## 198. *Nucleotides. Part XX.* Mononucleotides derived from Thymidine. Identity of Thymidylic Acid from Natural Sources with Thymidine-5′ Phosphate.*

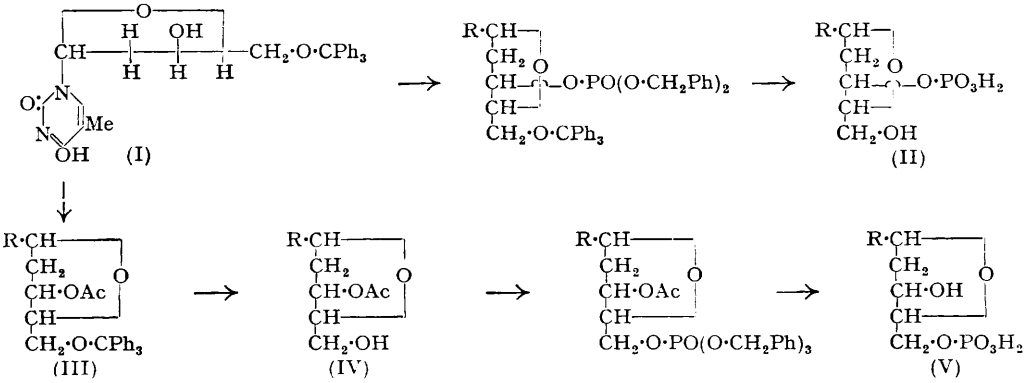
By A. M. Michelson and A. R. Todd.

Thymidine-3′ phosphate has been prepared by phosphorylation of 5′-trityl thymidine with dibenzyl phosphorochloridate (chlorophosphonate) and removal of protecting groups. Thymidine-5′ phosphate, prepared by a similar method *via* 3′-acetyl thymidine, was found to be identical with thymidylic acid obtained by enzymic hydrolysis of deoxyribonucleic acids.

With the successful development of methods for the isolation in quantity of the deoxyribonucleosides obtained by enzymic hydrolysis of deoxyribonucleic acids (Part XIV, Andersen, Dekker and Todd, *J.*, 1952, 2721) the way became clear for the synthesis of the simple deoxyribonucleotides. Their synthesis is an essential part of that section of our general programme of researches on nucleotides directed to the study of polydeoxyribonucleotides, but it has added importance in that only by unambiguous synthesis of the various deoxyribonucleoside phosphates can the postulated structure of the natural nucleotides be finally confirmed. The deoxyribonucleotides obtained by careful enzymic hydrolysis of deoxyribonucleic acids have, in the past, been rather inaccessible substances and as a result they have been but little studied. The four nucleotides, deoxyadenylic, deoxyguanylic, deoxycytidylic, and thymidylic acid (monophosphates of the respective nucleosides) have been isolated in crystalline form by Klein and Thannhauser (*Z. physiol. Chem.*, 1933, **218**, 173; 1934, **224**, 252; 1935, **231**, 96) and the existence of a fifth, deoxy-5-methylcytidylic acid, in enzymic digests of thymus nucleic acid has been convincingly demonstrated by Cohn (*J. Amer. Chem. Soc.*, 1951, **73**, 1539) although this has not been isolated in crystalline form. Until very recently it was considered that the phosphoryl group in the natural deoxyribonucleotides was in each case at $C_{(3')}$ of the deoxyribose residue, simply on an assumed analogy with the natural ribonucleotides from ribonucleic acids, which were formerly thought to be the 3′-phosphates of their respective ribonucleosides. The finding that enzymic hydrolysis of ribonucleic acids with ribonuclease followed by intestinal phosphatase gives substantial amounts of ribonucleoside-5′ phosphates (Cohn and

* Part XIX, preceding paper.

Volkin, *Nature*, 1951, **167**, 483) aroused some doubts, and Carter (*J. Amer. Chem. Soc.*, 1951, **73**, 1537) as a result of experiments on the action of purified 5′-nucleotidase on the natural deoxyribonucleotides brought forward strong evidence for the view that they were




in fact deoxyribonucleoside-5′ phosphates. A comparison of the ion-exchange behaviour of natural deoxyadenylic acid with that of the 2′-, 3′- and 5′-phosphates of adenosine also lends support to this view (Volkin, Khym, and Cohn, *J. Amer. Chem. Soc.*, 1951, **73**, 1535).

In the present investigation, the isomeric 3′- and 5′-phosphates of the natural deoxyribonucleoside thymidine (deoxy-5-methyluridine) have been synthesised. 5′-Trityl thymidine (I) (Levene and Tipson, *J. Biol. Chem.*, 1935, **109**, 623) was phosphorylated in the usual way with dibenzyl phosphorochloridate. The crude product, treated with hot acetic acid, gave a material consisting largely of thymidine-3′ benzyl phosphate, which, on hydrogenolysis, yielded thymidine-3′ phosphate (II; R = thymine), characterised as its crystalline barium and brucine salts. A small amount of a by-product was isolated, which had the analytical composition and stability of a dinucleoside phosphate; its formation was unexpected and its precise structure has not been determined.

Acetylation of 5′-trityl thymidine readily yielded 3′-acetyl 5′-trityl thymidine (III; R = thymine), converted by short heating with acetic acid into 3′-acetyl thymidine (IV; R = thymine). To provide further evidence that this compound had the structure allotted to it and that no acetyl migration had occurred in its preparation, it was treated with toluene-*p*-sulphonyl chloride in pyridine and the resulting toluene-*p*-sulphonyl derivative was heated with sodium iodide in acetone (Oldham–Rutherford reaction). Replacement of the toluene-*p*-sulphonyl group by iodine occurred smoothly, giving the crystalline 3′-acetyl 5′-deoxy-5′-iodothymidine. Phosphorylation of 3′-acetyl thymidine with dibenzyl phosphorochloridate, followed by removal of protecting groups, yielded thymidine-5′ phosphate (V; R = thymine) isolated as its barium and brucine salts.

The synthetic thymidine-5′ phosphate corresponded in its properties to natural thymidylic acid (Klein and Thannhauser, *loc. cit.*), and it was identical in paper chromatographic and ion-exchange characteristics with the natural material supplied by Dr. W. Cohn. The action of a preparation of rattlesnake (*Crotalus atrox*) venom containing 5′-nucleotidase on the synthetic materials and on several other nucleotides was examined. The venom had no action on thymidine-3′ phosphate, thymidine-3′ : 5′ diphosphate (Dekker, Michelson, and Todd, preceding paper), thymidine-3′ benzyl phosphate, or adenylic acid *a*; it dephosphorylated thymidine-5′ phosphate (natural and synthetic), thymidine-5′ benzyl phosphate, adenosine-5′ benzyl phosphate and cytidine-5′ phosphate.

We consider that these findings confirm Carter's views (*loc. cit.*) and establish that, at least in the case of natural thymidylic acid, the phosphoryl group is located at $C_{(5')}$.

## Experimental

*5′-Trityl Thymidine.*—Triphenylmethyl chloride (3·5 g.) was added to a solution of anhydrous thymidine (2·5 g.) in dry pyridine (50 c.c.) and the mixture left at room temperature for 1 week. It was then cooled to 0° and poured into ice-water (500 c.c.) with vigorous stirring, and the

precipitate washed with water and dried *in vacuo* ($P_2O_5$). The product was next dissolved in acetone (5 c.c.), dry benzene (35 c.c.) was added, and after filtration the acetone was boiled off, and the resulting solution cooled. 5′-Trityl thymidine separated as colourless needles (4 g., 80%), m. p. 128°, $[\alpha]_D^{13}$ +19·2° (*c*, 1·1 in 95% EtOH). Levene and Tipson (*J. Biol. Chem.*, 1935, **109**, 623) give m. p. 125°, $[\alpha]_D^{24}$ +11·4° (in $COMe_2$).

*Thymidine-3′ Phosphate.*—A solution of 5′-trityl thymidine (6·3 g.) in dry pyridine (65 c.c.) was cooled to just above its m. p., and dibenzyl phosphorochloridate (from 10 g. of dibenzyl phosphite) was added. The mixture was kept at this temperature with occasional shaking during 6 hours, then left at 0° overnight. Aqueous sodium carbonate (5 g. in 30 c.c. of water) was added, the mixture evaporated under reduced pressure, and the residue shaken with chloroform and aqueous sodium hydrogen carbonate. The chloroform layer was further washed with sodium hydrogen carbonate, then with water, dried ($Na_2SO_4$), and evaporated to a cream-coloured glass (A). A solution of (A) in ethanol was filtered, concentrated to small bulk (30 c.c.), and diluted with ether (250 c.c.). After several hours at 0° the precipitated gum was washed with ether and re-dissolved in ethanol–acetone. The filtered solution was evaporated, giving crude *5′-trityl thymidine-3′ dibenzyl phosphate*, a slightly yellowish glass (B) (7·8 g.) (Found, in material dried at 60°/1 mm. for 12 hours: C, 63·3; H, 5·5; N, 3·4. $C_{43}H_{41}O_8N_2P$ requires C, 69·3; H, 5·5; N, 3·8%).

The glass (B) (7·6 g.) was boiled in acetic acid (50 c.c. of 80%) for 7 minutes. Acetic acid was removed under reduced pressure, the residue neutralised with aqueous barium hydroxide, and triphenylmethanol removed by extraction several times with chloroform. Barium was removed from the aqueous solution by titration with sulphuric acid (rhodizonic acid) and centrifugation, and the solution concentrated to small bulk under reduced pressure and freeze-dried. The residue was purified by re-dissolving it in methanol, filtering and again evaporating; the residue was a glass (C) (3·3 g.). Paper-chromatographic examination showed that it contained four components. The major component, from its behaviour, appeared to be the expected thymidine-3′ benzyl phosphate; accompanying it were a small amount of free thymidine and very small amounts of free nucleotide (thymidine-3′ phosphate) and an unknown substance. Separation was achieved on an ion-exchange column (Dowex-2; chloride form); elution with 0·002N-hydrochloric acid removed the thymidine, 0·006N-acid the free nucleotide, 0·007N-acid the unknown substance, and 0·025N-acid eluted the thymidine-3′ benzyl phosphate as a very wide peak. (For repetition of such work the normalities given in the Table on p. 956 are recommended.)

The crude glass (C) (2·4 g.) was hydrogenated in aqueous ethanol (100 c.c. of 75%) at room temp./1 atm. over a mixture of palladium and palladised-charcoal catalysts. Catalyst was removed, and the filtrate concentrated to small bulk under reduced pressure, then brought to pH 7·5 with saturated aqueous barium hydroxide. The precipitate (mainly barium phosphate) was centrifuged off and well washed with water and the combined supernatant liquids were concentrated to small bulk under reduced pressure. Paper-chromatographic examination, using *n*-butanol–water (86 : 14) and *iso*propanol–water–ammonia (70 : 20 : 10) showed the presence of thymidine, thymidine-3′ phosphate, and a very small amount of an " unknown component " (derived, doubtless, from the unknown substance present in the original glass). Fractional precipitation of barium salts with ethanol having proved abortive as a method of separation, the solution was treated with aqueous lead acetate at pH 6·8. The lead salt precipitate was centrifuged off, washed well with water, and decomposed with hydrogen sulphide in the usual way, the product was converted into the barium salt, and the solution concentrated to 20 c.c. under reduced pressure. The barium salt which was precipitated on addition of ethanol (40 c.c.) was dried and dissolved in water, in which it was but moderately soluble, and the solution was concentrated to small volume (10 c.c.) and boiled for a few minutes. The granular precipitate was collected and purified by repetition of the process several times. *Barium thymidine-3′ phosphate* crystallised slowly from concentrated aqueous solution as colourless needles, $[\alpha]_D^{20}$ +7·3° (*c*, 1·5 in $H_2O$) (Found, in material dried at 110°/1 mm. for 12 hours: C, 24·1; H, 3·8; N, 5·7; P, 6·1. $C_{10}H_{13}O_8N_2PBa,2H_2O$ requires C, 24·3; H, 3·5; N, 5·7; P, 6·3%. Found, in material dried at 130°/5 × $10^{-5}$ mm. for 12 hours: C, 25·2; H, 3·4; N, 5·9. $C_{10}H_{13}O_8N_2PBa,H_2O$ requires C, 25·3; H, 3·2; N, 5·9%).

*Dibrucine thymidine-3′ phosphate*, prepared in the usual manner from the barium salt, recrystallised from aqueous ethanol (40%) as colourless needles which on heating softened at 172° and melted at 178° (Found, in air-dried material: C, 54·6; H, 6·7; N, 6·7. $C_{10}H_{15}O_8N_2P,2C_{23}H_{26}O_4N_2,7H_2O$ requires C, 54·4; H, 6·5; N, 6·8%).

The " unknown component " of the crude hydrogenation product was isolated by combining

all supernatant liquids from the lead salt precipitation and adding more lead acetate to them at pH 9·3. The mixture (*ca.* 300 c.c.) was heated to boiling and filtered. When the filtrate was cooled a gelatinous lead salt of the "unknown component" separated; it was collected and decomposed in the usual way and the free acid converted into the barium salt. The latter, which was very soluble in water, was purified by precipitation with acetone, forming a fine white powder (Found, in material dried at 130°/5 × $10^{-5}$ mm. for 12 hours: C, 39·3; H, 4·7; N, 9·2%). The substance was unaffected by rattlesnake venom and showed the stability towards acid and alkaline hydrolysis which would be expected of a diester of phosphoric acid [*barium* salt of dithymidine phosphate $(C_{20}H_{26}O_{12}N_4P)_2Ba$ requires C, 39·3; H, 4·2; N, 9·1%].

*3′-Acetyl 5′-Trityl Thymidine.*—A solution of anhydrous 5′-trityl thymidine (3·75 g.) in pyridine (35 c.c.) and acetic anhydride (8 c.c.) was kept at room temperature for *ca.* 20 hours, then cooled to 0° and poured into ice water (500 c.c.) with vigorous stirring. The white amorphous precipitate was washed with water and dried (4·0 g., 98%; m. p. *ca.* 90°). Recrystallised from benzene–light petroleum (b. p. 40—60°), *3′-acetyl 5′-trityl thymidine* formed rosettes of needles, m. p. 105°, $[\alpha]_D^{13}$ +18·6°, $[\alpha]_{Hg}^{13}$ +25·1° (*c*, 1·1 in 95% EtOH) (Found, in material dried for 10 hours at 70°/5 × $10^{-4}$ mm.: C, 73·9; H, 6·2; N, 4·7. $C_{31}H_{30}O_6N_2,C_6H_6$ requires C, 73·5; H, 6·0; N, 4·6. Found, in material sublimed at 200—220°/$10^{-5}$ mm.: C, 70·3; H, 5·7; N, 5·6. $C_{31}H_{30}O_6N_2$ requires C, 70·7; H, 5·7; N, 5·3%).

*3′-Acetyl Thymidine.*—A solution of 3′-acetyl 5′-trityl thymidine (3·7 g.) in acetic acid (12·5 c.c. of 80%) was heated under reflux for 10 minutes, cooled to room temperature, and diluted with ice-water (230 c.c.). The precipitate of triphenylmethanol was filtered off and the filtrate taken to dryness under reduced pressure at <30°, giving a crystalline mass. Recrystallised from acetone or acetone–light petroleum (b. p. 40—60°), *3′-acetyl thymidine* formed needles (1·8 g., 90%; m. p. 176°), $[\alpha]_D^{13}$ +0·7°, $[\alpha]_{Hg}^{13}$ +0·7° (*c* 1·43 in 95% EtOH) (Found, in material dried for 5 hours at 70°/5 × $10^{-4}$ mm.: C, 50·9; H, 5·5; N, 10·0. $C_{12}H_{16}O_6N_2$ requires C, 50·7; H, 5·6; N, 9·9%).

*3′-Acetyl 5′-Deoxy-5′-iodothymidine.*—A solution of anhydrous 3′-acetyl thymidine (0·65 g.) and toluene-*p*-sulphonyl chloride (0·49 g., 1·1 mol.) in dry pyridine (10 c.c.) was set aside at room temperature overnight, then poured into ice-water (140 c.c.) with vigorous stirring. The aqueous suspension was extracted three times with chloroform, and the combined extracts were washed with cold dilute sulphuric acid to remove pyridine, then with cold dilute sodium hydrogen carbonate solution, and finally with cold water, dried ($Na_2SO_4$), and evaporated under reduced pressure. The residual gum was dissolved in acetone and filtered to remove the slight turbidity and the solvent removed under reduced pressure to give a frothy glass (0·70 g.) of crude 3′-acetyl 5′-toluene-*p*-sulphonyl thymidine. This glass (0·64 g.) was dissolved in anhydrous acetone (25 c.c.), sodium iodide (0·70 g.) added, and the solution heated at 100° for 2 hours. Sodium toluene-*p*-sulphonate (0·250 g.; theor., 0·283 g.) was removed and the filtrate taken to dryness under reduced pressure. To the residue, water and chloroform were added, and the chloroform extract was washed with a little cold dilute sodium hydrogen carbonate solution, then with water, dried ($Na_2SO_4$), and taken to dryness under reduced pressure twice with acetone to remove traces of chloroform. The pale yellow frothy glass crystallised from methanol–ether–light petroleum (b. p. 40—60°) as colourless needles (0·43 g. ≡ 75%) of *3′-acetyl 5′-deoxy-5′-iodothymidine*, m. p. 131°, $[\alpha]_D^{13}$ −5·6°, $[\alpha]_{Hg}^{13}$ −6·9° (*c*, 1·6 in 95% EtOH) (Found, in material dried for 10 hours at 100°/5 × $10^{-4}$ mm.: C, 37·1; H, 3·7; N, 7·4. $C_{12}H_{15}O_5N_2I$ requires C, 36·6; H, 3·8; N, 7·1%).

*Thymidine-5′ Phosphate.*—Dibenzyl phosphorochloridate (from 5 g. of dibenzyl phosphite) was added to a solution of 3′-acetyl thymidine (2·01 g., dried at 110°/1 mm. for 12 hours) in anhydrous pyridine (25 c.c.) at −30°, and the mixture kept just above its f. p. for 3 hours and then left at room temperature overnight. Water (15 c.c.) and sodium carbonate (2·5 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 dissolved in a little ethanol, and ether (200 c.c.) was added to precipitate an oil. The oil was dissolved in acetone, and the solution filtered and evaporated under reduced pressure to a pale yellow glass (3·2 g.), consisting mainly of 3′-acetyl thymidine-5′ dibenzyl phosphate.

A solution of this (1·75 g.) in aqueous ethanol (50 c.c. of 75%) was hydrogenated at room temperature over a mixture of palladium and palladised-charcoal catalysts. Hydrogen uptake (178 c.c.) was complete in 1 hour. Catalyst was removed and the solution of 3′-acetyl thymidine-5′ phosphate brought to pH 11 with barium hydroxide and kept at 30° for 30 min. to hydrolyse

the acetyl group. The solution was then neutralised with carbon dioxide, boiled, and filtered. Lead acetate solution was then added, and the gelatinous lead salt of the nucleotide centrifuged off, well washed with water, and decomposed with hydrogen sulphide. The supernatant liquids from the precipitate of lead sulphide were concentrated under reduced pressure and finally lyophilised. The residue, which could not be crystallised, was dissolved in water (5 c.c.), neutralised with barium hydroxide, and filtered, and the barium thymidine-5′ phosphate precipitated by addition of two volumes of ethanol. After being washed with ethanol and then ether, and dried, the white amorphous solid was re-dissolved in water, clarified by centrifugation and again precipitated by adding two volumes of ethanol; the salt was washed with ethanol, then ether, and dried (0·72 g.) (Found, in material dried for 12 hours at 110°/1 mm.: C, 25·6, 25·6; H, 3·1, 3·2; N, 5·7, 6·0; P, 6·5. Calc. for $C_{10}H_{13}O_8N_2PBa$: C, 26·3; H, 2·8; N, 6·1; P, 6·8%), $[\alpha]_D^{17}$ −3·0° (*c*, 2·0 in water). Klein and Thannhauser (*Z. physiol. Chem.*, 1935, **231**, 96) give $[\alpha]_D^{21}$ −4·4° for natural barium thymidylate.

*Dibrucine thymidine-5′ phosphate* was prepared from the above barium salt in the usual manner. Recrystallised from aqueous ethanol (first 95% and then 60%) it formed rosettes of needles which softened at 140°, formed colourless globules at *ca.* 155° and melted to a clear liquid at *ca* 175° (Found, in air-dried material: C, 54·6; H, 6·3; N, 6·9. $C_{10}H_{15}O_8N_2P,2C_{23}H_{26}O_4N_2,7H_2O$ requires C, 54·4; H, 6·5; N, 6·8%).

*Thymidine-5′ Phosphate* via *Thymidine-5′ Benzyl Phosphate*.—A solution of 3′-acetyl thymidine-5′ dibenzyl phosphate (1·2 g.) in a mixture of dry benzene (5 c.c.) and 4-methylmorpholine (10 c.c.) was kept at 100° for 2 hours. Solvent was removed under reduced pressure, the residue dissolved in water (20 c.c.), and the solution made alkaline with barium hydroxide and kept at pH 11 for 1 hour at 30°. After neutralisation with carbon dioxide, the deep yellow solution was extracted 5 times with chloroform, and the chloroform extracts were discarded. The residual solution of crude barium thymidine-5′ benzyl phosphate (containing barium acetate) was made up to 100 c.c. and passed through a column of ion-exchange resin (IRC-50; acid form), and the effluent evaporated to 15 c.c. under reduced pressure, then freeze-dried. The residue was dissolved in methanol, and the solution filtered and taken to dryness to give a hygroscopic white glass (0·9 g.) which could not be crystallised (Found, in material dried at 40°/1 mm. for 12 hours: C, 49·2; H, 6·5; N, 7·7. *Thymidine-5′ benzyl phosphate* $C_{17}H_{21}O_8N_2P$ requires C, 49·5; H, 5·1; N, 6·8%). Paper chromatography in aqueous *n*-butanol and in *iso*propanol–ammonia systems indicated the presence of only one component absorbing in the ultra-violet region. The crude benzyl ester (0·5 g.) was hydrogenated in the usual manner, in aqueous ethanol, and worked up as barium thymidine-5′ phosphate (0·300 g.) (Found, in material dried at 110°/1 mm. for 12 hours: N, 6·0. Calc. for $C_{10}H_{13}O_8N_2PBa$: N, 6·1%).

*Action of Rattlesnake* (Crotalus atrox) *Venom on Thymidine Phosphates*.—To each nucleotide derivative (*ca.* 1 mg.) were added glycine buffer (0·3 c.c. of 0·25M; pH 9), magnesium chloride (0·1 c.c. of 0·1M), and rattlesnake venom (0·1 c.c. of a solution containing 20 mg. of dried *Crotalus atrox* venom in 1 c.c. of 0·1M-potassium chloride). The mixture was in each case incubated at 37° for 3 hours and then run on paper chromatograms with appropriate controls; the solvent systems were *n*-butanol–water (86 : 14) and *iso*propanol–water–ammonia (70 : 20 : 10).

*Separation of Mixtures of Thymidine-3′ and Thymidine-5′ Phosphate*.—Separation was achieved on an ion-exchange column (Dowex-2; formate form), elution being with a solution 0·05M with respect to sodium formate and 0·01M to formic acid. The elution diagram showed two peaks, the first corresponding to thymidine-5′ and the second to thymidine-3′ phosphate.

*Paper Chromatography of Thymidine Derivatives*.—Solvent systems used: I, *n*-butanol–water (86 : 14); II, *iso*propanol–ammonia–water (70 : 10 : 20); III, *n*-propanol–2N-hydrochloric acid (3 : 1). Results are tabulated.

| | Ascending $R_F$ values | | |
|---|---|---|---|
| | I | II | III |
| Thymidine | 0·43 | 0·62 | 0·73 |
| Thymidine-3′ phosphate | 0 | 0·075 | 0·78 |
| Thymidine-5′ phosphate | 0 | 0·068 | 0·69 |
| Natural thymidylic acid | 0 | 0·068 | 0·69 |
| Thymidine-3′ : 5′ diphosphate | 0 | 0 | 0·75 |
| Thymidine-3′ benzyl phosphate | 0·215 | 0·65 | — |
| Thymidine-5′ benzyl phosphate | 0·20 | 0·59 | — |
| 3′-Acetyl thymidine | 0·63 | — | — |
| 3′-Acetyl thymidine-5′ phosphate | 0·026 | 0·123 | — |
| 3′-Acetyl 5′-iodo-5′-deoxythymidine | 0·76 | — | — |
| " Unknown component " from thymidine-3′ phosphate preparation | 0 | 0·38 | — |

*Ion-exchange Characteristics of Thymidine Phosphates.*—Results are tabulated.

| | Optical-density ratio 280/260 mμ in 0·01N-HCl | Normality of HCl for removal from Dowex-2 resin |
|---|---|---|
| Thymidine-3′ phosphate ..... | 0·69 | 0·006 |
| Thymidine-5′ phosphate ..... | 0·71 | 0·006 |
| Thymidine-3′ benzyl phosphate ... | 0·77 | 0·08 |
| Thymidine-5′ benzyl phosphate ... | 0·79 | 0·03 |
| Thymidine-3′ : 5′ diphosphate ..... | 0·65 | 0·075 |
| " Unknown component " ..... | 0·68 | 0·009 |

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