Nucleotides Part XXXII. Synthesis of a Dithymidine Dinucleotide Containing a 3′ : 5′-Internucleotidic Linkage.*

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A dithymidine dinucleotide (V) has been synthesised by condensing 3′-*O*-acetylthymidine with thymidine 3′-(benzyl phosphorochloridate) 5′-(dibenzyl phosphate) and subsequently removing the protecting groups. This represents the first preparation of a dinucleotide by chemical means and since the synthetic material behaves towards enzymes exactly as the dinucleotidic fragments obtained by degrading deoxyribonucleic acids the postulate of a 3′ : 5′-internucleotidic linkage in the latter is further confirmed. Thymidine-3′ thymidine-5′ phosphate has also been prepared and by-products isolated include a dinucleoside pyrophosphate and a dinucleotide pyrophosphate.

In previous papers of this series we have described the preparation of the 3′- and 5′-phosphates of the natural deoxyribonucleosides thymidine (I; R = R′ = H) (Michelson and Todd, *J.*, 1953, 951), deoxycytidine (Michelson and Todd, *J.*, 1954, 34), deoxyadenosine and deoxyguanosine (Hayes, Michelson, and Todd, *J.*, 1955, 808), and have identified the

* Part XXXI, *J.*, 1955, 2206.

5′-phosphates with the deoxyribonucleotides obtained by enzymic degradation of deoxyribonucleic acids, thus confirming the structures proposed by Carter (*J. Amer. Chem. Soc.*, 1951, **73**, 1573). The formulation of the deoxyribonucleic acids as 3′ : 5′-linked linear polynucleotides (cf. ***inter al.***, Brown and Todd, *J.*, 1952, 52) is supported, *e.g.*, by this identification and by the isolation of the 3′ : 5′-diphosphates of thymidine and deoxycytidine from acid hydrolysates (Dekker, Michelson, and Todd, *J.*, 1953, 947). As a result of these studies it became possible to proceed to the next phase of our synthetic programme and to attempt the preparation of 3′ : 5′-linked dideoxyribonucleotides by unambiguous routes so that they could be compared in their behaviour with dinucleotides occurring in deoxyribonuclease digests of deoxyribonucleic acids. A number of such dinucleotides have been separated from deoxyribonuclease digests in solution, although not isolated in substance, and their behaviour towards various enzymes has been described (Markham and Smith, *Biochim. Biophys. Acta*, 1952, **8**, 350; Sinsheimer, *J. Biol. Chem.*, 1954, **208**, 444). Hitherto no synthesis of a dinucleotide by chemical as distinct from enzymic methods has ever been accomplished either in the ribo- or the deoxyribo-nucleotide series.

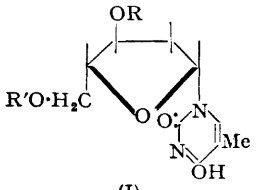
In exploratory work we endeavoured to prepare a dinucleoside phosphate containing the 3′ : 5′-internucleotidic linkage by treating the silver salt of deoxycytidine-3′ benzyl phosphate with 3′-*O*-acetyl-5′-deoxy-5′-iodothymidine under a variety of conditions; all attempts failed, however, and other methods had to be sought. Hayes, Michelson, and Todd (*loc. cit.*) prepared the monoacetyl derivatives of deoxyadenosine and deoxyguanosine by partial deacetylation of the corresponding 3′ : 5′-diacetyl compounds. The same method, applied to 3′ : 5′-di-*O*-acetylthymidine (I; R = R′ = Ac) yielded a mixture of 5′- and 3′-*O*-acetylthymidine which were separated by countercurrent distribution. When treated with *O*-benzylphosphorous *OO*-diphenylphosphoric anhydride in presence of 2 : 6-lutidine, 5′-*O*-acetylthymidine yielded 5′-*O*-acetylthymidine-3′ benzyl phosphite [I; R = PHO(O·CH_2Ph), R′ = Ac] as an unstable resin which could be converted into 5′-*O*-acetyl thymidine-3′ benzyl phosphorochloridate [I; R = PClO(O·CH_2Ph), R′ = Ac] by reaction with *N*-chlorosuccinimide. No attempt was made to purify the phosphorochloridate; instead it was prepared in solution from the phosphite, and it was used immediately to minimise decomposition. In small-scale preliminary experiments the phosphorochloridate appeared to react satisfactorily with 3′-*O*-acetylthymidine, giving a product containing material with the paper-chromatographic behaviour expected of a dinucleoside phosphate. Reaction of the phosphorochloridate with salts of *OO*-diphenylphosphoric acid or of trifluoroacetic acid to give mixed anhydrides, followed by treatment of these with 3′-*O*-acetylthymidine proved ineffective although a method of this type had been successfully employed in this laboratory by Dr. R. H. Hall for preparing di(ribonucleoside-5′) phosphates.

3′-*O*-Acetylthymidine was therefore allowed to react with crude 5′-*O*-acetylthymidine-3′ benzyl phosphorochloridate in presence of excess of 2 : 6-lutidine. Acetyl and benzyl groups were removed by hydrolysis and the product subjected to anion-exchange chromatography (Dowex 2 resin; chloride form). Elution with 0·01N-hydrochloric acid gave a mixture of thymidine-3′ phosphate and thymidine-3′ thymidine-5′ phosphate (II) * which were separated from one another by fractional precipitation of their calcium salts. Further elution with 0·1N-hydrochloric acid yielded di(thymidine-3′) P^1P^2-pyrophosphate (III) isolated as its calcium salt. The structure allotted to each of these products follows from their mode of preparation, analysis, and properties. The production of some dinucleoside pyrophosphate was not unexpected since hydrolysis of a phosphorochloridate in presence of base is a well-known method for preparing symmetrical pyrophosphates.

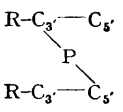
The successful synthesis of thymidine-3′ thymidine-5′ phosphate encouraged us to undertake the synthesis of a true dinucleotide by an analogous route. 3′-*O*-Acetylthymidine-5′ dibenzyl phosphate was deacetylated with methanolic ammonia and the thymidine-5′ dibenzyl phosphate so obtained was treated with *O*-benzylphosphorous *OO*-diphenylphosphoric anhydride to give the corresponding 3′-phosphite [I; R = PHO(O·CH_2Ph), R′ = PO(O·CH_2Ph)$_2$]. This was not exhaustively purified but was treated directly with

* In formulæ (II—V) the expression R–$C_{3'}$–$C_{5'}$ has been adapted as an abbreviation for thymidine, $C_{3'}$ and $C_{5'}$ being the only sites in the deoxyribose residue bearing hydroxyl groups; P symbolises a phosphate and –P–O–P– a pyrophosphate residue.

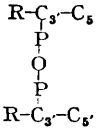
N-chlorosuccinimide, and the crude phosphorochloridate obtained was brought into reaction with 3′-*O*-acetylthymidine in presence of 2 : 6-lutidine. One benzyl group is readily removed by acid hydrolysis from a phosphoric triester containing one or more benzyl groups; a second benzyl group is not easily removed in this way and it is usually preferable to perform this operation by catalytic hydrogenation. Since the fully protected dinucleotide formed by the reaction described would contain two phosphoric triester groupings, one (the internucleotidic linkage) bearing one, and the other (the terminal group) bearing two



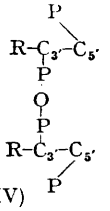
(I)



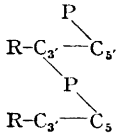
(II)



(III)



(IV)



(V)

benzyl groups, the crude reaction product was treated first with acid to remove two benzyl groups, then with barium hydroxide to effect deacetylation, and was finally hydrogenated as barium salt to complete the debenzylation. Paper chromatography showed that in addition to a trace of thymidine-5′ phosphate the product contained thymidine-3′ : 5′ diphosphate and two other substances, apparently a dinucleotide and a dinucleotide pyrophosphate. When an aqueous solution of the mixed barium salts was brought to pH 7·7 the dibarium salt of the dinucleotide pyrophosphate (IV) separated as a white precipitate. Although it contained no other nucleotide material, this salt could not be completely freed from barium phosphate and so gave unsatisfactory analytical values; the identity of its main constituent is, however, not in doubt. The mother-liquor remaining after removal of the dinucleotide pyrophosphate was subjected to anion-exchange chromatography (Dowex 2 resin; chloride form). Elution with 0·08N-hydrochloric acid gave a mixture of thymidine-3′ : 5′ diphosphate (I; R = R′ = PO_3H_2) and the dithymidine dinucleotide (V). These were separated by making use of the fact that calcium thymidine-3′ : 5′ diphosphate is much less soluble in hot water than in cold. The calcium salt of the dinucleotide (V) crystallised from water in clusters of needle-like hydrated prisms. This salt, like all the others described in this paper, could be dehydrated at 110° but was extremely hygroscopic when anhydrous; accordingly all analyses were carried out on the hydrated salts after allowing them to come to equilibrium in air.

The various thymidine derivatives described in this paper can be characterised and distinguished by paper chromatography. Distinction is also possible by paper electrophoresis in two solvents, 0·1M-potassium dihydrogen phosphate and 0·1M-disodium hydrogen phosphate; the former solvent suppresses the dissociation of secondary phosphoric groups so that migration is dependent only on the number of primary phosphoric dissociations and molecular weight, whereas in the alkaline system both primary and secondary dissociations play a part. Towards the mixture of enzymes contained in rattlesnake venom (*Crotalus atrox*) and in prostate extract the synthetic compounds behaved in the expected manner. The results are summarised in the Table. The complexities of nomenclature encountered in polynucleotides make it desirable that some abbreviated description of general applicability be devised. In the Table such an abbreviated description is used; in it T = thymidine residue, P = phosphate, and P–P = pyrophosphate, and the points of attachment of phosphate or pyrophosphate groups to thymidine are indicated by numbers. Thus the dinucleotide (V) is adequately defined by the expression T5′–P–3′T5′–P; this system of abbreviation may be applied generally in the nucleotide and polynucleotide field.

The results in the Table accord with the view that rattlesnake venom contains a 5′-nucleotidase and a phosphodiesterase, both of which are highly specific, together with a pyrophosphatase of much lower specificity (cf. Christie, Elmore, Kenner, Todd, and Weymouth, *J.*, 1953, 2947). It is worthy of note that the phosphodiesterase of rattlesnake

venom appears to be specific for esters of nucleoside-5′ phosphates; thus although it will convert both thymidine-3′ thymidine-5′ phosphate and thymidine-5′ benzyl phosphate into thymidine-5′ phosphate it is quite without action on thymidine-3′ benzyl phosphate.

Substrate	Products formed by action of: Crude rattlesnake venom	Crude prostatic phosphatase
T3′–P	No action	T
T5′–P	T	T
P–3′T5′–P	No action	T
T3′–P–5′T	T	T *
T5′–P–3′T5′–P	T	T3′–P–5′T + T
T3′–P–P–3′T	T3′–P *	T *
P–5′T3′–P–P–3′T5′–P	P–3′T5′–P *	T3′P–P–3′T + T

* Unchanged starting material also present.

Prostate extract shows phosphomonoesterase and phosphodiesterase as well as weak pyrophosphatase activity.

Purified prostate phosphomonoesterase converted the synthetic dinucleotide into thymidine-3′ thymidine-5′ phosphate, and the dinucleotide pyrophosphate into di(thymidine-3′) P^1P^2-pyrophosphate; a small amount of thymidine was also produced in the latter case. Purified rattlesnake venom phosphodiesterase converted the synthetic dinucleotide smoothly into 2 mols. of thymidine-5′ phosphate, and when applied to thymidine-3′ thymidine-5′ phosphate it gave a mixture of thymidine and thymidine-5′ phosphate although the rate of hydrolysis was much slower in this case. The results of these experiments with purified enzymes which were kindly carried out by Dr. R. Markham are the same as those reported for the dinucleotides obtained in solution after digestion of deoxyribonucleic acids with deoxyribonuclease (Markham and Smith, *loc. cit.*; Sinsheimer, *loc. cit.*) and support the view that these hydrolysis products are indeed 3′ : 5′-linked dinucleotides.

The preparation of the dinucleotide (V) represents the first chemical synthesis of a dinucleotide containing the internucleotidic linkage characteristic of the nucleic acids. The methods employed for its preparation should, with some modification, be capable of extension to the synthesis of tri- and higher poly-nucleotides of known constitution, products which would be of value in further studies on the structure of deoxyribonucleic acids and the mechanism of their hydrolytic degradation. Further work in this direction will be reported later.

Experimental

The names used below for binuclear compounds are to be regarded as temporary expedients pending evolution of a comprehensive nomenclature.

Partial Deacetylation of 3′ : 5′-*Di*-O-*acetylthymidine*.—A solution of 3′ : 5′-di-*O*-acetylthymidine (11·3 g.) Michelson and Todd, *J.*, **1955**, 816) in ethanol (1 l.) containing saturated methanolic ammonia (165 c.c.) was kept at room temperature for 9 hr., then evaporated under reduced pressure. The residual gum was separated into its four components by countercurrent distribution in an automatically operated 100-stage machine with ethyl acetate–water (100 transfers). Paper chromatography was used to determine the location of each product in the series of fractions obtained from the apparatus; the contents of appropriate fractions were then combined and evaporated and the products recrystallised. The results are summarised in the accompanying Table. All products were identified by m. p. and mixed m. p.

Peak fraction	Fractions combined	Product	Wt. (g.)	M. p.	Recryst. from
5	1—12	Thymidine	0·92	183°	MeOH
28	16—37	5′-*O*-Acetylthymidine	2·75	146	$COMe_2$–C_5H_{12}
45	38—60	3′-*O*-Acetylthymidine	1·95	176	,,
85	70—95	3′ : 5′-Di-*O*-acetylthymidine	3·18	125	EtOH

5′-O-*Acetylthymidine*-3′ *Benzyl Phosphite*.—5′-*O*-Acetylthymidine (0·74 g., 1 mol.) in methyl cyanide (10 c.c.) was added to a solution of *O*-benzylphosphorous *OO*-diphenylphosphoric

anhydride [from 1 g. (2 mols.) of benzyl dihydrogen phosphite] (Corby, Kenner, and Todd, *J.*, 1953, 3669) in dry benzene (20 c.c.). 2 : 6-Lutidine (0·66 c.c., 2 mols.) was added and the mixture set aside at room temperature overnight, moisture being excluded. Solvents were removed under reduced pressure, the residual clear gum was dissolved in chloroform, and the solution washed with water, sodium hydrogen carbonate, potassium hydrogen sulphate, and again water, then dried (Na_2SO_4). The dried solution was concentrated to small bulk and *cyclo*hexane was added. The precipitated *phosphite* was purified by redissolving it in chloroform and again precipitating it with *cyclo*hexane; dried *in vacuo* it formed a glassy foam (1·06 g.) (Found, in material dried at room temp./10^{-5} mm.: C, 51·4; H, 5·7; N, 5·9. $C_{19}H_{23}O_8N_2P$ requires C, 52·0; H, 5·3; N, 6·4%). On ascending chromatograms [Whatman No. 1; *n*-butanol–water (86 : 14)] the product had R_F 0·66. The phosphite decomposed slowly at room temperature.

Thymidine-3′ Thymidine-5′ Phosphate and Di(thymidine-3′) P¹P²-Pyrophosphate.—*N*-Chlorosuccinimide (0·49 g.) was added to a solution of 5′-*O*-acetylthymidine-3′ benzyl phosphite (1·5 g.) in a mixture of dry benzene (10 c.c.) and methyl cyanide (2 c.c.). After 3 hr. at room temperature the mixture was added to a solution of 3′-*O*-acetylthymidine (1 g.) in methyl cyanide (12 c.c.) containing 2 : 6-lutidine (2 c.c., 5 mols.) and the whole was left at room temperature for 36 hr., moisture being excluded. The mixture was evaporated under reduced pressure, the last traces of 2 : 6-lutidine being removed at a pressure of 0·1 mm. The residue was heated in water (76 c.c.), ethanol (20 c.c.), and 0·4N-sulphuric acid (4 c.c.) under reflux for 1 hr. before being concentrated to half-volume under reduced pressure. Saturated aqueous barium hydroxide was added to pH 10, the mixture left for 20 hr. at room temperature, and then dilute sulphuric acid was added to neutrality. The hot suspension was filtered, the combined filtrate and aqueous washings were concentrated to small bulk under reduced pressure and brought to pH 7·8 with aqueous barium hydroxide, and ethanol (10 vols.) was added. The precipitated mixed barium salts were collected, washed with ethanol, then ether, and dried; paper chromatography indicated that they contained thymidine-3′ phosphate, di(thymidine-3′) *P¹P²*-pyrophosphate, and thymidine-3′ thymidine-5′ phosphate. The salts were dissolved in water (50 c.c.) and run on to a column of Dowex 2 resin (chloride form; 6·5 × 3 cm.). Elution with 0·01N-hydrochloric acid removed thymidine-3′ phosphate and the dinucleoside phosphate together; the dinucleoside pyrophosphate was then eluted with 0·1N-hydrochloric acid. Appropriate fractions were combined, neutralised with saturated aqueous calcium hydroxide, and concentrated to small bulk under reduced pressure and, after careful adjustment to pH 7·4 with calcium hydroxide, ethanol (50 c.c.) was added to precipitate the calcium salt as a white amorphous powder. The crude calcium salt of the dinucleoside pyrophosphate weighed 350 mg. and that of the mixed simple phosphate 850 mg.

Calcium di(thymidine-3′) pyrophosphate (cf. III). The crude calcium salt (300 mg.) was dissolved in water (10 c.c.), ethanol (15 c.c.) was added, and the mixture set aside at 0° for 3 hr., then centrifuged. The clear supernatant liquid was concentrated under reduced pressure to small bulk (1 c.c.), and ethanol (50 c.c.) was added. The *calcium salt* of the dinucleoside pyrophosphate separated as a white powder (125 mg.) which was collected, washed with ethanol and ether, and dried (Found, in air-dried material: C, 31·6; H, 4·5; N, 7·7; P, 8·3%; ratio N/P = 2·05, thymidine/P = 1·14. $C_{20}H_{26}O_{15}N_4P_2Ca,4H_2O$ requires C, 32·5; H, 4·6; N, 7·6; P, 8·4%; N/P = 2·0, thymidine/P = 1·0). In this, as in all other cases, thymidine/P ratios were determined by estimation of thymidine by ultraviolet absorption (using $\varepsilon = 9300$ at 267 mμ) in N/20-hydrochloric acid, phosphorus being determined in an aliquot part of the same solution.

Calcium thymidine-3′ thymidine-5′ phosphate (cf. II). The crude mixed calcium salts (800 mg.) were dissolved in water (10 c.c.), the solution was filtered, and ethanol (15 c.c.) added. The mixture was left for 3 hr. at room temperature and the precipitated *calcium thymidine-3′ phosphate* (200 mg.) was centrifuged off. The salt was purified by dissolving it in a minimum of cold water, filtering the solution, and boiling the filtrate; the salt separated as a white crystalline precipitate (Found, in air-dried material: C, 29·9; H, 4·3; N, 6·6; P, 7·4. $C_{10}H_{13}O_8N_2PCa,2H_2O$ requires C, 30·3; H, 4·3; N, 7·1; P, 7·8%).

The supernatant liquid left after removal of the calcium salt by centrifugation was concentrated to small bulk (1 c.c.) under reduced pressure, boiling ethanol (9 c.c.) was added, and the mixture poured into a stirred mixture of acetone (15 c.c.) and ethanol (15 c.c.). The white precipitate of *calcium thymidine-3′ thymidine-5′ phosphate* (495 mg.) was collected by centrifugation, washed with acetone, then ether, and dried [Found, in air-dried material: C, 34·6; H, 5·5; N, 8·1; P, 4·7%; ratio N/P = 3·82, thymidine/P = 1·97. $(C_{20}H_{26}O_{12}N_4P)_2Ca,12H_2O$ requires C, 35·6; H, 5·5; N, 8·3; P, 4·6%; N/P = 4·0, thymidine/P = 2·0].

Thymidine-5′ Dibenzyl Phosphate.—A solution of crude 3′-*O*-acetylthymidine-5′ dibenzyl

phosphate (3·3 g.) (Michelson and Todd, *J.*, 1953, 951) in ethanol (20 c.c.) was added to saturated methanolic ammonia (20 c.c.), and the mixture was left overnight at room temperature. The mixture was then evaporated under reduced pressure and the residue shaken with chloroform and water. The aqueous layer, which contained a little thymidine-5′ benzyl phosphate, was discarded, and the chloroform layer washed with a small quantity of water, dried (Na_2SO_4), and concentrated to small bulk. *cyclo*Hexane was added and the precipitated pale yellow gum was purified by again dissolving it in chloroform and reprecipitating it with *cyclo*hexane. Dried *in vacuo*, *thymidine-5′ dibenzyl phosphate* formed a solid foam (2·06 g.) (Found, in material dried at room temp./10^{-5} mm.: C, 57·3; H, 5·8; N, 5·3. $C_{24}H_{27}O_8N_2P$ requires C, 57·3; H, 5·4; N, 5·6%).

The Dinucleotide (V) *and Di(thymidine-5′ Phosphate)-3′ P^1P^2-Pyrophosphate* (IV).—A solution of thymidine-5′ dibenzyl phosphate (2 g.) in methyl cyanide (15 c.c.) containing 2 : 6-lutidine (1·05 c.c.) was added to *O*-benzylphosphorous *OO*-diphenylphosphoric anhydride (from 1·55 g. of benzyl dihydrogen phosphite) dissolved in dry benzene (30 c.c.). The mixture was kept for 20 hr. at room temperature, then evaporated under reduced pressure to a viscous syrup. Chloroform (50 c.c.) was added and the solution washed successively with water, solutions of sodium hydrogen carbonate and of potassium hydrogen sulphate, and water, and dried (Na_2SO_4), then concentrated to small bulk. The crude thymidine 3′-(benzyl phosphite) 5′-(dibenzyl phosphate) was precipitated as a viscous gum (2·5 g.) by addition of *cyclo*hexane, then reprecipitated from chloroform with *cyclo*hexane and dried at room temperature/10^{-3} mm.; but on account of its instability it was not further purified.

The crude phosphite (2·48 g.) was treated in dry benzene (10 c.c.) and methyl cyanide (2 c.c.) with *N*-chlorosuccinimide (0·55 g.), and after 3 hr. at room temperature added to a solution of 3′-*O*-acetylthymidine (1·15 g.) in methyl cyanide (13 c.c.) containing 2 : 6-lutidine (2·3 c.c., 5 mols.). The mixture was kept at room temperature for 36 hr., moisture being excluded. Solvents were removed under reduced pressure and the residue was refluxed in ethanol (22·5 c.c.), water (9 c.c.), and 0·4N-sulphuric acid (4·5 c.c.), then cooled to room temperature. Saturated aqueous barium hydroxide was added to pH 10, and the mixture left for 20 hr., then neutralised with dilute sulphuric acid. The precipitated barium salts were removed by filtration through Supercel, the combined filtrate and washings were concentrated to small bulk and adjusted to pH 7·8, and ethanol (10 vols.) was added. The precipitate was centrifuged off and dissolved in water (50 c.c.), and the solution brought to pH 4 with sulphuric acid. Barium sulphate was removed by centrifugation and the supernatant liquid and washings were shaken with hydrogen at room temperature and atmospheric pressure in presence of palladium oxide and palladised charcoal. When hydrogen uptake ceased, catalyst was removed, and the filtrate concentrated to 50 c.c. under reduced pressure and adjusted to pH 7·7 with barium hydroxide. The precipitated barium salt (A) was collected by centrifugation and worked up separately from the supernatant liquid (B).

(A) The precipitate was dissolved in a minimum of cold 0·1N-hydrochloric acid (*ca.* 3 c.c.), clarified by centrifugation, and poured into ethanol (2 vols.). The precipitated barium salt of the pyrophosphate (IV) (50 mg.) was contaminated with some inorganic phosphate which could not be separated from it but it contained no other nucleotidic material (Found, in air-dried material: C, 17·1; H, 2·8; N, 4·4; P, 11·6. Calc. for $C_{20}H_{26}O_{21}N_4P_4Ba_2,6H_2O$: C, 20·6; H, 3·3; N, 4·8; P, 10·7%).

(B) The aqueous supernatant liquid (B) (pH 7·7) was put on a column of Dowex 2 resin (chloride form; 6·5 × 2 cm.), the column washed with water, and the nucleotidic material eluted with 0·08N-hydrochloric acid, an automatic fraction collector being used. Appropriate fractions were combined, neutralised with aqueous calcium hydroxide, and taken to small volume under reduced pressure. Ethanol (100 c.c.) was added and the precipitated calcium salts (1·1 g.) were collected, washed with ethanol, then ether, and dried. Paper chromatography showed that the precipitate contained the salts of both thymidine-3′ : 5′ diphosphate and the desired dinucleotide (V), together with a trace of thymidine-5′ phosphate. The crude salt (1 g.) was therefore dissolved in cold water (50 c.c.) by prolonged shaking, and the solution boiled for 10 min., then filtered hot from the granular precipitate of *calcium thymidine-3′ : 5′ phosphate* (490 mg.) which was further purified by repeating its precipitation from aqueous solution by boiling (Found, in air-dried material: C, 22·4; H, 3·5; N, 5·3; P, 11·5. $C_{10}H_{12}O_{11}N_2P_2Ca_2,3H_2O$ requires C, 22·6; H, 3·4; N, 5·3; P, 11·6%).

The filtrate from the initial precipitation of the above salt was cooled and put on a column of Dowex 2 resin (chloride form; 5 × 1·5 cm.), the column cleared of mononucleotide by washing with 0·015N-hydrochloric acid, and the dinucleotide then eluted with 0·04N-hydrochloric acid.

Appropriate fractions were combined, neutralised with aqueous calcium hydroxide, concentrated to small bulk (*ca.* 10 c.c.) under reduced pressure, adjusted to pH 7·4 with calcium hydroxide, and filtered into ethanol (2 vols.). The white solid which separated was reprecipitated from ethanol as a granular powder (360 mg.). Recrystallised from water, the *calcium salt* of the dinucleotide (V) formed clusters of colourless prismatic needles, $[\alpha]_D^{20}$ 7·0° (*c*, 1·7 in H_2O) [Found, in air-dried material: C, 29·3; H, 4·6; N, 6·5; P, 8·0. $(C_{20}H_{25}O_{15}N_4P_2)_2Ca_3,14H_2O$ requires C, 29·7; H, 4·8; N, 6·9; P, 7·7. Found, in material dried for 12 hr. at 110°/1 mm.: P, 8·8. $(C_{20}H_{25}O_{25}N_4P_2)_2Ca_3$ requires P, 9·1%).

Characterisation of Thymidine Nucleotides and Related Compounds.—For abbreviations in the Table, see p. 2633.

(a) *Paper chromatography.* R_F values are given for ascending chromatograms on Whatman No. 1 paper with (A) *iso*propanol–water–ammonia (7 : 2 : 1) and (B) *n*-propanol–2N-hydrochloric acid (3 : 1).

(b) *Paper electrophoresis.* Experiments run for 12 hr. at 150 v on Whatman No. 1 paper in (i) 0·1M-disodium hydrogen phosphate and (ii) 0·1M-potassium dihydrogen phosphate. Movement towards the anode in all cases measured in cm. from a central base line.

(c) *Ultraviolet absorption.* Spectra of all compounds examined showed identical positions of maximum and minimum in N/50-hydrochloric acid ($\lambda_{max.}$ 267 mμ; $\lambda_{min.}$ 235 mμ) and in N/50-sodium hydroxide ($\lambda_{max.}$ 267 mμ; $\lambda_{min.}$ 245 mμ). The optical density ratio at 280/260 mμ was determined in each case.

	R_F values		Electrophoresis (cm.)		Optical density ratio 280/260 mμ	
	A	B	Na_2HPO_4	KH_2PO_4	N/50-HCl	N/50-NaOH
T3′–P	0·075	0·78	6·8	7·2	0·675	0·660
T5′–P	0·068	0·69	6·8	7·2	0·710	0·695
P–3′T5′–P	0	0·76	10·4	11·8	0·675	0·685
T3′–P–5′T	0·28	0·59	2·4	5·1	0·675	0·625
T3′–P–P–3′T	0·16	0·47	4·8	9·1	0·685	0·650
T5′–P–3′T5′–P	0·034	0·54	6·8	9·1	0·690	0·660
P–5′T3′–P–P–3′T5′–P ...	0	0·51	9·8	14·1	0·690	0·645

Enzyme Experiments.—Experiments using rattlesnake venom (*C. atrox*) were carried out according to Michelson and Todd (*J.*, 1953, 951). The crude prostatic phosphatase was prepared as a freeze-dried extract by the method of Beale, Harris, and Roe (*J.*, 1950, 1397); the thymidine derivative (*ca.* 1 mg.) was added to 0·1M-ammonium citrate (0·3 c.c.; pH 5) and the enzyme (0·1 c.c. of a solution containing 100 mg. of freeze-dried extract per 5 c.c.), and the mixture was incubated for 3 hr. at 37°.

In all enzyme experiments the products of reaction were identified by paper chromatography in 3 solvent systems [A and B above, and *n*-butanol–water (86 : 14)] and by paper electrophoresis as above.

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