[CONTRIBUTION FROM THE DEPARTMENT OF ANATOMY, MARQUETTE UNIVERSITY SCHOOL OF MEDICINE]

Natural 3'-Deoxyribomononucleotides[1]

BY LEW CUNNINGHAM

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Deoxyribonucleic acid was digested by an enzyme produced by *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) and the resulting mononucleotides were isolated and characterized. In addition to their resistance to 5'-nucleotidase action, these nucleotides are identical with synthetic deoxynucleoside 3'-phosphates in their ultraviolet spectrum and in their behavior in several chromatographic systems. However, they are resistant to 3'-nucleotidase and relatively stable in the presence of cerium hydroxide, while on an anion-exchange column, deoxyadenosine 3'-phosphate moves with adenosine 2'-phosphate rather than adenosine 3'-phosphate.

It was reported[2] that mononucleotides released from deoxyribonucleic acid by the action of micrococcal nuclease[3] differ from the familiar natural deoxyribomononucleotides in having phosphate esterified at the 3' rather than the 5'-position. This was inferred from negative results with a 5'-nucleotidase. The presence of singly esterified phosphate at the 3'-position was also indicated by the results of hydrolysis of a dinucleotide isolated following micrococcal nuclease action.[4]

It appeared desirable to confirm the specificity for the 5'-phosphodiester bond, assigned to this enzyme, by positive identification of the 3'-mononucleotides. Various ion-exchange and paper chromatographic systems were used, depending upon the mononucleotide to be characterized. In the case of deoxycytidylic acid, the ultraviolet spectrum was sufficient for identification of the isomer.

The 5'-isomers of the deoxymononucleotides are attacked by the same 5'-nucleotidase preparation which dephosphorylates 5'-ribomononucleotides,[5] and it was hoped at first that a 3'-nucleotidase could be used, similarly, to confirm the point of esterification of the second series of deoxymononucleotides as well. However, the presence of the hydroxyl at the 2'-position seems to have a greater effect upon the behavior of 3'-esters than on the behavior of compounds esterified at the more remote 5'-position. Attempts to dephosphorylate the 3'-deoxymononucleotides specifically have so far failed. Furthermore, in ion-exchange chromatographic systems, which do not separate corresponding 5'-phosphorylated ribo- and deoxyribonucleosides, 3'-deoxyribomononucleotides were found to precede their ribomononucleotide analogs.

Materials and Methods

Partially degraded herring sperm nucleic acid was obtained from the Nutritional Biochemical Co., Cleveland, Ohio. According to the suppliers, it had been prepared by the method of Levene.[6] A 20% solution, made 0.1 *N* with respect to NaOH, was stored for 24 hr. at room temperature. Then the *p*H was adjusted to 5.2 with acetic acid. An equal volume of 95% ethanol was added[7] and nucleic acid was allowed to settle out overnight. The supernatant fluid was decanted and the sediment dissolved in three ml. of 0.1 *N* NaOH per g. of the original nucleic acid preparation. This solution was dialyzed at 5°, first against three liters of 0.1 *N* NaCl, replaced every second day for ten days, and then against distilled water, replaced also every second day for ten days. The final dialyzed material contained 1.7 mg./ml. phosphorus (0.055 *M* with respect to phosphorus) and the ϵ_{260}^{8} value was 7,800. A disadvantage of this substrate is its content of substantial quantities of deoxyuridine,[9] presumably replacing deoxycytidine.

The 5'-isomers of deoxyribomononucleotides were obtained from the California Foundation for Biochemical Research, Los Angeles, California, and had been prepared by digestion with pancreatic deoxyribonuclease followed by digestion with purified snake venom phosphodiesterase.[10] Synthetic deoxycytidine[11,12] 3'-phosphate and barium thymidine 3'-phosphate were the gift of Sir Alexander Todd.

Cerium hydroxide was formed by mixing a solution of cerium (cerous) nitrate (Amend Drug and Chemical Co., New York) with ammonium hydroxide–ammonium chloride buffer at *p*H 8.5, as described by Bamann.[13] Crude venom of the diamond-back rattlesnake (*Crotalus adamanteus*) was obtained from Ross Allen Reptile Institute, Silver Springs, Florida. Two preparations of the 3'-nucleotidase[14,15] of common rye grass were used. One was supplied by Dr. N. Kaplan. The second was prepared from moistened seeds (allowed to germinate for three days at room temperature in a large, loosely covered glass dish) by extraction, precipitation with ammonium sulfate and dialysis as described by Shuster and Kaplan (steps 1 and 2).[15]

The phosphorus content of the mononucleotides was determined by the method of King[16] (no perchloric acid being added after the heating) after drying to constant weight at 60°. Ammonium content was determined with Nessler reagent.[17]

Chromatography.—In general, paper[18] and ion-exchange[19] chromatography was carried out by procedures now standard. Except as otherwise indicated, ion-exchange methods were taken from the work of Cohn and associates.[19] Whatman #1 paper was used for paper chromatography except

(1) A brief report was presented at the 131st meeting of the American Chemical Society, Miami, Florida, April, 1957.

(2) L. Cunningham, B. W. Catlin and M. Privat de Garilhe, THIS JOURNAL, **78**, 4642 (1956).

(3) Although this enzyme was previously called a deoxyribonuclease, Dr. J. D. Smith has pointed out in a personal communication that preparations of the enzyme, when activated by calcium, attack ribonucleic acid, the nature of the digestion products indicating a specificity similar to that shown with deoxyribonucleic acid.

(4) M. Privat de Garilhe, L. Cunningham, Ulla-Riitta Laurila and M. Laskowski, *J. Biol. Chem.*, **224**, 751 (1957).

(5) C. E. Carter, THIS JOURNAL, **73**, 1537 (1951).

(6) P. A. Levene and L. W. Bass, "The Nucleic Acids," Chemical Catalogue Co., New York, N. Y., 1931, p. 298.

(7) J. D. Smith and G. R. Wyatt, *Biochem. J.*, **49**, 144 (1951).

(8) E. Chargaff and S. Zamenhof, *J. Biol. Chem.*, **173**, 327 (1948).

(9) W. Anderson, C. A. Dekker and A. R. Todd, *J. Chem. Soc.*, 2721 (1952).

(10) R. O. Hurst, A. W. Marko and G. C. Butler, *J. Biol. Chem.*, **204**, 807 (1953).

(11) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 951 (1953).

(12) A. M. Michelson and A. R. Todd, *ibid.*, 34 (1954).

(13) E. Bamann and H. Trapmann, *Biochem. Z.*, **326**, 237 (1955).

(14) L. Shuster and N. O. Kaplan, *J. Biol. Chem.*, **201**, 535 (1953).

(15) L. Shuster and N. O. Kaplan, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Academic Press, New York, N. Y., 1955, p. 551.

(16) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(17) P. B. Hawk, B. L. Oser and W. H. Summerson, "Practical Physiological Chemistry," 12th Ed., Blakiston, New York, N. Y., 1953, p. 1229.

(18) G. R. Wyatt, in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Vol. I, Academic Press, New York, N. Y., 1955, Chapter 7.

(19) W. E. Cohn, ref. 18, Chapter 6; W. E. Cohn, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. III, Academic Press, New York, N. Y., 1957, pp. 743–746.

as otherwise indicated. The isopropyl alcohol-ammonia mixture contained isopropyl alcohol–58% NH_4OH–water 70:6:24.[12]

Preparation of Mononucleotides.—A solution was prepared having per milliliter 50 micromoles of deoxyribonucleic acid phosphorus (about 14 mg. of nucleic acid), 0.025 mg. of the sodium salt of thymol blue and 0.01 mmole of calcium acetate, adjusted to *p*H 8.5 by the addition of 0.1 *N* NaOH. One million units per ml. of trichloroacetic acid–ethanol purified nuclease from strain SA-B[2] was added and incubation carried out at 45°, with additions of more alkali to maintain *p*H about 8.5 until it was judged that enzyme action, as indicated by acid formation, had ceased.[2] A digest of the dialyzed deoxyribonucleic acid containing 47.5 mmoles of phosphorus (about 13.5 g. nucleic acid) was fractionated on a column of Dowex-1-2x formate, 15 × 5 cm., eluting with 0.03 *M* ammonium formate buffer, *p*H 4.5. The mononucleotides were concentrated on Dowex-1-8x formate at high *p*H and extracted with small quantities of ammonium formate solution at low *p*H, then neutralized with ammonium hydroxide. Ammonium formate was nearly quantitatively removed by vacuum sublimation.

Dephosphorylation of Mononucleotides.—The conditions for digestion with crude snake venom were[11,12]: 0.15 *M* glycine (*p*H 9), 0.02 *M* $MgCl_2$, 0.02 *M* KCl, substrate 2 mg./ml., venom 4 mg./ml., 37°, 24 hr. Rye grass 3'-nucleotidase was used as directed by Shuster and Kaplan,[15] and units were calculated by their method.[15] Incubation with cerium hydroxide was carried out as described by Bamann,[13] incubating at 37° for 24 hr.

Results

In the chromatography, deoxyuridylic acid overlapped substantial portions of the deoxycytidylic and thymidylic peaks, so that yields of the latter mononucleotides were low.

Although inorganic phosphorus could not be detected in the digest, small quantities of ultraviolet absorbing material, largely nucleosides (from terminal residues of the polynucleotide chains?) emerged before the deoxycytidylic acid. No peak for 5-methyldeoxycytidylic acid was observed. Deoxyguanylic acid appeared in relatively smaller quantity than in previous digests.[2]

Data from the isopropyl alcohol–ammonia and ion-exchange chromatographic experiments, especially, tended to rule out contamination of the mononucleotides with other types of nucleic acid degradation products. Results from all chromatography in which separation of mononucleotide isomers was achieved indicated that there was no contamination by the 5'-phosphates. All the samples were completely resistant to dephosphorylation by crude snake venom. Most of the preparations appeared to be mixtures of the mono- and diammonium salts.

Deoxyadenosine 3'-ammonium phosphate, 650 mg., had 8.2% phosphorus (calculated for diammonium salt, phosphorus 8.4%) and ammonium ion 1.45 moles per mole of phosphorus. In water at *p*H 6.5, optical density ratios were: 250/260, 0.79; 250/270, 1.17; 280/260, 0.14; ϵ 260 was 15,600. With the diphenylamine reagent[20] color development was slower than with the 5'-isomer but the final optical density after ten minutes' heating was only 10% less. The R_f, descending, on Whatman #2, in isopropyl alcohol–ammonia was 0.35 (of the 5'-isomer also 0.35). A good separation of the isomers was obtained with 80% saturated ammonium sulfate–2% isopropyl alcohol[21] (*p*H adjusted to 6.0 with added ammonium hydroxide), descending, as shown in Table I.

TABLE I

R_f VALUES OF ADENINE MONONUCLEOTIDES IN AMMONIUM SULFATE–ISOPROPYL ALCOHOL

Point of esterification	Ribo	Deoxy
3'	0.16	0.15
2'	.25	..
5'	.27	0.21

The deoxyadenylic acid isomers were chromatographed on Dowex 1, 8x, chloride, 12.5 cm. × 0.9 cm.[2], eluting with 0.002 *M* HCl, *p*H 2.8. Volkin, *et al.*,[22] had shown that in this system 5'-adenylic acid and 5'-deoxyadenylic acid move together, followed by 2'-adenylic acid and 3'-adenylic acid, in that order. Two peaks emerged, in the positions of the first two peaks shown in Fig. 1. The material of the first peak, but not the second, was de-

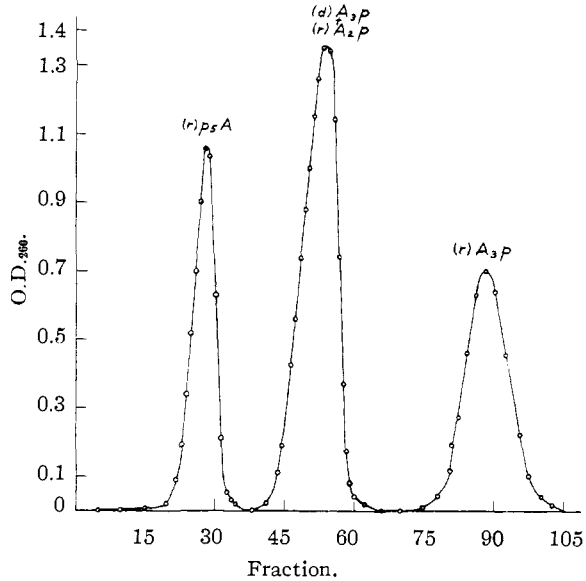

Fig. 1.—Chromatography of 1.7 mg. of deoxyadenosine 3'-phosphate, and the following isomers of adenylic acid: 2', 1.9 mg.; 3', 1.9 mg.; 5', 1.6 mg. Conditions are given in the text. (d)A3p is deoxyadenosine 3'-phosphate, (r)A2p is adenosine 2'-phosphate, etc.

phosphorylated by snake venom. The ratio of the *R* value of the slower mononucleotide to that of the faster was 0.53 (from the number of fractions to the peaks). This may be compared to the ratio of 0.59 found with synthetic deoxyadenylic acid isomers, in a similar chromatographic system, by Hayes, Michelson and Todd.[23] When the experiment was repeated with the 3'-isomer alone, no ultraviolet absorbing material was found in the position of the first peak. When (Fig. 1) the three possible ribomononucleotides and 3'-deoxyadenylic acid were chromatographed together, the deoxymononucleotide (identified by the Dische reaction) emerged in the same peak as 2'-adenylic acid

(20) Z. Dische, *Mikrochem.*, **8**, 4 (1930).

(21) R. Markham and J. D. Smith, *Biochem. J.*, **49**, 401 (1951).

(22) E. Volkin, J. X. Khym and W. E. Cohn, THIS JOURNAL, **73**, 1533 (1951).

(23) D. H. Hayes, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 808 (1955).

Deoxyguanosine 3′-ammonium phosphate, 35 mg., had 6.5% phosphorus (calculated for monoammonium salt, phosphorus 8.5%) and ammonium ion 1.1 moles per mole phosphorus. In water at *p*H 6.5 optical density ratios were: 250/260, 1.2; 250/270, 1.46; 280/260, 0.67; ϵ 260 was 12,000. After some unsuccessful attempts to separate the deoxyguanylic isomers in KH_2PO_4–isoamyl alcohol,[24] a modification was developed which gave results comparable to those obtained elsewhere with the synthetic isomers[23] (Table II).

TABLE II

R_f, DEOXYGUANYLIC ISOMERS, IN 5% KH_2PO_4

	3′	5′	3′/5′
Natural	0.51	0.55	0.93
Synthetic[23]	0.60	0.65	0.92

The paper was cut in such a way as to form a narrow tail which entered a dish containing 5% KH_2PO_4 and drew the solution upward. The spots of mononucleotide were placed well above the lower end of the sheet of paper, in the region which was protected against excessive imbibition of the aqueous fluid. Although the development of the chromatogram was thus much retarded, the spots were compact and results were reproducible. The use of the organic layer seemed to offer no advantage and was discontinued.

Thymidine 3′-ammonium phosphate, 337 mg., had 9.0% phosphorus (calculated for monoammonium salt, phosphorus 9.1%) and ammonium ion 1.5 moles per mole phosphorus. In water at *p*H 6.5 the optical density ratios were: 250/260, 0.663; 250/270, 0.63; 280/260, 0.70; ϵ 260 was 8,700. The R_f, descending, in isopropyl alcohol–ammonia was 0.24 (of the 5′-isomer 0.23). The R_f, descending, in 2 *N* HCl–1-propanol 1:3,[25] was 0.82, as compared with 0.84 for the synthetic 3′-isomer, and 0.74 for the (natural) 5′-isomer; ratio of R_f of 5′- to 3′-isomer, 0.89. Michelson and Todd[25] with this solvent, ascending, obtained R_f values having a ratio of 0.885 for the isomers.

Deoxycytidine 3′-(ammonium?) phosphate, 40 mg., had 9.4% phosphorus (calculated for deoxycytidylic acid, phosphorus 10.1%) and ammonium ion 0.2 mole per mole phosphorus. In water at *p*H 6.5, optical density ratios were: 250/260, 0.84; 250/270, 0.706; 280/260, 0.93; ϵ 260 was 8,700. The R_f, ascending, in isopropyl alcohol–ammonia was 0.12 (of synthetic deoxycytidine 3′-phosphate, 0.12; of 5′-deoxycytidylic acid, 0.12).

On Dowex 1, 8x, formate, 7 cm. × 0.9 cm.^{2}, eluting with 0.01 *M* formic acid, *p*H 3.0, deoxycytidylic acid isomers were separated (Fig. 2), while a mixture of natural and synthetic 3′-deoxycytidylic acids emerged as a single peak. The 280/260 ratios for each deoxyribomononucleotide were the same as those found by Michelson and Todd[12] in 0.015 *M* formic acid. It is interesting to note that these authors found that 2′- and especially 3′-cytidylic acid moved more slowly than 3′-deoxycytidylic acid on Dowex-1-formate. In contrast, cytidine and deoxycytidine 5′-phosphates moved at very nearly the same speed.

(24) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(25) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2632 (1955).

Dephosphorylation by Cerium Hydroxide.—In confirmation of Bamann's report,[13] the 5′-isomer of adenylic acid was relatively resistant to the action of the rare earth "phosphatase model" system as compared to 3′-adenylic acid (or to 5′-deoxyadenylic acid, in agreement with previous findings[26] in the cytidylic series). There was no significant difference in the susceptibility of the two isomers of deoxyadenylic acid to dephosphorylation (Table III). The other 5′-deoxymononucleotides, similarly, were attacked at about the same rate as the corresponding 3′-deoxynucleotides.

TABLE III

DEPHOSPHORYLATION OF VARIOUS ADENINE MONONUCLEOTIDES BY CERIUM HYDROXIDE, %/HR.

Ribo	
3′	2.9
5′	0.5
Deoxyribo	
3′	1.1
5′	1.3

Dephosphorylation by 3′-Nucleotidase.—The preparation of this enzyme[14,15] which was supplied by Dr. Kaplan attacked 3′-adenylic acid very much more rapidly than 5′-adenylic acid (Table IV). On the contrary, 3′-deoxyadenylic acid was attacked only about twice as fast as its 5′-isomer (Table IV), and similar resistance was found with the other 3′-deoxymononucleotides. An attempt was made, without success, to abolish the difference between the rate of release of inorganic phosphate from the 3′-phosphates of adenosine and of deoxyadenosine, by increasing the substrate concentration (Table V).

TABLE IV

RYE GRASS 3′-NUCLEOTIDASE ACTIVITY WITH VARIOUS ADENINE-CONTAINING MONONUCLEOTIDES

Ribo	Units
3′	7.2
5′	0.07
Deoxyribo	
3′	0.17
5′	0.07

TABLE V

ACTION OF 3′-NUCLEOTIDASE WITH VARYING SUBSTRATE CONCENTRATIONS[a]

Adenosine 3′-phosphate, μmole/ml.		Deoxydenosine 3′-phosphate, μmole/ml.	
Present	Cleaved	Present	Cleaved
4.2	0.65	5.0	0.05
26.0	.85	24.0	.07
42.0	.97	97.0	.09

[a] Crude enzyme, 1.25 mg./ml., ten minutes, 37°.

The data shown in Table V were obtained with the crude enzyme prepared in this Laboratory. The substrate concentrations of 4.2 and 5.0 micromoles per milliliter were practically the same as those used for the determinations shown in Table IV, but the rate of attack on the deoxymononucleotide was relatively lower (Table IV) when Dr. Kaplan's preparation was used. This evidence of fractionation of different phosphatase activities

(26) M. Shimomura and F. Egami, *Bull. Chem. Soc. Japan*, **26**, 263 (1953).

suggested that the 3′-nucleotidase itself, if sufficiently purified, would prove to be entirely specific for ribose compounds.

Discussion

In certain respects, the deoxyribomononucleotides released by micrococcal nuclease were shown to resemble the corresponding 2′- rather than the 3′-ribomononucleotides. On the other hand, the nature of the sugar exerts little or no influence upon certain optical properties. The optical density ratio, 280/260, at *p*H 3 or below, is 2.0 for cytidine,[27–29] or deoxycytidine[12] 3′-phosphate and 2.1 for the 5′-phosphates; there is very little scatter in the data reported in the literature or in the values obtained (Fig. 2) at the summits of the elution peaks. The infrared spectra[12] of cytidine and deoxycytidine 5′-phosphates are also closely similar, as are those of 3′-deoxycytidylic acid and one form of 3′-cytidylic acid, while that of 2′-cytidylic acid differs from any of the others. These comparisons are of particular interest, inasmuch as recent X-ray diffraction studies of Alver and Furberg[30] have confirmed the generally accepted assignment of the point of esterification of cytidine 3′-phosphate.

Enzyme preparations from bovine thymus[31] and spleen[32] attack phosphodiester bonds in deoxyribonucleic acid with the formation of singly esterified phosphate at the 3′-position, as indicated by studies of the fragments, including mononucleotides, with snake venom enzymes. This activity may be due to the presence in these different tissues of a single enzyme (deoxyribonuclease II).[33,34] This nuclease differs[31,32,33] from micrococcal nuclease in respect to most properties so far studied.

Although, for some reason which remains to be explained, the bacterial and mammalian enzymes fail to degrade deoxyribonucleic acid completely to

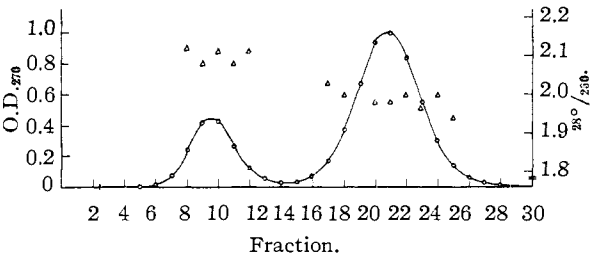

Fig. 2.—Chromatography of 0.25 mg. of 5′-deoxycytidylic acid and 0.64 mg. of 3′-deoxycytidylic acid. 280/260 values are indicated by the triangles. Conditions are given in the text.

mononucleotides, their digestion products do provide further direct evidence for the presence of the 3′-phosphodiester bond in deoxyribonucleic acid. The existence of this bond was first indicated by the isolation,[35] from acid hydrolysates, of pyrimidine derivatives identified by synthesis[36] as nucleoside 3′,5′-diphosphates. However, the yield of these nucleotides is also rather low,[37] presumably because of the prevalence of pyrimidine tracts[38–40] in the polynucleotide chains. Some method of production of 3′-deoxymononucleotides in as high a yield (over 90%)[10,41] as the 5′-isomers would be desirable.

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I have profited from conversations with Dr. M. Laskowski and Dr. P. M. Roll.

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(27) W. E. Cohn, THIS JOURNAL, **72**, 2811 (1950); see also in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Vol. I, Academic Press, New York, N. Y., 1955, p. 513.

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(30) E. Alver and S. Furberg, *Acta Chem. Scand.*, **11**, 188 (1957).

(31) Ulla-Riitta Laurila and M. Laskowski, *Federation Proc.*, **16**, 209 (1957).

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(36) C. A. Dekker, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 947 (1953).

(37) H. S. Shapiro and E. Chargaff, *Biochem. Biophys. Acta*, **23**, 451 (1957).

(38) C. Tamm, H. S. Shapiro, R. Lipshitz and E. Chargaff, *J. Biol. Chem.*, **203**, 673 (1953).

(39) A. S. Jones and D. S. Letham, *J. Chem. Soc.*, 2573 (1956); A. S. Jones, D. S. Letham and M. Stacey, *ibid.*, 2579, 2584 (1956).

(40) W. E. Cohn and E. Volkin, *Biochem. Biophys. Acta*, **24**, 359 (1957).

(41) R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.*, **198**, 293 (1952).