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Reactions of Nucleosides on Polymer Supports. Synthesis of Thymidylylthymidylylthymidine*

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ABSTRACT: A procedure is described for synthesizing thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine on an insoluble polymer support. The support is a carboxylated styrene popcorn polymer. In the acid chloride form it reacts with 5′-*O*-monomethoxytritylthymidine, forming an ester link at the 3′-*O* position of the nucleoside. Subsequent cleavage of the monomethoxytrityl group by acid and condensation of the liberated 5′-hydroxyl group with 5′-*O*-monomethoxytritylthymidine 3′-phosphate afford a dinucleoside phosphate derivative on the support. On repetition of these steps and treatment with alkali to break the ester link holding the nucleotide product to the support, thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine is obtained in 51% yield based on thymidine originally bound to the support.

Since introduction of the technique of synthesizing oligonucleotides on polymer supports (Letsinger and Mahadevan, 1965, 1966) several laboratories have reported work in the area. A major advantage of the support technique is that the products in a multistep synthesis may be separated easily from excess reagents and soluble by-products at each step. In the original procedure 5′-*O*-trityldeoxycytidine was joined to an insoluble polystyrene-type popcorn polymer by reaction of the 4-amino group with an acid chloride function on the support. Nucleotide units were added by successive phosphorylation of the 3′-hydroxyl group with β-cyanoethyl phosphate and dicyclohexylcarbodiimide, activation of the phosphate with mesitylenesulfonyl chloride, and condensation of the active phosphate with a nucleoside at the 5′-oxygen position.

Recently Hayatsu and Khorana (1966) and Cramer *et al.* (1966) described a synthetic procedure in which the initial nucleoside was joined through the 5′-hydroxyl group to a triarylmethyl derivative of a polymer that was soluble in pyridine. The chain was lengthened from the 3′ position by condensation with

* From the Department of Chemistry, Northwestern University, Evanston, Illinois. *Received January 20, 1967.* This research was supported by the Division of General Medical Sciences, National Institutes of Health (GM-10265), and by Public Health Service predoctoral fellowship awards to M. H. C. (5-F1-GM-23-558) and D. M. J. (5-F1-GM-23-577). Part VI in series on Nucleotide Chemistry. Part V: Letsinger and Ogilvie (1967).

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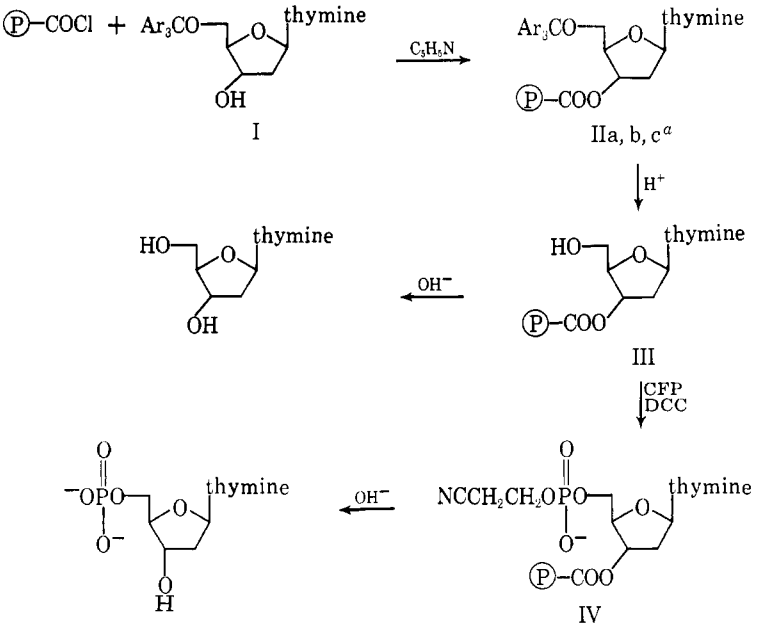
SCHEME I: Reaction of Thymidine on Support Polymer.



[a] Ar_3C = (a) trityl, (b) monomethoxytrityl, and (c) dimethoxytrityl.

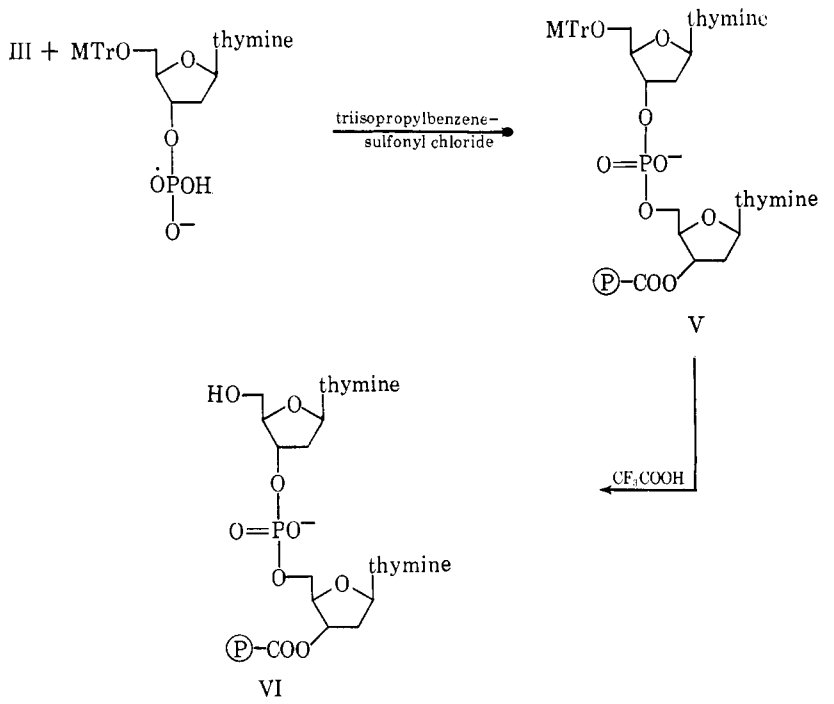
3′-*O*-acetylthymidine 5′-phosphate. On hydrolytic cleavage of the acetate, a new 3′-hydroxyl group was liberated for the next cycle. Separation of the polymer from the solvent was effected in this case by precipitation with water followed by filtration. Essentially the same chemical steps have also been used with an insoluble polymer (Melby and Strobach, 1967).

Concurrent with the synthetic experiments with deoxycytidine, a study was initiated to test the feasibility of building oligonucleotide chains on polymer supports from the 5′-hydroxyl terminus of the bound nucleoside. The approach that was envisaged involved reaction of the acid chloride form of the support polymer (Ⓟ–COCl)[1] with the 3′-hydroxyl of a 5′-*O*-protected nucleoside derivative, cleavage of the 5′-*O*-blocking group, and condensation of the 5′-hydroxyl with a suitable phosphate derivative. In developing this approach we first carried out a number of experiments to determine conditions for joining nucleoside derivatives through the 3′-hydroxyl position to the support and for removing nucleotidic material from the support. Triarylmethyl groups were selected to protect the 5′-hydroxyl positions since the derivatives are readily prepared and the triarylmethyl groups may be removed satisfactorily from the pyrimidine nucleotide derivatives.

Although infrared spectroscopy (Letsinger *et al.*, 1964) provides a means for determining when nucleosidic material has been added to or removed from the support polymer, the technique is not generally suitable for following the synthesis of an oligonucleotide quantitatively. The products for a given chemical step were, therefore, determined by cleaving the covalent bonds holding the nucleosidic and nucleotidic material to the support, washing the support, separating the soluble components by paper chromatography or electrophoresis, and assaying the materials spectrophotometrically. Cleavage was effected initially with mixtures of ammonium hydroxide and pyridine, the organic solvent being used to swell the polymer; however, this combination did not prove very effective. Much nucleotide material remained on the support even after several separate treatments. Attention was then turned to the use of sodium hydroxide solutions. It was found that 0.5 M sodium hydroxide in 50% aqueous dioxane at room temperature was quite satisfactory. With it 93% of the thymidine was removed from the polymer–thymidine sample (III) within 16 hr and all of the thymidine was liberated within 2 days. There is danger that deamination may occur when nucleotides bearing amino groups are subjected to alkaline treatment; however, no evidence for deamination was found when deoxycytidine derivatives were treated with 50% aqueous dioxane solutions of sodium hydroxide under these conditions (Letsinger and Mahadevan, 1966). Ethanol was a poor solvent for the cleavage reaction. Thus, only 43% of the thymidine was released when the polymer–thymidine was treated with 0.1 M sodium hydroxide in ethanol at reflux for 5 hr.

[1] Abbreviations used: T, thymidine; pT, thymidine 5′-phosphate; Tp, thymidine 3′-phosphate; TpT, thymidylyl-(3′–5′)-thymidine; TpTpT, thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine; DCC, dicyclohexylcarbodimidine; CEP, β-cyanoethyl phosphate; Ⓟ–, inert portion the polymer support; MTr, monomethoxytrityl.

SCHEME II: Sequence for Addition of One Nucleotide Unit.



The hydroxyl groups in polymer III (Scheme I) reacted satisfactorily with the common phosphorylating reagents. Phosphorylations of III derived from the monomethoxytritylthymidine derivative IIb yielded thymidine 5′-phosphate as the sole mononucleotide; however, chromatographic data indicated that both thymidine 3′-phosphate and thymidine 5′-phosphate were obtained from samples of III that had been prepared from the dimethoxytritylthymidine derivative IIc. The latter result is consistent with the assumption that some of the dimethoxytrityl groups had been lost during the reaction with the acid chloride and that the resulting thymidine had coupled to the support through the 5′-*O* position. In support of this explanation is the observation that alkaline hydrolysis of IIc afforded thymidine as well as dimethoxytritylthymidine. Since the dimethoxytrityl group was not satisfactory in this system, all further work was carried out with samples of III prepared from the monomethoxytritylthymidine derivative IIb.

Ninety per cent of the thymidine in III was phosphorylated when the polymer was treated with β-cyanoethyl phosphate and dicyclohexylcarbodiimide for a period of 6 days. With mesitylenesulfonyl chloride as the activating agent in place of dicyclohexylcarbodiimide a 76% yield of thymidine 5′-phosphate was obtained from a reaction run for 4 hr. About the same yield (73%) of thymidine 5′-phosphate was realized by phosphorylating with phosphorus oxychloride in the presence of imidazole and triethylamine.

Attempts to condense the cyanoethyl derivative of the polymer–thymidine (IV) with 5′-*O*-monomethoxytritylthymidine were not promising. With mesitylenesulfonyl chloride as the condensing agent a mixture of products was obtained which contained methoxytritylthymidylylthymidine and thymidine 5′-phosphate in approximately a 5:3 mole ratio. The reaction of the activated 5′-phosphodiester with the 3′-hydroxyl group is, therefore, less satisfactory than the corresponding reaction of an activated 3′-phosphodiester with a 5′-hydroxyl group (Letsinger and Mahadevan, 1966).

Direct condensation of a nucleoside 3′-phosphate with the 5′-hydroxyl of the polymer derivative proved superior for adding nucleotide units to the chain. This method is illustrated by the synthesis of thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine. The phosphorylation reaction is similar to that utilized in the preliminary studies, with 5′-*O*-monomethoxytritylthymidine 3′-phosphate being used in place of β-cyanoethyl phosphate. The nucleotide derivative was obtained by reaction of thymidine 3′-phosphate with monomethoxytriphenylmethyl chloride in pyridine. In pilot runs carried to the dinucleoside phosphate stage it was found that somewhat better yields of the phosphodiester were obtained by using 2,4,6-triisopropylbenzenesulfonyl chloride (72 ± 2% yield) in place of mesitylenesulfonyl chloride (66 ± 3% yield), and that the monomethoxytrityl group could be removed very rapidly from the nucleotide derivatives on the polymer with trifluoroacetic acid in benzene (see Hayatsu and Khorana, 1966). Consequently these modifications were introduced.

The sequence for adding a nucleotide unit to the chain is outlined in Scheme II. The two steps involved

were condensation of 5′-*O*-monomethoxytritylthymidine 3′-phosphate with the 5′-hydroxyl of the terminal nucleoside on the support, and cleavage of the monomethoxytrityl ether to liberate a new 5′-hydroxyl group. The time required for addition of one nucleotide unit, including periods for washing the polymer, was 1 day. For preparation of the trinucleoside diphosphate the cycle was repeated and the products were stripped from the support by alkaline hydrolysis. The three major products obtained were thymidine, thymidylylthymidine, and thymidylylthymidylylthymidine. On the basis of the thymidine units originally bound to the support the over-all yields of the compounds for the two-cycle synthesis were 33, 16, and 51%, respectively. The absorbance of these materials (measured at 267 mμ) constituted 76% of the total absorbance of the substances eluted from the support. The other ultraviolet-absorbing material (in part, thymidine phosphate) was probably held to the insoluble polymer by pyrophosphate-type links.

Thymidylylthymidylylthymidine prepared in this manner was homogeneous on paper chromatography and electrophoresis. Complete degradation by snake venom and spleen phosphodiesterase indicated that the nucleotide units were joined in the natural 3′–5′ linkage. The ultraviolet and infrared spectra and the elemental analysis were also consistent with the proposed structure.

This synthetic procedure is more rapid and gives better yields than the procedure which builds from trityldeoxycytidine bound to be an insoluble support (Letsinger and Mahadevan, 1965, 1966). The yields are better than those reported for support synthesis by Cramer *et al.* (1966) and by Melby and Strobach (1967) but are somewhat lower than the yields reported by Hayatsu and Khorana (1966) for syntheses conducted in solution.

At the present stage of development the procedure utilizing 5′-*O*-monomethoxytritylthymidine 3′-phosphate and an insoluble polymer support provides a convenient method for synthesizing short-chain oligonucleotides derived from thymidine. The method can probably be used for deoxycytidine derivatives as well. For extension to the synthesis of oligonucleotides containing deoxyguanosine and deoxyadenosine, however, blocking groups which do not require acidic conditions for removal will have to be used.

Experimental Section

General Methods. Paper chromatography was conducted by the descending technique on Whatman 3MM paper. The solvent systems were (A) isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2), (B) 1-butanol–acetic acid–water (5:2:3), and (C) 1-propanol–2 M hydrochloric acid (3:1). The solvents were prepared on a volume basis.

Paper electrophoresis was performed using Whatman 3MM paper with an applied potential of about 40 v/cm. A Savant flat-plate electrophoretic chamber and 2000-v power supply were used. The buffers were ammonium bicarbonate (0.05 M, pH 7.55), ammonium formate–formic acid (0.05 M in ammonium ion, pH 3.6), and potassium dihydrogen phosphate–disodium hydrogen phosphate (0.0033 and 0.063 M, respectively, pH 8.0). Nucleosides and nucleotides were observed by fluorescence on paper in ultraviolet light (254 mμ). Compounds containing monomethoxytrityl or dimethoxytrityl groups were detected by spraying the papers with 10% aqueous perchloric acid and allowing them to dry at room temperature. Compounds containing monomethoxytrityl appeared yellow-orange; those containing the dimethoxytrityl group appeared red-orange.

Preparative separation of nucleotide mixtures was accomplished using a DEAE-cellulose column (4 × 30 cm) containing about 60 g of DEAE-cellulose (Calbiochem, 0.93 mequiv/g). Column effluents were monitored with a Gilson UV-2651F absorption meter coupled to a Texas Instruments Rectiriter recorder. A Gilson Model VL fractionator was used to collect 12–13-ml fractions. The DEAE-cellulose column was run at a rate of 10 fractions/hr.

Infrared spectra were obtained with a Baird Associates Model AB2 infrared recording spectrophotometer. The samples were prepared in potassium bromide disks. Ultraviolet spectra were obtained with a Cary 14 recording spectrophotometer or a Beckman DU manual spectrophotometer. Elemental analyses were performed by the Micro-Tech Laboratories, Skokie, Ill.

Solvents and Reagents. Reagent grade pyridine was distilled from *p*-toluenesulfonyl chloride, redistilled from calcium hydride, and stored over Linde Molecular Sieves. Triethylamine was distilled from calcium hydride and stored over barium oxide. Benzene was dried by distillation and dioxane was purified according to Fieser (1957). Monomethoxytrityl chloride (mp 119–121°) (Marvel *et al.*, 1944), dimethoxytrityl chloride (mp 112–114°) (Hogenkamp and Oikawa, 1964), 5′-*O*-tritylthymidine (mp 159–160°) (Weimann and Khorana, 1962), 5′-*O*-monomethoxytritylthymidine (mp 102–105°) (Schaller *et al.*, 1963), 5′-*O*-dimethoxytritylthymidine (mp 124–126°) (Schaller *et al.*, 1963), mesitylenesulfonyl chloride (mp 56°) (Wang and Cohen, 1957), and 2,4,6-triisopropylbenzenesulfonyl chloride (mp 95–96°) (Lohrman and Khorana, 1966) were prepared by procedures described in the literature. Barium β-cyanoethyl phosphate was converted to the pyridinium salt by ion exchange with Dowex 50 (pyridinium form) by the method of Tener (1961).

Alkaline Cleavage and Analysis. The standard procedure used in removing products from the support was to stir the polymer for 16 hr with 0.5 M sodium hydroxide in 50% aqueous dioxane. The mixture was then neutralized with Dowex 50 resin in the pyridinium form, the resin and support polymer were separated from the solution by filtration through glass wool, and the resin was washed several times with 50% aqueous pyridine. The combined filtrate and washings were evaporated to dryness below 35° and then redissolved in water. A portion of the solution was

TABLE I: Products from Cleavage of Partially Phosphorylated Thymidine–3′-Polymer.

Products	R_F in Solvent A	Fraction of Total Ultraviolet-Absorbing Products	% Removed in Alkaline Hydrolysis		
			1st Cycle[a]	2nd Cycle	3rd Cycle
Thymidine	0.67	0.56	93	4	3
Thymidine 5′-phosphate	0.13	0.36	96	2	2
A	0.41	0.048	67	16	17
B	0.16	0.032	81	12	9

[a] In each cycle the polymer was stirred for 16 hr in 0.5 M NaOH in 50% aqueous dioxane.

applied to a 42 × 57 cm sheet of Whatman 3MM paper on a 10-cm line and developed with solvent A or C. Bands visible in ultraviolet light were eluted with water and the optical densities of the resulting solutions were measured at 267 mμ. For calculation of the amounts of nucleosides or nucleotides present, the optical densities of solutions eluted from blanks cut from regions of the paper near the product spots were determined and substracted from the observed values. Corrections for the blanks were relatively small; *e.g.*, for 5 μmoles of thymidine the absorbance of the blank was 2% that of the thymidine. As a test of the elution procedure five thymidine solutions containing from 0.3 to 9.9 μmoles of thymidine were spotted on Whatman 3MM paper and developed with solvent A. Elution and spectrophotometric analysis indicated recovery of 98.8 ± 1.4% of the thymidine for these samples (9700 was used as the extinction coefficient for thymidine).

The efficiency of the hydrolytic method may be illustrated with the cleavage of a sample of thymidine–3′-polymer (III) which had been partially phosphorylated by reaction with phosphorus oxychloride in the presence of imidazole. As shown in Table I all nucleosidic and nucleotidic material was eluted by three alkaline cleavage cycles, and 96% of the thymidine 5′-phosphate and 93% of the thymidine were removed in the first alkaline cycle. Two other materials (A and B) which were not identified but have R_F values corresponding to dithymidine pyrophosphate and dithymidine phosphate, respectively, were removed somewhat less readily. Reproducibility in the alkaline cleavage reactions was also satisfactory; duplicate cleavage experiments on two supports bearing thymidine and thymidine 3′-phosphate (60% of the thymidine had been phosphorylated in one case; 86% in the other) yielded consistent results (±0.7% for each component). It was also shown in a control reaction that neither thymidine 5′-phosphate nor thymidylyl-(3′–5′)-thymidine was degraded under the alkaline conditions used for the cleavage reactions.

Several experiments were carried out to test the practicality of cleaving nucleotidic material from the support with ammonical solutions. In a typical case a 5-g sample of thymidine–3′-polymer which had been phosphorylated with phosphorus oxychloride was suspended in 60 ml of pyridine–concentrated ammonium hydroxide (1:1, v/v) and heated on a steam bath with stirring for 4 hr. During this time 150 ml of concentrated ammonium hydroxide was slowly added dropwise. The polymer was separated by filtration, washed thoroughly with pyridine, methanol, and ether, and the combined solution was evaporated under vacuum. The nucleotidic material amounted to 0.116 g and by spectral analysis of paper chromatograms was 80% thymidine phosphate and 19% thymidine. In three subsequent treatments the amounts of material removed were 0.094, 0.044, and 0.060 g. Analysis of the third portion indicated 30% thymidine and 68% thymidine phosphate. Assay of another sample of the polymer by the sodium hydroxide method showed 0.778 g of nucleotidic material/5 g of polymer sample. The product analyzed as 56% thymidine and 38% thymidine phosphate. Clearly the ammonical cleavage reaction is very slow and results in preferential release of thymidine phosphate relative to thymidine.

Thymidine–3′-Polymer (III). The support polymer used in these studies was prepared by polymerizing styrene (52.0 g), *p*-vinylbenzoic acid (11.4 g), and *p*-divinylbenzene (0.12 g) in the absence of a catalyst at 50° (Letsinger and Mahadevan, 1966). Titration with alkali showed 1.38 mequiv of acid/g. The carboxy polymer was converted to the acid chloride form by treatment with excess thionyl chloride in benzene at reflux for 5 hr, following which the polymer was washed with benzene and stored over phosphorous pentoxide *in vacuo*. Infrared absorption attributable to the carboxyl group in Ⓟ–COOH was completely replaced by bands at 5.65 (strong) and 5.75 μ (weak) in Ⓟ–COCl.

5′-*O*-Triarylmethylthymidine was joined to the support by reaction in pyridine. In a typical case 7.15 g (13.9 mmoles) of 5′-*O*-monomethoxytritylthymidine was stirred with 10.0 g (13.8 mmoles of acid chloride) of the acid chloride polymer in 70 ml of dry pyridine for 2 days. The polymer was removed from the solution and stirred with 25 ml of pyridine and 5 ml of methanol for 9 hr in order to esterify the remaining acid chloride groups. At this stage the polymer weighed

13.1 g (the weight increase corresponds to esterification of 65% of the acyl groups in the polymer) and showed a strong band at 5.8–6.1 μ in the infrared spectrum. Analysis of a small portion by the alkaline cleavage technique gave on chromatography in solvent A a single spot at R_F 0.87 corresponding to monomethoxytritylthymidine (positive test with acid spray).

A portion (1.2 g) of the 5′-*O*-monomethoxytrityl 3′-polymer was suspended in 30 ml of 80% aqueous acetic acid and heated at reflux for 3 hr. The insoluble polymer (0.96 g) was separated from the yellow solution, washed with acetic acid and benzene, and dried under vacuum. Chromatography in solvent A of the base cleavage product obtained from a small portion (~20 mg) of the resulting polymer showed a single spot (R_F 0.67), corresponding to thymidine. Quantitative assay of this material indicated 0.71 mequiv of thymidine/g of polymer. Considering the theoretical weight change of the polymer for conversion of the acid chloride to the thymidine derivative, this corresponds to 0.61 thymidine unit/initial carboxyl group on the support of polymer.

From a similar set of reactions carried out with 5′-*O*-dimethoxytritylthymidine, alkaline cleavage of the dimethoxytritylthymidine–3′-polymer (prior to treatment with acid) yielded thymidine as well as the expected dimethoxytritylthymidine, the ratio being ~1:3.

Phosphorylation Experiments. The results of three experiments on the phosphorylation of thymidine–3′-polymer are reported in Table II. Reaction conditions for each are described below.

(1) A mixture of 21 mmoles of pyridinium β-cyanoethyl phosphate and 8.65 g (42 mmoles) of dicyclohexylcarbodiimide in 40 ml of pyridine was stirred for 18 hr; then 3 g of thymidine–3′-polymer was added and the mixture was stirred for an additional 7 days at room temperature. Following filtration, the solid was washed with benzene and with absolute ethanol, heated on a steam bath for 8 hr with 80% aqueous pyridine, and separated by filtration. It was then washed successively with dioxane, methanol (1:1), benzene, and ether, and dried over phosphorus pentoxide. The final polymer weighed 2.92 g. Data for the product analysis for a sample that was cleaved by alkali are presented in Table II. The only phosphate obtained was thymidine 5′-phosphate (R_F 0.80 in solvent C). In a similar phosphorylation experiment in which the thymidine–3′-polymer sample had been prepared from 5′-*O*-dimethoxytritylthymidine rather than 5′-*O*-monomethoxytritylthymidine, a mixture of thymidine 5′-phosphate (R_F 0.80) and thymidine 3′-phosphate (R_F 0.86, solvent C) was obtained.

(2) A mixture of β-cyanoethyl phosphate (0.145 mmole), 0.095 g (0.432 mmole) of mesitylenesulfonyl chloride, and 0.043 g of thymidine–3′-polymer in 1 ml of pyridine was stirred for 4 hr at room temperature. The solid was separated by centrifugation and worked up as in part 1.

(3) Phosphorus oxychloride (0.87 ml, 9.4 mmoles in 3 ml of pyridine was added over a period of 15 min to 2.25 g of imidazole and 4 ml of triethylamine in 18 ml of pyridine at −16°. After 1.5 hr 0.5 g of thymidine–3′-polymer was added and the mixture was stirred at ~−16° for 2 days. The solid was separated by filtration, washed with pyridine, stirred with 50% aqueous pyridine for 13 hr, and worked up as in 1.

TABLE II: Phosphorylation Experiments on Thymidine–3′-Polymer.

Expt	Reagent	Time	Products	%[a]
1	CEP + DCC	6 days	pT	90
			T	9
			A[b]	~0.5
2	CEP + mesitylene-sulfonyl chloride	4 hr	pT	76
			T	17
			A[b]	7
3	$POCl_3$ + imidazole	49 hr	pT	73
			T	15
			A[b]	1
			B[c]	11

[a] Mole %. [b] A, probably a dinucleoside monophosphate; R_F in solvent A, 0.41; R_E pH 8.1, relative to pT, 0.24. [c] B, probably a dinucleoside pyrophosphate. R_F in solvent A, 0.16; R_E pH 8.1, relative to pT, 0.75. B hydrolyzes in 1 M hydrochloric acid at 100° (30 min) to pT.

Condensation of 5′-O-Monomethoxytritylthymidine with Phosphorylated Polymer–Thymidine. A sample of polymer–thymidine III was phosphorylated with β-cyanoethyl phosphate and dicyclohexylcarbodiimide as in 1 of the previous experiment. Analysis by alkaline hydrolysis of a small portion showed that 82% of the thymidine was phosphorylated. The cyanoethyl phosphoryl derivative of the polymer thymidine IV (0.170 g, 0.088 mmole of thymidine units) was stirred with 0.082 g (0.36 mmole of mesitylenesulfonyl chloride in 2 ml of pyridine for 23 hr; then 0.183 g (0.35 mmole) of 5′-*O*-monomethoxytritylthymidine in 1.5 ml of pyridine was added and the mixture was stirred for an additional 76 hr. The red-brown polymer was separated, washed well, dried, and analyzed by the alkaline cleavage procedure. The results are shown in Table III. Minor products A–C were not characterized.

5′-O-Dimethoxytritylthymidine 3′-Phosphate. To an anhydrous mixture of 20 mmoles of pyridinium β-cyanoethyl phosphate and 1 g of pyridinium Dowex 50 resin in 20 ml of dry pyridine was added a solution of 12.4 g (60 mmoles) of dicyclohexylcarbodiimide and 2.18 g (4.00 mmoles) of 5′-*O*-dimethoxytritylthymidine in 20 ml of pyridine. The mixture was sealed and stirred in the dark for 2 days; then it was cooled to 0°, mixed with 40 ml of water, and stirred for another 12 hr. Precipitated dicyclohexylurea was filtered from the solution and washed with 50% aqueous

TABLE III: Products from Condensation of Thymidine with IV.

Product	R_F (solvent A)	% of Ultraviolet-Absorbing Material Eluted
MTr-TpT	0.81	50
Thymidine	0.70	20
A	0.56	6
B	0.44	3
C	0.30	6
Thymidine 5′-phosphate	0.18	15

pyridine. The combined filtrate and wash (about 125 ml) were extracted several times with hexane (50 ml) and then extracted four times with 100-ml portions of chloroform. On combination of the chloroform extracts and evaporation with repeated additions of dry pyridine, a gum was obtained which was dissolved in 25 ml of pyridine and reprecipitated by dropwise addition into 500 ml of rapidly stirred ether. The cyanoethyl 5′-*O*-dimethoxytritylthymidine 3′-phosphate thus obtained was collected as a white solid by centrifugation and washed twice with ether. For removal of the cyanoethyl group this material was dissolved in 30 ml of pyridine and added to 70 ml of 1 N aqueous sodium hydroxide at 0°. The solution was allowed to warm up and was stirred at room temperature for 30 min. Sodium ions were removed by treatment with pyridinium Dowex 50 resin and the solution was concentrated to a small volume under reduced pressure with several additions of pyridine. Dilution with water and lyophilization produced 1.71 g (61%) of crude 5′-*O*-dimethoxytritylthymidine 3′-phosphate. Chromatography in solvent A showed approximately 95% of the material at R_F 0.49 (positive for dimethoxytrityl) and trace amounts at R_F 0.10 (probably thymidine 3′-phosphate) and near the solvent front (dimethoxytritanol). On electrophoresis at pH 7.55 the major product (R_F 0.49) moved half the distance of thymidine 5′-phosphate. This preparation was repeated on a tenfold scale with the same results (61% yield).

5′-O-Monomethoxytritylthymidine 3′-Phosphate. Since a good quantity of 5′-*O*-dimethoxytritylthymidine 3′-phosphate was available from the previously described reaction, it was used as a source of thymidine 3′-phosphate for synthesis of 5′-*O*-monomethoxytritylthymidine 3′-phosphate. Thus, a solution of 2.00 g of 5′-*O*-dimethoxytritylthymidine 3′-phosphate in 15 ml of 80% aqueous acetic acid was allowed to stand for several hours at room temperature; then the solution was evaporated to dryness under vacuum. The gum was taken up in a mixture of 50 ml of water and 50 ml of ether and the layers were separated. The water layer was extracted several times with fresh portions of ether and then centrifuged to remove a small amount of suspended solid. On lyophilization, the water layer yielded a white solid, which was mixed with pyridine and evaporated to dryness two times. The product was homogeneous on electrophoresis and on chromatography in solvent C, which served to distinguish between thymidine 3′- and 5′-phosphates. For conversion to the monomethoxytrityl derivative, the gum was mixed with 3.0 g of monomethoxytrityl chloride in 20 ml of pyridine. The solution was stirred in the dark for 4 days. Then the precipitate was removed by centrifugation and the solution was added dropwise to 2 l. of ether with stirring. Pyridinium 5′-*O*-monomethoxytritylthymidine 3′-phosphate precipitated. It was collected by centrifugation, washed with ether, and dried *in vacuo;* weight 1.90 g. Electrophoresis at pH 8.0 showed a single spot (positive for the monomethoxytrityl group) with R_E 0.58 compared to thymidylic acid. Chromatography in solvent A showed the product to be essentially pure with R_F 0.51. A trace of impurity (negative for a trityl-type compound) moving slightly faster than thymidine and a trace of mono-*p*-methoxytriphenylcarbinol at the solvent front were also present.

Synthesis of Thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine. A solution of pyridinium 5′-*O*-monomethoxytritylthymidine 3′-phosphate (600 mg) was prepared in dry pyridine (15 ml) and triethylamine (5 ml), and the solvents were removed at room temperature *in vacuo* to leave the triethylammonium salt of the blocked nucleotide as a pale yellow gum. The evaporation process was repeated twice to ensure dryness; then the gum was dissolved in a solution containing 840 mg of 2,4,6-triisopropylbenzenesulfonyl chloride in 15 ml of pyridine at 0°. After the solution had warmed to room temperature, 314 mg of thymidine–3′-polymer (0.71 mmole of thymidine/g, prepared from monomethoxytritylthymidine) was added and the mixture was stirred for 7 hr. The solid was then separated by centrifugation, washed twice with fresh pyridine, and stirred with pyridine–water (95:5) for ~16 hr. It was then washed with methanol and benzene and treated with a 5% solution of trifluoroacetic acid in benzene (15 ml) for 15 min to remove the monomethoxytrityl group. Formation of the triarylmethyl carbonium ion was indicated by appearance of a deep yellow color. After the polymer had been washed repeatedly with benzene and ether, a second acid treatment produced very little color. The polymer was washed with benzene, methanol, pyridine, and ether, and then was dried *in vacuo*.

For addition of the third nucleotide unit the polymer bearing the dinucleotide VI was added to a fresh pyridine solution of triethylammonium-5′-*O*-monomethoxytritylthymidine 3′-phosphate, prepared from 600 mg of the pyridinium salt as described above; and the entire sequence of steps used for the first coupling reaction was repeated. The final polymer weighed 390 mg.

Hydrolytic cleavage from the support was effected by stirring a portion of the polymer (351 mg) with 15

TABLE IV: Fractionation of Products from Preparation of TpTpT.

Peak	Fractions Pooled	ODU (267 mμ)	R_F		R_E (rel to TpTpT, pH 8)
			Solvent A	Solvent B	
I	29–45	370	0.67[a]		
II	150–170	325	0.38	0.45	0.56
III	178–195	60	0.47	0.46	1.11
			0.56	0.62	0.56
IV	245–260	210	0.10	0.46	1.47
V	278–305	1630	0.16	0.32	1.0
VI	355–373	265	0.10[b]	0.32[c]	1.54 (1.09)[d]
VII	400–416	135	0.047[b]	0.21[c]	1.20 (1.04)[d]
VIII	432–445	68	0.013[b]	0.21[c]	1.58 (1.07)[e]

[a–d] Very minor components were also found: (*a*) at R_F 0.85, 0.78, and 0.84; (*b*) at R_F 0.84; (*c*) near the solvent front; (*d*) at R_E indicated in parentheses. [e] About 40% of product at R_E 1.07 and 60% at R_E 1.58.

ml of 0.5 M sodium hydroxide in 50% aqueous dioxane for 12 hr. This process was repeated two times in order to assure complete removal of the nucleotides. At this stage the recovered polymer showed no carbonyl bands in the 5.6–6.0-μ region. The alkaline solution was initially red-violet; however, the color disappeared on standing. Sodium ions were removed by stirring the alkaline solution with pyridinium Dowex 50 resin. The resin was removed from the solution and washed with 50% aqueous pyridine, and the washings and neutralized solution were combined and stripped to a small volume below 25°. In this operation a small portion of the solution was inadvertently lost. Following treatment with 3 ml of concentrated ammonium hydroxide the remaining solution was evaporated. The residual gum was dissolved in 50 ml of 0.01 M ammonium bicarbonate in aqueous ethanol (10% ethanol) and applied to the top of the DEAE-cellulose column (4 × 30 cm, bicarbonate form). The sample was washed into the column with 650 ml of the 0.01 M bicarbonate buffer; then a linear salt gradient was begun using 2 l. of 0.01 M ammonium bicarbonate in 10% ethanol solution in the mixing chamber and 2 l. of 0.25 M ammonium bicarbonate in 20% ethanol in the reservior. When this gradient expired, another was run using 2 l. of 0.25 M ammonium bicarbonate (20% ethanol) in the mixing chamber and 2 l. of 0.50 M ammonium bicarbonate (20% ethanol) in the reservior. Common tubes from the fractionation were pooled and concentrated to dryness *in vacuo* below 35°. The resulting gums were dissolved in distilled water and the evaporation repeated to assist in the removal of ammonium bicarbonate; then the solids were dissolved in water and lyophilized to yield dry powders. Data for the fractionation and the products are collected in Table IV.

The products from peaks I, II, and IV correspond to thymidine, thymidylyl-(3′–5′)-thymidine, and thymidylic acid, respectively. The material in peak V (61 mg) was homogeneous on electrophoresis and on paper chromatography, moving identically with a sample of thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine prepared independently by Dr. Clifford L. Leznoff by detritylation of the 5′-*O*-trityl derivative which had been made by the method of Jacob and Khorana (1965). The material in peaks IV and VI–VIII may well have been held to the polymer through pyrophosphate-type linkages at the phosphate bridges in the trinucleoside diphosphate chain. In support of this idea is the fact that the same substances were slowly liberated into solution when the polymer was subjected to the action of aqueous pyridine prior to treatment with sodium hydroxide, and the material balance for the components in I, II, and V agreed well with that calculated on the basis of thymidine originally bound to the support (see below).

The white powder from peak V gave an acidic solution (pH ~2) when dissolved in water. The possibility that ammonia was lost during lyophilization or storage under vacuum was tested by titrating a solution of 12.30 mg of the solid in 1.0 ml of water with 1.99×10^{-2} M sodium hydroxide. The solution gave a sharp end point at 7.45×10^{-3} mequiv of base (pH of solution 6.5), corresponding to the loss of about 30% of the ammonia from the salt of TpTpT.

For analysis, a portion of the material from peak V was further purified by passage through a G-25 Sephadex column (1.5 × 120 cm, packed as a liquid suspension in 0.02 M ammonium bicarbonate). The column was eluted with 0.02 M ammonium bicarbonate, 12–13-ml fractions being collected. Most of the material (~99%) was eluted in fractions 16 and 17. A trace impurity was eluted in fraction 19. Fractions 16 and 17 were pooled, lyophilized, redissolved in water, and lyophilized again to ensure removal of residual ammonium bicarbonate, and the solid product was stored *in vacuo* over phosphorus pentoxide. The theoretical values for the analysis are calculated for the trihydrate of thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine with 1.5 equiv of ammonia bound as the salt. The presence of less than 2 equiv of ammonia is consistent with

the titrimetric data reported above.

Anal. Calcd for $C_{30}H_{40}N_6O_{19}P_2 \cdot 3H_2O \cdot 1.5NH_3$: C, 38.73; H, 5.47; N, 11.29. Found: C, 38.47; H, 5.17; N, 10.94.

Ultraviolet spectra were obtained in acidic, neutral, and alkaline solution for the sample purified by G-25 Sephadex gel filtration. The extinction coefficients (calculated on the basis of a molecular weight of 930, which corresponds to the formula consistent with the analytical data) are summarized in Table V. Values

TABLE V: Ultraviolet Spectra of Thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine.

Solvent (M)	λ_{max}	ϵ_{max}	λ_{min}	ϵ_{min}
HCl (0.01)	266	25,200	235	6,600
Water	266	25,400	235	8,200
NaOH (0.01)	266	22,000	247	15,000

of 25,400 (Gilham and Khorana, 1958) and 25,800 (Jacob and Khorana, 1965) for λ_{max} 266 (neutral solution) have been reported previously.

The characterization of the compound in peak V was completed by a study of its hydrolysis. Material purified by gel filtration was incubated with snake venom phosphodiesterase and was found to hydrolyze completely to 5′-thymidylic acid and thymidine in a ratio of 1.86:1 (calcd ratio 2:1). Material obtained directly from the DEAE-cellulose separation was hydrolyzed completely by spleen phosphodiesterase to 3′-thymidylic acid and thymidine in a ratio of 1.7:1 (calcd ratio 2:1) The enzyme assays were conducted in a manner similar to that described by Razzell and Khorana (1959, 1961). Thus a solution of 0.2–0.5 mg of the oligonucleotide derivative was incubated with 0.2 mg of spleen phosphodiesterase (Nutritional Biochemicals Corp.) in a mixture of 0.05 ml of 1 M tetrasodium pyrophosphate (adjusted to pH 6.0 with phosphoric acid) and 0.05 ml of 1 M ammonium acetate (adjusted to pH 6.0 with acetic acid) at 37° for 4 hr. The resulting solution was spotted as a 5–10-cm band on a sheet of Whatman 3MM paper and developed in solvent A. Appropriate spots and blanks were cut from the paper and eluted with distilled water. The solutions were made up to specific volumes and the absorbances were measured at 267 mμ. For degradations by snake venom phosphodiesterase 500 units of Russel's viper venom phosphodiesterase (Calbiochem Co.) was dissolved in 2.5 ml of 0.33 M Tris buffer adjusted to pH 9.1 with hydrochloric acid. Samples of 0.2–0.5 mg of nucleotidic material were dissolved in 0.1 ml of the enzyme solution and incubated at 37° for 7 hr. Analyses were conducted by the method described for the spleen enzyme.

For determining the yield of TpTpT in the synthesis another portion of the trinucleotide–polymer derivative (27.6 mg, 7.1% of the total polymer from the synthesis) was hydrolyzed with alkali and the products were separated by chromatography on DEAE-cellulose as before. Since the amount of thymidine in the initial 314-mg sample of thymidine–3′-polymer amounted to 223 μmoles and the per cent of the final polymer analyzed was 7.1%, a total of 0.071 × 223 μmoles = 15.8 μmoles of products would be expected. In fact, 50 optical density units of thymidine, 44 optical density units of TpT, and 205 optical density units of TpTpT were obtained. In terms of extinction coefficients of 9,700, 18,500, and 25,400 for thymidine, TpT, and TpTpT, respectively, these values correspond to 5.16 μmoles of thymidine, 2.38 μmoles of TpT, and 8.06 μmoles of TpTpT. The total of 15.6 μmoles agrees well with the theoretical amount (15.8 μmoles). The other ultraviolet-absorbing products obtained from the reaction (see, *e.g.*, Table III) probably stemmed from the arenesulfonyl chloride and the excess triarylthymidine 3′-phosphate which had reacted with the polymer derivative at the nucleophilic oxygen of the phosphodiester groups (forming pyrophosphates or mixed anhydrides which were later hydrolyzed). On the basis of the reasonable assumption that these minor components did not contain thymidine from the original thymidine–3′-polymer, 51% of the thymidine in III was converted to TpTpT in this synthesis.

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Isolation and Physicochemical Characterization of a Lipoprotein Fraction from Bovine Milk*

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ABSTRACT: An ultracentrifugally and electrophoretically homogeneous low-density lipoprotein fraction was isolated from disrupted microsomes from bovine milk. Microsomes were obtained by sedimentation (14,000*g*) from buttermilk and subsequently disrupted by passage through a high-pressure cell at 8000 psi. Adjustment of the density of the medium with NaBr permitted isolation of the low-density lipoprotein by flotation at 104,000*g*. The lipoprotein floated in salt solution at a density of 1.063 g/cm^3, and analysis of the flotation patterns obtained with the ultracentrifuge yielded a flotation constant, $S_f^0 = 26.2$, with a k value of 39.5 ml/g describing the concentration dependence of the flotation coefficients, S_f. Further analysis of the flotation data yielded the values 1.0496 ± 0.0015 g/cm^3 for the density of the lipoprotein in its hydrated form, 232 A for the average molecular diameter, and 3.9 × 10^6 for the approximate average molecular weight of the lipoprotein. Analysis by moving-boundary electrophoresis yielded the values $\mu = -4.6 \times 10^{-5}$ cm^2 sec^{-1} v^{-1} in phosphate buffer (pH 6.60, $\mu = 0.1$) and $\mu = -5.48 \times 10^{-5}$ cm^2 sec^{-1} v^{-1} in Veronal buffer (pH 8.6, $\mu = 0.1$). The gross chemical composition of the lipoprotein was 12.87% protein, 87.13% total lipid, 52.02% phospholipid, and 35.11% neutral lipid. The lipoprotein was unique in its high phospholipid content and extremely low cholesterol value. The fatty acid distribution of the lipoprotein was typical of fats from animal sources and did contain small amounts of short-chain fatty acids. The density of the lipoprotein in its anhydrous form, as calculated from the compositional data, was 0.983 g/cm^3.

Milk fat exists as a microscopic, immiscible emulsion of liquid fat in an aqueous phase of milk plasma at body temperature. Because of the surface forces inherent in this system, the fat takes the form of finely dispersed spheres stabilized by a third phase oriented at the fat–plasma interface, commonly referred to as the fat globule membrane. The structure of these fat globules, the identity of the materials that stabilize this natural emulsion, and the nature of the stabilizing forces have received much attention in many laboratories. Work in this area of research has been extensively reviewed by King (1955) and more recently by Brunner (1965).

In freshly secreted milk the lipoprotein complex of the fat globule membrane represents the principal protein–lipid interaction product. However, many studies have been reported on various fractions of the fat globule membrane complex in terms of their physical characteristics as well as chemical composition. Fractionations were accomplished by differential centrifugation (Alexander and Lusena, 1961), treatment with detergents (Alexander and Lusena, 1961; Harwalkar and Brunner, 1965; Hayashi *et al.*, 1965; Hayashi and Smith, 1965), or precipitation by controlling ionic strength and pH (Herald and Brunner, 1957). The fractions mentioned in the literature are often heterogeneous (Brunner and Thompson, 1961; Jackson *et al.*, 1962; Ramachandran and Whitney, 1960; Thompson, 1960) and usually have not been clearly defined by ultracentrifugation because they are insoluble in the solvents commonly employed for study with the ultracentrifuge.

This paper reports on the isolation of a soluble, homogeneous, low-density lipoprotein fraction from the fat globule membrane complex of bovine milk by the application of techniques usually employed for the investigation of serum and cellular lipoprotein complexes. The physicochemical characterization of the lipoprotein by chemical analysis, flotation in

* From the Department of Chemistry, University of Maryland, College Park, Maryland, and from the Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C. 20250. *Received February 13, 1967.* A preliminary report was presented at the 150th National Meeting of the American Chemical Society in Atlantic City, N. J., Sept 1965.

† This paper is part of a dissertation presented by E. B. to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the Ph.D. degree.

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