197. *Nucleotides. Part XIX.* Pyrimidine Deoxyribonucleoside Diphosphates.*

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Mild acid hydrolysis of herring sperm deoxyribonucleic acids yields pyrimidine nucleoside diphosphates as originally claimed by Levene and Jacobs (*J. Biol. Chem.*, 1912, **12**, 411). Thymidine-3′ : 5′ diphosphate and deoxycytidine-3′ : 5′ diphosphate have been isolated and found to be identical with synthetic products prepared by phosphorylating thymidine and deoxycytidine. Evidence has been obtained pointing to the presence of deoxy-5-methylcytidine nucleotides in the acid hydrolysates.

The isolation of pyrimidine nucleotides from mild sulphuric acid hydrolysates of thymus nucleic acid was first claimed by Levene and Mandel (*Ber.*, 1908, **41**, 1905) in 1908. Levene and Jacobs (*J. Biol. Chem.*, 1912, **12**, 411) subsequently presented evidence for the existence in acid hydrolysates of fish sperm nucleic acid of both the monophosphates and diphosphates of the pyrimidine nucleosides and were able to obtain the barium salt of thymidine diphosphate (diphosphothymidine) in crystalline form. Hydrogenation of this compound followed by mild acid hydrolysis gave dihydrothymine and a reducing phospho-sugar. Levene (*ibid.*, 1921, **48**, 119) later simplified the isolation procedure for the pyrimidine nucleoside phosphates, but, although his conditions for hydrolysis remained the same, he was now able to isolate only the diphosphates.

* Part XVIII, *J.*, 1952, 3681.

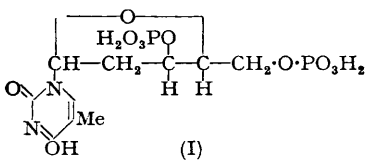
Thannhauser and Ottenstein (*Z. physiol. Chem.*, 1921, **114**, 39), using a saturated aqueous solution of picric acid for hydrolysis of thymus nucleic acid, were likewise able to obtain pyrimidine nucleotides which, as brucine salts, gave analytical values corresponding to those required by the brucine salts of the mono- and the di-phosphates. The same results were obtained with methanolic hydrochloric acid hydrolysis by Thannhauser and Blanco (*ibid.*, 1926, **161**, 116) although these authors noted that this method produced more diphosphate and less monophosphate.

These pyrimidine nucleotides were originally described as hexose derivatives but, subsequently to Levene and London's (*J. Biol. Chem.*, 1929, **83**, 793) elucidation of the structure of the sugar obtained from thymus nucleic acid as 2-deoxy-D-ribose, the nucleotides were recognised as phosphoric acid esters of pyrimidine deoxyribosides (Levene and Bass, " Nucleic Acids," Chem. Catalog Co., New York, 1931, p. 204).

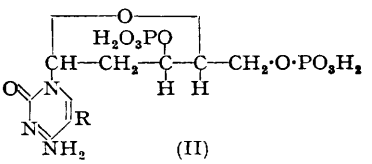
Bredereck and Caro (*Z. physiol. Chem.*, 1938, **253**, 170) questioned the results of Levene and Jacobs (*loc. cit.*) and Thannhauser and Blanco (*loc. cit.*). Using either method of hydrolysis, they isolated primarily the monophosphates of the pyrimidine nucleosides. Although they did not exclude the diphosphates as possible components of acid hydrolysates they stated that conclusions as to the structure of deoxyribonucleic acid should not be based on diphosphate formation under these conditions of cleavage. In a reply to this paper, Levene (*J. Biol. Chem.*, 1938, **126**, 63) criticised Bredereck and Caro (*loc. cit.*) for basing their conclusions on the analyses of brucine salts and amorphous barium salts, the latter having been identified by their barium content only. Levene at this time also repeated the elementary analysis of thymidine diphosphate and cystosine deoxyriboside diphosphate and reported figures which were more in accord with the calculated values.

In connection with the general problem of nucleic acid structure, it was clearly desirable to establish whether or not pyrimidine nucleoside disphosphates were substantial products of acid hydrolysis of deoxyribonucleic acids. This we have done by means of ion-exchange chromatography (Cohn, *J. Amer. Chem. Soc.*, 1950, **72**, 1471). Commercial herring sperm deoxyribonucleic acid was hydrolysed by 2 hours' refluxing with 2% sulphuric acid and the mixture of nucleotide barium salts isolated by hot precipitation, as described by Levene (*loc. cit.*). On chromatography on an anion-exchange resin (Dowex-2; chloride form) these salts yielded two main fractions, corresponding to thymidine diphosphate (I) and deoxycytidine diphosphate (II; R = H). The fore-fractions of the latter were always contaminated with small amounts of a substance which was almost certainly a deoxy-5-methylcytidine derivative, possibly deoxy-5-methylcytidine diphosphate (II; R = Me) from the higher optical density ratio (280/260 mμ), analogous to that of deoxy-5-methylcytidine monophosphate reported by Cohn (*J. Amer. Chem. Soc.*, 1951, **73**, 1539). There appeared to be no more than traces of other substances present in the crude barium salts.

Partial fractionation by ion-exchange chromatography of the mother-liquors remaining after hot precipitation of the mixed barium salts of the diphosphates gave some further indication of the nature of the other products obtained by acid hydrolysis of the nucleic acid. Although confirmation by isolation of the individual substances is lacking, the elution positions and optical density ratios indicate the presence of monophosphates of thymidine and deoxycytidine, as well as the diphosphates and small amounts of more complex products, probably in the main dinucleotides of mixed composition. The ratio of monophosphate to diphosphate in the case of the deoxycytidine indicates that the diphosphate is by far the most abundant deoxycytidine derivative in the hydrolysate, a finding which in part confirms Levene's observations (*loc. cit.*).



(I)



(II)

Thymidine was phosphorylated with dibenzyl phosphorochloridate (chlorophosphonate) in the usual way, catalytic hydrogenation of the reaction product giving a mixture of

thymidine monophosphates and thymidine diphosphate, which could be separated either by means of an anion-exchange column or by boiling a concentrated aqueous solution of the barium salts; the barium salt of the diphosphate separating as a granular precipitate. Attempts to separate the monophosphate fraction into two components (the 3′- and the 5′-phosphates) under conditions which separate satisfactorily mixtures of the three isomeric uridine phosphates (Cohn and Volkin, *Nature*, 1951, **167**, 483) were unsuccessful, only one peak being obtained on elution. The synthetic phosphate which from its mode of preparation and stability we formulate as thymidine 3′ : 5′-diphosphate (I), was shown to be identical with the thymidine diphosphate isolated from the deoxyribonucleic acid hydrolysis by comparison of the dibarium salts (infra-red spectra) and tetrabrucine salts (*X*-ray powder photography). It is of interest that, like adenosine-2′(3′) : 5′ diphosphate (Kornberg and Pricer, *J. Biol. Chem.*, 1950, **186**, 557), thymidine-3′ : 5′ diphosphate is not attacked by bull semen phosphatase. An analogous phosphorylation of deoxycytidine yielded deoxycytidine-3′ : 5′ diphosphate, the brucine salt of which was identical with the brucine salt of the deoxycytidine diphosphate obtained from deoxyribonucleic acid.

In Part X (Brown and Todd, *J.*, 1952, 52) it was observed that, if deoxyribonucleic acids are regarded as essentially 3′ : 5′-linked polynucleotides, then a certain amount of pyrimidine nucleoside-3′ : 5′ diphosphates would be expected to arise on acid hydrolysis by a degree of random fission. Although the experiments described in this paper are qualitative rather than quantitative, the amount of the diphosphates produced is surprisingly high for them to have been formed solely by random fission. It suggests indeed that they may arise in part from those positions in the polynucleotide chain where a pyrimidine nucleoside residue is adjacent to a purine nucleoside residue. The initial step in mild acid hydrolysis of the nucleic acid is probably removal of purine residues, leaving free deoxyribose residues attached through phosphate groups to other similar residues and to pyrimidine nucleoside residues. In the latter case further hydrolysis might well lead to rupture between the deoxyribose molecule and phosphorus so that pyrimidine nucleosides bearing two phosphate groups would be produced. It is hoped to return to this point in a later paper, and to examine its significance in connection with the sequence of nucleoside residues in the polynucleotide chain.

Experimental

Separation of Thymidine Diphosphate and Deoxycytidine Diphosphate from Acid Hydrolysates of Herring Sperm Deoxyribonucleic Acid.—An aqueous solution of the barium salts (1·3 g.), obtained after hydrolysis and hot precipitation by Levene's method (*loc. cit.*), was run slowly on to a column (10 cm. × 5 sq. cm.) of Dowex-2 anion-exchange resin (mesh size 200—400) in the chloride form, and the column washed with water until barium ion was no longer removed. Elution was continued with 0·008N-hydrochloric acid (approx. 2 c.c./min.), and the eluate collected in 20-c.c. fractions in an automatically operated fraction-collector. The progress of elution was followed by estimating the optical-density ratio of the fractions at 260 and 280 mμ. After the first major component (deoxycytidine diphosphate, optical-density ratio 280/260 mμ = 2·05) had been eluted, the second component (thymidine diphosphate, optical-density ratio = 0·65) was removed from the column with 0·075N-hydrochloric acid.

The fractions containing deoxycytidine diphosphate were united and concentrated at room temperature under reduced pressure to small bulk (50 c.c.), and the product was isolated by freeze-drying this solution. The residue was dissolved in a little water, and a solution of excess of brucine in methanol added. After evaporation to dryness under reduced pressure, the resultant crystalline mass was recrystallised twice from 80% ethanol and once from 35% ethanol, giving large hydrated needles of the *tetrabrucine* salt (325 mg.) of deoxycytidine-3′ : 5′ diphosphate, sinters at 180°, melts at 185° (Found, in air dried material: C, 51·2; H, 6·7; N, 6·6; P, 2·5; loss at 130°/5 × 10^{-5} mm., 18·2. $C_9H_{15}O_{10}N_3P_2,4C_{23}H_{26}O_4N_2,23H_2O$ requires C, 51·0; H, 6·9; N, 6·5; P, 2·6; $23H_2O$, 17·4. Found, in material dried at atmospheric pressure over $CaCl_2$: C, 57·5; H, 6·3. $C_9H_{15}O_{10}N_3P_2,4C_{23}H_{26}O_4N_2,8H_2O$ requires C, 57·5; H, 6·4. Found, in material dried at 130°/5 × 10^{-5} mm. for 12 hours: C, 61·7; H, 5·8; N, 7·6. $C_9H_{15}O_{10}N_3P_2,4C_{23}H_{26}O_4N_2$ requires C, 61·8; H, 6·1; N, 7·8%). Material regenerated from the brucine salt had the following spectral characteristics: $\lambda_{max.}$ 280 mμ; $\lambda_{min.}$ 240—241 mμ; optical-density ratios 240/260 mμ = 0·25, 280/260 mμ = 2·05 in 0·05N-HCl. $\lambda_{max.}$ 271—272 mμ;

$\lambda_{min.}$ 250—251 mμ; optical-density ratios 240/260 mμ = 0·96, 280/260 mμ = 0·995 in 0·05-N-NaOH.

The fractions containing thymidine diphosphate were united and neutralised with saturated aqueous barium hydroxide. Excess of lead acetate solution (20%) was then added, and the lead salt collected by centrifugation, washed several times with water, and finally decomposed in the usual manner with hydrogen sulphide at room temperature. After aeration, the solution of thymidine diphospate was treated with a slight excess of aqueous barium hydroxide, then neutralised with carbon dioxide (final vol. approx. 500 c.c.). Inorganic matter was removed by centrifugation and the supernatant liquid concentrated under reduced pressure to 15 c.c. The solution was then boiled for several minutes, causing the separation of a white granular precipitate which was collected and dried (570 mg.) (Found, in crude material dried at 110°/1 mm. for 12 hours: C, 19·3; H, 2·7. Calc. for $C_{10}H_{12}O_{11}N_2P_2Ba_2$: C, 17·8; H, 1·8%). Purification was accomplished by redissolution in the minimum amount of cold water, removal of a small amount of insoluble material at the centrifuge, concentration *in vacuo*, and reprecipitation by heat. This process was repeated several times. The purified dibarium thymidine-3′ : 5′ diphosphate consisted of tiny, colourless, striated globules (Found: C, 18·1; H, 2·3; N, 3·9; P, 8·9. Calc. for $C_{10}H_{12}O_{11}N_2P_2Ba_2$: C, 17·8; H, 1·8; N, 4·2; P, 9·2%). Light absorption: $\lambda_{max.}$ 266 mμ; $\lambda_{min.}$ 232—233 mμ; optical-density ratios 240/260 mμ = 0·35, 280/260 mμ = 0·65 in 0·05N-HCl. $\lambda_{max.}$ 266 mμ; $\lambda_{min.}$ 244 mμ; optical-density ratios 240/260 mμ = 0·75, 280/260 mμ = 0·63 in 0·05N-NaOH.

The *tetrabrucine* salt crystallised in small needles from 35% ethanol; it softens at 176° and melts at 182—184° [Found, in air-dried material: C, 53·0; H, 6·7; N, 6·1; P, 2·6; loss at 130°/5 × 10^{-5} mm., 14·6. $C_{10}H_{16}O_{11}N_2P_2,4C_{23}H_{26}O_4N_2,18H_2O$ requires C, 53·2; H, 6·8; N, 6·1; P, 2·7; $18H_2O$, 14·1. Found, in anhyd. material (slight decomp.): C, 60·4; H, 6·2. $C_{10}H_{16}O_{11}N_2P_2,4C_{23}H_{26}O_4N_2$ requires C, 61·9; H, 6·1. Found, in material dried at 1 atm. over $CaCl_2$: C, 54·3; H, 7·2. $C_{10}H_{16}O_{11}N_2P_2,4C_{23}H_{26}O_4N_2,14H_2O$ requires C, 54·9; H, 6·6. (Levene, *loc. cit.*, reports the tetrabrucine salt as crystallising with $14H_2O$.) Found, in material dried at 110°/1 mm. for 18 hours: C, 58·2; H, 6·1; N, 6·7. $C_{10}H_{16}O_{11}N_2P_2,4C_{23}H_{26}O_4N_2,7H_2O$ requires C, 58·2; H, 6·4; N, 6·7%].

Action of Bull Semen Phosphates on Thymidine-3′ : 5′ Diphosphate (cf. Carter, *J. Amer. Chem. Soc.*, 1951, **73**, 1537).—Thymidine-3′ : 5′ diphosphate (prepared from the barium salt) and adenosine-5′ phosphate were separately dissolved in veronal buffer (pH 6·2) to give approx. 0·2% solutions. The enzyme solution was undiluted, centrifuged, bull semen. Substrate (0·25 c.c.), 0·01M-magnesium chloride solution (0·05 c.c.), and enzyme (0·1 c.c.) were incubated at 37° for 1 hour. Inorganic-phosphorus determinations by Allen's method (*Biochem. J.*, 1940, **34**, 858) showed complete dephosphorylation of the adenosine-5′ phosphate and negligible dephosphorylation of the thymidine-3′ : 5′ diphosphate.

Phosphorylation of Thymidine.—A solution of thymidine (2·23 g.; dried for 12 hours at 110°/1 mm.) in dry pyridine (30 c.c.) at −40° was treated with dibenzyl phosphorochloridate (from 10 g. of dibenzyl phosphite; Atherton, Openshaw, and Todd, *J.*, 1945, 382) and kept just above the m. p. of the mixture for 6 hours, and then left at room temperature overnight. Water (20 c.c.) and sodium carbonate (5 g.) were added, and the mixture was evaporated to dryness under reduced pressure. The residue was dissolved in chloroform, washed with aqueous sodium hydrogen carbonate and then with water, and dried (Na_2SO_4); removal of the solvent under reduced pressure gave a gum which was evaporated twice with ethanol and finally dissolved in a small volume of ethanol. Addition of ether (300 c.c.) gave a gummy precipitate which was washed with ether by decantation, dissolved in acetone, and evaporated to a cream-coloured glass (5·6 g.) under reduced pressure.

A solution of the glass (4 g.) in aqueous ethanol (200 c.c. of 50%) was hydrogenated at room temperature and atmospheric pressure, at a mixture of palladium and palladised charcoal catalysts. Catalyst was removed by filtration and the solution concentrated to small volume under reduced pressure. Water (100 c.c.) was added, then saturated barium hydroxide solution to pH 8. After neutralisation with carbon dioxide and centrifugation, the solution was evaporated to small bulk *in vacuo* and boiled for several minutes. The granular precipitate formed was filtered off and the process repeated after concentration of the filtrate, to give a total of 1·35 g. of crude barium salt of thymidine-3′ : 5′ diphosphate. The final mother-liquors were examined on an anion-exchange column (Dowex-2, chloride form) and found to contain mainly mononucleotide material. The synthetic barium thymidine-3′ : 5′ diphosphate was purified as described for the natural nucleotide (Found: C, 18·1; H, 2·1; N, 3·9; P, 8·8. Calc. for $C_{10}H_{12}O_{11}N_2P_2Ba_2$: C, 17·8; H, 1·8; N, 4·2; P, 9·2%). Infra-red spectra of the natural and

the synthetic barium salt were identical. The tetrabrucine salt softened at 176°, and melted at 182—184° undepressed in admixture with the tetrabrucine salt of natural thymidine diphosphate (Found, in air-dried material: C, 53·1; H, 7·0; N, 6·1. $C_{10}H_{16}O_{11}N_2P_2,4C_{23}H_{26}O_4N_2,18H_2O$ requires C, 53·2; H, 6·8; N, 6·1%). *X*-Ray powder photographs of the natural and the synthetic tetrabrucine salt were identical.

Phosphorylation of Deoxycytidine.—A solution of deoxycytidine (0·97 g.) in dry pyridine (50 c.c.) was treated with dibenzyl phosphorochloridate (from 5 g. of dibenzyl phosphite) and worked up as in the phosphorylation of thymidine. After hydrogenation, the mixture was adjusted to pH 9 and run on to a Dowex-2 column (chloride form). Mononucleotide material was removed with 0·005N-hydrochloric acid, then the diphosphate eluted with 0·009N-acid. From the fractions containing deoxycytidine-3′ : 5′ diphosphate the *tetrabrucine* salt was prepared as described for the natural product; it had m. p. 185° after sintering at 180°, undepressed in admixture with the brucine salt of natural deoxycytidine-3′ : 5′ diphosphate (Found, in air-dried material: C, 50·9; H, 6·4; N, 6·5. $C_9H_{15}O_{10}N_3P_2,4C_{23}H_{26}O_4N_2,23H_2O$ requires C, 51·0; H, 6·9; N, 6·5%). *X*-Ray powder photographs of the natural and synthetic specimens were identical, as were the infra-red spectra.

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