ranged from 27–33%. By contrast, when these experiments were repeated on portions of the above eluted fractions following deliberate degradation of the DNA therein, by storage at 22° for 1 week, no increase in A_{260} following DNAse treatment could be detected.

Protein and Protein–DNA Mixture.—In Fig. 2, A illustrates the chromatography of 20.0 mg. of bovine plasma albumin, while B illustrates the results from a mixture containing 20.0 mg. of bovine plasma albumin and 2.0 mg. of calf thymus DNA. The exact nature of albumin fraction 4 is unknown. As calculated from the spectrophotometric data (A_{280}) the total recovery of albumin in the eluted fractions amounted to 100 ± 1% of that placed on the column. It is apparent in B of Fig. 2, both from the order of magnitude of A_{280}/A_{260} of each fraction and from the relative order of desorption of each fraction from the column, that fractions 1, 2, 3 and 4 (tube numbers 1–10, 11–20, 21–30 and 31–40, respectively) are albumin, while fractions 6 and 7 (tube numbers 51–60 and 61–70, respectively) are DNA. In the eluates concerned with fraction 6, and fraction 7, (DNA peaks), 65 and 35 micrograms of albumin, respectively, were found. In terms of per cent. of albumin (20 mg.) in the original albumin–DNA mixture placed on the column, the above values are 0.33 and 0.17%, respectively. No DNA was detected in any of the four albumin fractions. It is apparent that in these fractions there was little cross contamination of the DNA by albumin or of albumin by DNA. It should be noted that fraction 4 (albumin) is desorbed from the column by 0.06 *M* phosphate eluent from this mixture, instead of by 0.08 *M* phosphate eluent as was the case in the absence of admixed DNA (Fig. 2A). The elution schedule in Fig. 2 was slightly different from that in Fig. 1. This difference may account in part for the altered shape of the DNA peaks in Fig. 2B.

Polyadenylic Acid.—Figure 3A illustrates the results obtained by the chromatography of polyadenylic acid on a standard column of CPM. It is evident that polyadenylic acid is heterogeneous and that the largest amount is desorbed as a major peak by 0.06 *M* phosphate eluent. All values of A_{280}/A_{260} for fractions of this major peak and for those fractions measured from two minor peaks fall within the limits of 0.34 ± 0.01. As calculated from the spectrophotometric data (A_{260}) the total recovery of polyadenylic acid in the eluted fractions was 85.5%.

Figure 3B, illustrates the chromatography of a mixture of polyadenylic acid (2.0 mg.) with calf thymus DNA (2.0 mg.). From their position on the chromatogram and the order of magnitude of A_{280}/A_{260} values for fractions of peaks 1, 2 and 3 (eluted by 0.04, 0.06 and 0.08 *M* phosphate buffers, respectively), it is apparent that these three peaks represent polyadenylic acid. All of these values of A_{280}/A_{260} lie between the limits of 0.32 ± 0.10. By the same criteria it is apparent that fractions of peaks, 4, 5 and 6 (eluted by 0.10, 0.12 and 0.14 phosphate buffers, respec-

tively) are composed of a mixture of DNA and polyadenylic acid. This result would be anticipated from the data shown in Fig. 1 (DNA alone) and in Fig. 3A (polyadenylic acid alone). The values A_{280}/A_{260} of fractions included in peaks 4, 5 and 6 are scattered within the limits of 0.48 ± 0.06. The A_{280}/A_{260} value for a solution of this polyadenylic acid of like concentration is 0.32 and for this sample of DNA, 0.54. No DNA was found in fractions of peaks 1, 2 and 3. In fractions of peaks 4, 5 and 6 72% of the DNA originally in the mixture placed on the column was recovered. This was distributed as follows: 25.8% in peak 4, 36.7% in peak 5, and 9.4% in peak 6.

Discussion

To be of use for the separation and purification of macromolecules, a calcium phosphate adsorbent must be able to adsorb and to desorb such molecules under the influence of appropriate eluents. For this adsorbent to be useful for purposes of *column* chromatography, the individual particles must be of sufficient size and/or favorable shape as to allow adequate eluent flow rates through columns of the same. In contrast to the coarser crystal size of acid and neutral calcium phosphates, small crystal size is a striking feature of the chemically precipitated basic calcium phosphates and apatites.[35] Both the average crystal diameter (range 150–300 Å.)[36] and the fine flat needle-like external structure[37] of precipitated calcium apatites favor such dense packing as to prevent adequate flow rates through columns of these crystals. The electron microphotographs of Hayek, *et al.*,[37] indicate that acid precipitated ($CaHPO_4$) calcium phosphate crystals[38] are much coarser grained by comparison with the above and in external form are triclinic approaching a cuboidal form. Tiselius and coworkers,[3] circumvented these unfavorable conditions by converting preformed coarser-grained brushite ($CaHPO_4 \cdot 2H_2O$) crystals into a semi-hydroxylapatite (approaching $Ca_{10}(PO_4)_6(OH)_2$) of particle size suitable for column chromatography. This was accomplished by boiling the former in NaOH solution, followed by washing and heating the crystals for various lengths of time in a series of phosphate buffers. This preparation of calcium phosphate has found application in the column chromatography of proteins by Tiselius[3] and by Li[39] and of DNA by Semenza.[11] The latter author was able to separate DNA (previously adsorbed in 0.05 *M* phosphate buffer +1.0 *M* NaCl) into two chromatographic peaks, desorbed, respectively, by 0.30 and by 0.35 *M* phosphate buffer, *p*H 6.3, each in 1.0 *M* NaCl solution. Maximum resolution was obtained with a load of 0.25 mg. of DNA per column (16 × 50 mm.), which is 1/8 of the load resolved by the present authors' method.

Characteristics of this calcium phosphate preparation which recommend it for column chromatography are: ease and reproducibility of preparation; high eluent flow rates through packed columns; stability; and the degree of chromatographic resolution obtained, (a) at or near physiological concentrations (ionic strength and *p*H) of the eluents and (b) for the size of load placed on the column.

(35) J. J. Weikel, W. F. Neumann and I. Feldman, THIS JOURNAL, **76**, 5202 (1954).

(36) A. S. Posner, A. F. Doris and A. Perloff, *Natl. Bur. Standard Tech. Bull.*, **41**, 88 (1947).

(37) E. Hayek, F. Mullner and K. Noller, *Monat. Chem.*, **82**, 959 (1951).

(38) The present authors have refrained from applying the term "gel" ("(a) a jelly or (b) a solid gelatinous form in which a colloidal system is sometimes obtained as distinguished from the liquid form of sol"; ref., "Chambers Technical Dictionary," The Macmillan Co., New York, N. Y., 1956, p. 371; Van Nostrand Chemists Dictionary, D. Van Nostrand Co., Inc., New York, Toronto, London, 1953, p. 321, etc.), to any of these aqueous suspensions or packed preparations of calcium phosphate crystals.

(39) C. H. Li, *J. Biol. Chem.*, **229**, 157 (1957).

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Cupric Chelates of Pyridoxylvaline and Pyridoxylidenevaline[1]

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These two chelates have been compared to clarify a previously observed association in the 3-hydroxypyridine system between (a) the formation of stable linkage to the phenolic oxygen and (b) a lowering of the pK_a of the pyridine N. The results show that the pK lowering depends upon the extent of electron displacement occasioned by the linkage and not upon its presence or absence. In Cu pyridoxylvaline, Cu is strongly linked to the phenolic O with only a small displacement of the pK; only upon deprotonation does the spectrum show a strong new deformation of the electron distribution.

Tentative structures attributed to metal chelates of pyridoxylidene amino acids showed the metal bonded to the Schiff base at 3 points (like Fig. 1, structure II), although alternative formulations left the carboxylate group free, probably to assist in explaining how *α-decarboxylation* can be catalyzed.[2] A study of a group of chelates of pyridoxylidenevaline led, however, to the conclusion that if any one of the 3 groups remains free, it will tend to be the phenolic group.[3] A downward shift of the pK_a of the pyridinium N by 3 or more *p*H units is believed to signal covalent linkage of the phenoxide ion to the metal (Cu(II), Fe(III)), eliminating the usual powerful inductive effect of the phenolic group of the 3-hydroxypyridines on the pyridinium dissociation. A small shift ((Ni-(II), Mn(II), Fe(II), Zn) was interpreted to repre-

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(2) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954).

(3) H. N. Christensen, *ibid.*, **79**, 4073 (1957)