ether (IIf) with m.p. 164–166°, $[\alpha]D^{20}$ −114° (dioxane), ultraviolet maximum at 268 mμ (log ε 4.31). Acid hydrolysis yielded 82% of testosterone (If) with m.p. 152–154°, $[\alpha]^{20}D$ +109.7°, ultraviolet maximum at 242 mμ (log ε 4.25).

Progesterone 3-Benzylsulfoxidoenol Ether (XVIa).—The oxidation of the thioenol ether IIa (3.00 g.) was carried out on the steam-bath by heating for 15 minutes with 350 cc. of dioxane, 20 cc. of 30% hydrogen peroxide and 5 cc. of sodium carbonate solution. After dilution with water, the sulfoxide was extracted with a mixture of methylene chloride and ether and recrystallized from methylene chloride–methanol; yield 1.7 g. (48%), m.p. 200–201°, $[\alpha]^{20}D$ −94.3° (dioxane), ultraviolet maximum at 258 mμ (log ε 4.32).

Anal. Calcd. for $C_{28}H_{36}O_2S$: C, 77.02; H, 8.31; S, 7.33. Found: C, 77.45; H, 8.51; S, 7.30.

Desoxycorticosterone Acetate 3-Benzylsulfoxidoeno-Ether (XVIb).—A solution of 5.0 g. of desoxycorticosterone acetate 3-benzylthioenol ether (IIb) in 300 cc. of dioxane was allowed to stand at room temperature for 48 hours with 20 cc. of 30% hydrogen peroxide. The usual work-up followed by recrystallization from acetone–methanol produced 2.6 g. (50%) of desoxycorticosterone acetate 3-benzylsulfoxidoenol ether (XVIb) with m.p. 171–173° (dec.), $[\alpha]^{20}D$ −82.8° (dioxane), ultraviolet maximum at 258 mμ (log ε 4.32).

Anal. Calcd. for $C_{30}H_{38}O_4S$: C, 72.84; H, 7.74; S, 6.46. Found: C, 72.89; H, 8.10; S, 6.40.

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[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

The Preparation of Desoxynucleotides[1]

BY E. VOLKIN, J. X. KHYM AND W. E. COHN

Desoxynucleotides have been isolated in good yield from enzyme digests of DNA by the method of ion-exchange chromatography. The techniques of enzymatic digestion, ion-exchange separation of the mononucleotides and the preparation of these as solid products are described. The mononucleotides have been analyzed spectrophotometrically and with respect to nitrogen and phosphorus composition; some preliminary biological characterizations of these products have been carried out.

Introduction

Ever since the double enzymatic hydrolysis procedure of Klein[1a] offered the possibility of preparing quantities of mixed nucleotides from desoxyribonucleic acid (DNA) with comparative ease, the problem of obtaining the individual compounds in pure form has been one of separation from the mixtures obtained. In view of the speed, precision, yield and mild conditions which characterize the ion-exchange separation of the analogous ribonucleotide hydrolysis mixtures,[2] the adaptation of this method to the resolution of the enzymatically produced mixture was sought as a means of making the desoxy compounds readily available for chemical and biological study. In this communication, such a combined procedure is described, together with preliminary characterizations of the nucleotides thus prepared.

Experimental

Material.—Polymerized DNA was prepared from calf thymus by the method of Mirsky and Pollister.[3] Desoxyribonuclease was prepared from calf pancreas by McCarty's procedure[4] and stored in the frozen-dried state. The activity of the enzyme was comparable to that prepared by McCarty. Alkaline intestinal phosphatase, prepared from calf intestinal mucosa according to Schmidt and Thannhauser[5] without purification by alumina or kaolin adsorption, was obtained from the Armour Laboratories. The dried material contained fifteen phosphatase units per mg. Although this preparation was highly contaminated with adenosine deaminase, the latter enzyme did not interfere with the procedure, since it specifically attacks the nucleoside.[6]

Enzymatic Digestion.[7]—To 10 g. of thymus DNA, dissolved in 2 l. of water, was added 70 cc. of 0.1 *M* magnesium chloride. The solution was adjusted to *p*H 7.2 and 25 mg. of desoxyribonuclease, dissolved in water and previously adjusted to the same *p*H, was added to the DNA solution with vigorous stirring. As the reaction progressed, the solution was maintained at *p*H 7.2 by the addition of 0.5 *N* sodium hydroxide from a buret. The reaction was allowed to proceed to completion and required 13 cc. of 0.5 *N* sodium hydroxide. Since with this amount of enzyme the hydrolysis takes between seven and eight hours, the digest is generally placed overnight in the cold before subsequent phosphatase hydrolysis.

The digest is then made approximately 0.005 *M* with respect to sodium arsenate by adding 20 cc. of 0.5 *M* sodium arsenate and is then adjusted to *p*H 8.4. Four grams of intestinal phosphatase, dissolved in 50 cc. of water and adjusted to the same *p*H, was added to the nuclease-treated DNA and the reaction followed by titration with 0.5 *N* sodium hydroxide as above. This hydrolysis procedure requires 45 cc. of 0.5 *N* sodium hydroxide and takes nine to ten hours for completion.

The reaction is stopped, and at the same time the digest is made ready for ion-exchange separation, by the addition of concentrated ammonia to a final concentration of 1 *M* ammonium hydroxide.

Ion-Exchange Separation.—The enzymatic digest of 10 g. of DNA in about 2 l. of solution at *p*H *ca.* 10 is absorbed on a strong-base chloride-form fine-mesh ion-exchange column 33 sq. cm. × 6.0 cm. in height. This column is then washed with the following: (1) water, to remove ammonia; (2) 0.01 *M* ammonium chloride (12–15 l.), until the *p*H of the effluent falls to 7, to remove bases and nucleosides; (3) 0.01 *M* hydrochloric acid (15–20 l.) to remove all nucleotides. Polynucleotides and undigested nucleic acid are left on the column; this is the principal purpose of the preliminary step, which may be omitted if desired.

The 0.01 *M* hydrochloric acid solution is made alkaline with ammonium hydroxide and adsorbed on the separation column, 33 sq. cm. × 12 cm. of the same material. The following reagents are then used in succession: (1) 0.01 *M* ammonium chloride until the *p*H falls to 6; (2) 0.001 *M* hydrochloric acid to remove desoxycytidylic acid (and 5-methylcytidylic acid)[8]; (3) 0.002 *M* hydrochloric acid to remove desoxyadenylic acid; (4) 0.003 *M* hydrochloric acid to remove thymidylic acid; (5) 0.005 *M* hydrochloric acid

(1) Work performed under Contract W-7405-eng-26 for the Atomic Energy Commission.

(1a) W. Klein, *Z. physiol. Chem.*, **218**, 164 (1933).

(2) W. E. Cohn, THIS JOURNAL, **72**, 1471 (1950).

(3) A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.*, **30**, 117 (1946–1947).

(4) M. McCarty, *ibid.*, **29**, 123 (1945–1946).

(5) G. Schmidt and S. J. Thannhauser, *J. Biol. Chem.*, **149**, 369 (1943).

(6) H. M. Kalckar, *ibid.*, **167**, 461 (1947).

(7) C. A. Zittle, L. A. Wells and W. G. Batt, *Arch. Biochem.*, **13**, 395 (1947).

(8) W. E. Cohn, THIS JOURNAL, **72**, 2811 (1950); **73**, 1539 (1951).

to remove desoxyguanylic acid. The effluent from the column was passed through a specially designed cell in a Beckman spectrophotometer connected to a recording milliammeter; this permits continuous recording of the optical density (hence nucleotide content) of the column effluents. A recording *p*H meter was also inserted into the effluent stream to detect the critical *p*H changes. Identification of peaks was made first by spectrophotometry, subsequently by analyses of the products for nitrogen, phosphorus, desoxysugar and base component.

The nucleotides, collected as described above in volumes of 12–20 l. of dilute acid, were concentrated by reabsorbing each, at high *p*H with ammonium hydroxide, on separate, smaller anion-exchange columns containing about 1 ml. of resin per 130 mg. of nucleotide to be absorbed. Following a water wash, the nucleotides were eluted with 0.01 *M* hydrochloric acid; the purine nucleotides were quickly neutralized with ammonium hydroxide to avoid acid hydrolysis. Further concentration by vacuum distillation was sometimes used to achieve solutions from which the nucleotides could be precipitated by alcohol plus ether or by barium plus ammonium hydroxide

Results

The method described is composed of three successive steps and the results of each step will be presented in order.

Enzymatic Hydrolysis.—It has been stated[7] that the hydrolysis of DNA by desoxyribonuclease followed by alkaline phosphatase in the presence of arsenate results in the quantitative degradation of DNA to mononucleotides, which infers that the end-point of this reaction is characterized by a base consumption of one equivalent per mole of DNA phosphate. Ion-exchange analysis of a number of such digests does not support this view but rather

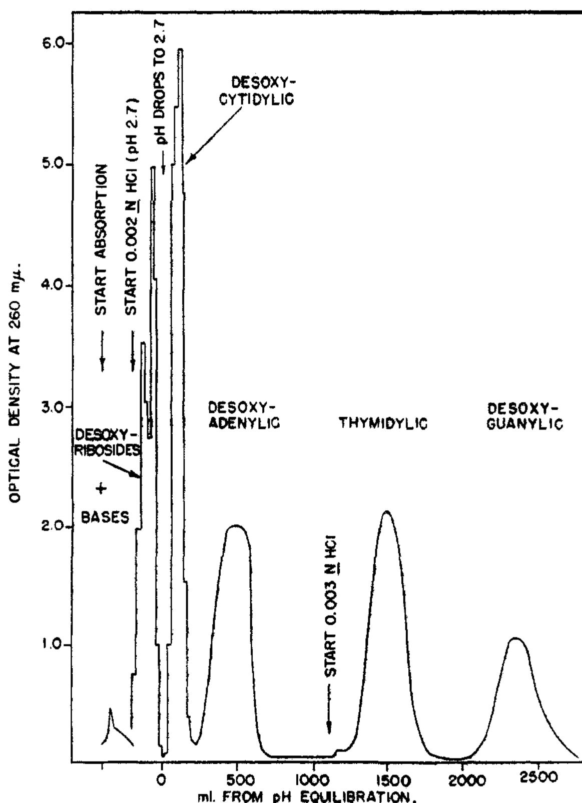

Fig. 1.—Separation of nucleotides from enzymatic digest of 150 mg. of desoxyribonucleic acid; column: Dowex-1, 200–400 mesh, 8 cm. × 0.72 sq. cm. Solution: HCl as shown, 1 ml./min.; recovered and identified: ~90%.

indicates that some mononucleotide material has been further degraded before all DNA has been converted to mononucleotides. Table I, which summarizes the data on the composition of several 5- to 10-g. digests before separation was begun, reveals that enzymatic hydrolysis to the extent of one base equivalent consumed per mole DNA phosphate yields about 65% mononucleotides, while continuation of the hydrolysis results in excessive breakdown of mononucleotides (Table I, DNA-6). It should be stated that excessive amounts of mononucleotide breakdown products in the digest coincide, in our experience, with anomalous ion-exchange separations. Thus, desoxycytidylic acid (and some thymidylic acid) may be displaced from the resin, together with bases and nucleosides, by the initial 0.01 *M* ammonium chloride wash, which necessitates further ion-exchange separations of the ammonium chloride effluent to recover these nucleotides. This situation seems to be due to a high ionic strength in the digest, usually manifested in advance by a high buffer capacity in the shift of *p*H from 7.2 to 8.4; it has been encountered only when the enzymatic hydrolysis has been carried too far.

TABLE I

COMPOSITION OF ENZYMATIC DIGESTS (5–10 G.) IN PER CENT. OF TOTAL EXTINCTION, FROM ION-EXCHANGE ANALYSIS (FIG. 1)

	DNA-3		DNA-4		DNA-5		DNA-6	
Eq. acid liberated/mole P	*ca.* 1		1.0		1.0		1.4	
Bases, nucleosides[a]	14		8		12		40	
Desoxycytidylic acid	11	69	8	66	9	63	10	49
Desoxyadenylic acid	23		21		18		16	
Thymidylic acid	23		20		21		15	
Desoxyguanylic acid	12		17		15		8	
Residue[b]	16		27		26		11	

[a] Mostly inosine and xanthine; some thymidine, cytidine, guanosine; traces of hypoxanthine, xanthosine. [b] Polynucleotides, polyphosphates, etc.

The presence of polynucleotides in hydrolysates which presumably represent complete breakdown of DNA to nucleotides is not clearly understood; the release of tertiary phosphate linkages (*p*K 12–12.5) in the hydrolysis of nucleotides should not be measurable at the *p*H employed. Since nucleosides, bases and polynucleotides are readily separable from the mononucleotides by the ion-exchange process,[2] the yields of 50–70% mononucleotides are quite adequate for preparative purposes.

Ion-Exchange Separation and Concentration.—The method of analysis is nearly identical with that described by Cohn for ribonucleotides[2] and is demonstrated in Fig. 1. For rapid surveys, all mononucleotides are eluted together with 0.005 to 0.01 *N* hydrochloric acid (bases plus nucleosides being first removed with 0.01 *M* ammonium chloride). The material remaining on the column is designated "polynucleotide" since known polynucleotide material behaves in this manner[9]; however, polyphosphates, if present, would be included in this fraction.[10] The ion-exchange separation of the mononucleotides from a 20-g. digest is shown in

(9) C. E. Carter and W. E. Cohn, THIS JOURNAL, **72**, 2604 (1950).
(10) W. E. Cohn and C. E. Carter, *ibid.*, **72**, 4273 (1950).

Fig. 2, which is the automatically recorded spectrophotometric transmission data. The individual peaks were collected, made alkaline with ammonia, reabsorbed on smaller columns, eluted from these with 0.01 *N* hydrochloric acid and promptly neutralized. These concentrates, upon analysis, showed no ultraviolet absorbing material other than the main nucleotide component.

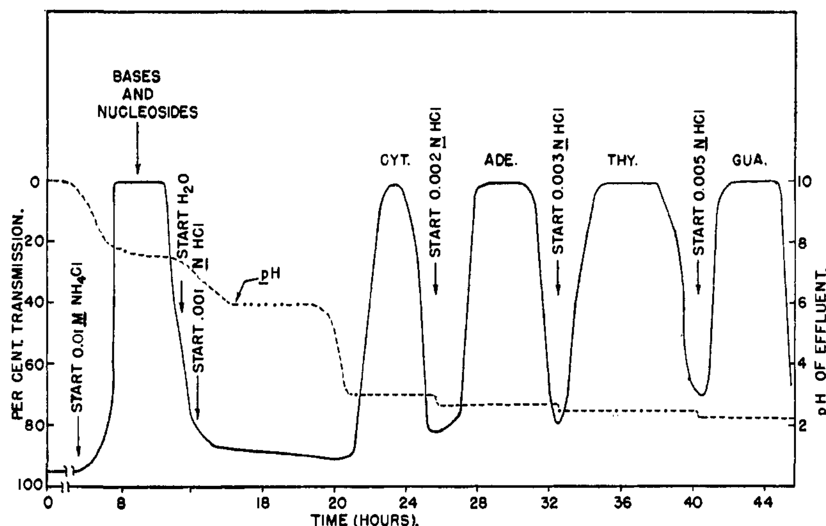

Fig. 2.—Ion-exchange separation of nucleotides from enzymatic digest of 10 g. of DNA; bed: 12 cm. × 33 sq. cm., Dowex-1, 250–500 mesh; eluent: HCl as shown, 1 ml./sq. cm./min.

Crystallization or Precipitation.—As the yield data in Table II indicate, achieving a solid product was the most costly single step in the entire procedure. The solid desoxynucleotides (or alkaline barium precipitates containing them) prepared have the analytical values given in Table III. While the analyses on the free acids agree reasonably well with the theoretical, it is evident that the corresponding barium salts are low in nitrogen and phosphorus. It should be noted, however, that the nitrogen:phosphorus ratios of these latter compounds are more in keeping with the theoretical, indicating that these solids are contaminated with inorganic barium salts, presumably the carbonate as carbon dioxide was not excluded from the reagents used.

TABLE II

YIELDS THROUGH SEPARATION PROCEDURE (DNA-5 AND DNA-6) IN ARBITRARY SPECTROPHOTOMETRIC UNITS

	Initial hydrolysate	After ion-exchange sepn.	After ion-exchange concn.	As final solids
Bases, nucleosides	30			
Desoxycytidylic acid (DNA-5)	25	16	13	6.4
Desoxyadenylic acid (DNA-5)	46	49	41	28
Desoxyadenylic acid (DNA-6)		19	18	11[a]
Thymidylic acid (DNA-5)	55	55	48	10[a]
Thymidylic acid (DNA-6)		25	25	15.5[a]
Desoxyguanylic acid (DNA-5)	39	39	31	20
Desoxyguanylic acid (DNA-6)		14	11	8[a]
Unaccounted for (DNA-5)	66			

[a] Isolated as barium salt.

Discussion

Klein and Thannhauser[11,12,13] obtained highly purified, crystalline desoxyribonucleotides from enzymatically hydrolyzed DNA by extensive chemical purification methods. The ion-exchange method of purification described here affords a simple and relatively rapid technique for separating the mononucleotides free of each other by a single or two-step operation. Likewise, analysis by ultraviolet spectrophotometry is a sensitive method for the detection of many other impurities.[14] For many purposes, it should be possible to omit preparation of the solid and to use the final column concentrate, which usually contains about 20 mg. of nucleotide per ml. of dilute (0.01–0.1 *M*) chloride solution. It is one advantage of the ion-exchange separation technique that it operates without recourse to precipitation or crystallization; if the identification of the product and the establishment of its purity may be done upon a solution, precipitation steps can be omitted.

One of the major points of interest in connection with the desoxynucleotides concerns the presence or absence of the isomerism now known to occur in the four ribose nucleotides.[2,8] Neither Fig. 2 nor, more particularly, Fig. 1 gives any evidence of isomers, nor have repeated efforts to find such been rewarded. Heating desoxycytidylic acid in acid, under conditions which will convert either ribocytidylic acid A or B to a mixture of both, yields no evidence of isomers. We are forced to the conclusion that the type of isomerism found in the ribose nucleotides does not exist in the desoxynucleotides derived as indicated above.

The absence of isomerism may be related to the position of the phosphate residue. Whereas the B-ribonucleotides are undoubtedly 3′-phosphates,[15] the desoxynucleotides we have prepared are hydrolyzed by the specific 5′-phosphatase[16] from bull semen, indicating a 5′-phosphate structure.[17] It is possible that this difference in point of esterification is related to the absence of isomerism in the desoxy series.

The similarity between desoxynucleotides and the corresponding 5′-ribose compounds is also em-

(11) W. Klein and S. J. Thannhauser, *Z. physiol. Chem.*, **218**, 173 (1933).
(12) W. Klein and S. J. Thannhauser, *ibid.*, **224**, 252 (1934).
(13) W. Klein and S. J. Thannhauser, *ibid.*, **231**, 96 (1935).
(14) W. E. Cohn, unpublished data.
(15) D. G. Doherty, C. E. Carter and W. E. Cohn, unpublished data.
(16) L. A. Heppel, *Federation Proc.*, **9**. 184 (1950); J. T. Mann, *Biochem. J.*, **39**, 345 (1945).
(17) C. E. Carter THIS JOURNAL, **73**, 1537 (1951).

TABLE III

ANALYTICAL DATA ON SOLID DESOXYNUCLEOTIDES

		N	P	E[c]	Mols base[d] per mol P	M.p., °C.	At pH 2 250/260	At pH 2 280/260
Desoxycytidylic acid	Found	12.8	9.6	19.4	1.06	185–187	0.43	2.12
	Theor.[b]	13.7	10.1	22.3[a]				
	Ratio	0.935	0.95	0.87				
Desoxyadenylic acid	Found	19.3	8.5	41		142.5	.81	0.24
	Theor.[b]	21.1	9.4	44[a]				
	Ratio	0.915	0.905	0.93				
Barium desoxyadenylate	Found	6.6		14	1.05		.81	.23
	Theor.[b]	21.1		44[a]				
	Ratio	0.31		0.32				
Barium thymidylate	Found	5.5	5.5	14.5			.64	.74
	Theor.[b]	8.7	9.6	26[a]				
	Ratio	0.63	0.57	0.56				
Barium thymidylate	Found	5.4	5.3	15	1.01		.65	.72
	Theor.[b]	8.7	9.6	26[a]				
	Ratio	0.62	0.55	0.58				
Desoxyguanylic acid	Found	20.4	7.9	31.5			1.02	.70
	Theor.[b]	20.1	8.9	34[a]				
	Ratio	1.01	0.89	0.93				
Barium desoxyguanylate	Found	11.6	4.9	18	1.01		0.98	.70
	Theor.[b]	20.1	8.9	34[a]				
	Ratio	0.57	0.55	0.53				

[a] Calculated from ribose analogs and from thymidine. [b] No correction for Ba, H_2O, etc.; values given are those of the free nucleotide. [c] Extinction at 260 mμ, pH 2, per mg. [d] From extinction of hydrolyzed nucleotide. Purine nucleotides were hydrolyzed with 0.1 N HCl (see text); pyrimidine nucleotides were hydrolyzed with 72% $HClO_4$ [A. Marshak and H. J. Vogel, *Federation Proc.*, 9, 85 (1950)].

phasized by their ion-exchange behavior. Desoxyadenylic is easily separated from both A and B ribonucleic adenylic acids, as is shown in Fig. 3, and the separation of the corresponding cytidylic acids has been described elsewhere.[8] However,

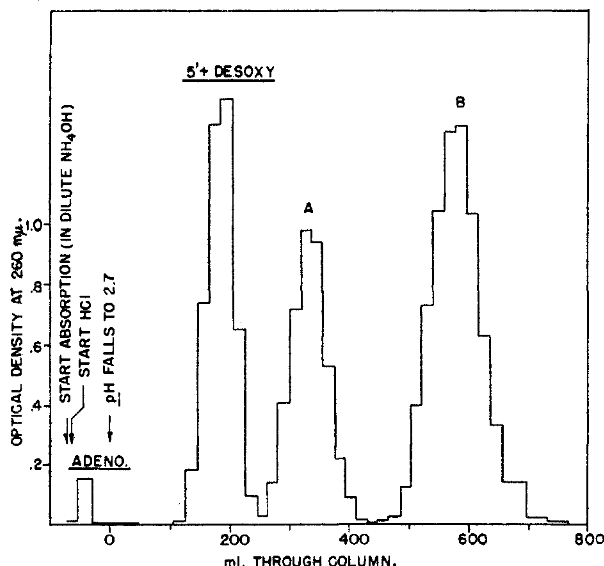

Fig. 3.—Ion-exchange separation of desoxyadenylic acid, adenosine-5'-phosphate, adenylic acids A and B; bed: 6.2 cm. × 0.88 sq. cm., Dowex-1, 200–500 mesh, Cl^- form; eluent: 0.0021 N HCl, 0.5 ml./min.

muscle adenylic acid, the 5'-ester, is eluted at precisely the same place as desoxyadenylic acid and no separation has yet been achieved.[14]

In spite of the resemblances between the adenosine-5'-phosphate and desoxyadenylic acid indicated above, their stability to acid distinguishes them completely. Desoxyadenylic acid is converted to adenine at the rate of 2% per hour in 0.01 N hydrochloric acid at room temperature (20% per hour in 0.1 N hydrochloric acid); the three ribose-adenylic acids and the pyrimidine nucleotides (ribo- and desoxyribo-) are unaffected by these conditions.[14]

Preliminary tests of the biological activity of the desoxynucleotides described previously have been made by several investigators. All four are about as effective in replacing vitamin B_{12} as thymidine and other desoxyribosides for both *Lactobacillus lactis*[18] and *Lactobacillus leichmanii*.[18,19] Thymidylic acid alone will replace thymidine in the *Lactobacillus arabinosus* assay, but is less active.[20] It will also replace folic acid.[20] None of the desoxynucleotides can replace vitamin B_{12} for *Euglena gracilis*, which cannot use DNA or its nucleosides as B_{12} replacements.[21]

OAK RIDGE, TENNESSEE RECEIVED OCTOBER 5, 1950

(18) W. Shive, M. E. Sibley and L. L. Rogers, THIS JOURNAL, 73, 867 (1951).

(19) H. P. Broquist, private communication.

(20) W. Shive, personal communication.

(21) J. O. Lampen, private communication.