the presence of Ag-B antigens interferes with the ability of node cells to induce tolerance of other than Ag-B factors. The MLI occurs only between Ag-B incompatible cells (*13*).

A deficiency of one or more of the Ag-B specificities present in marrow, lymph node, and skin cells seems a more likely explanation for the tolerogenic shortcoming of thymocytes, since lymphocytes from rats inoculated at birth with these cells are fully reactive in vitro. Furthermore, high degrees of tolerance of skin grafts have never been induced by inoculation of these cells (*3*). It may be pertinent that in mice the content of H-2 isoantigen in thymus cells is significantly reduced in the presence of thymus-specific antigens (*14*). Another, although less likely, possibility is that thymocytes fail to reach or persist in the appropriate organs of the host for tolerance to be induced.

Although all available evidence suggests that skin grafts are more exacting in their histocompatibility requirements than homografts of other organs—kidney, liver, heart, and so forth (*15*)—it remains to be determined whether the poor ability of lymph node cells and thymocytes to induce tolerance when tested with skin grafts applies to homografts of other organs as well. This question is currently under investigation.

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16. Supported by PHS grant AI-07001. W.K.S. and D.B.W. are PHS career development awardees. D.M.L. is an NIH postdoctoral fellow. The technical assistance of J. Cavallaro is acknowledged.

13 November; revised 15 December 1969

Oligodeoxyribonucleotides: Chemical Synthesis in Anhydrous Base

Abstract. *The sugar hydroxyl of a methoxytrityldeoxyribonucleoside reacts with a methoxytrityldeoxyribonucleoside phosphorofluoridate in dimethylformamide and potassium tertiary butoxide to yield the protected dinucleoside monophosphate. The reaction is fast and specific, and is used in a stepwise synthesis to prepare trinucleoside diphosphate and tetranucleoside triphosphate.*

Condensing agents such as dicyclohexylcarbodiimide (*1*), mesitylene sulfonyl chloride (*2*), and triisopropylbenzene sulfonyl chloride (*3*) are used usually for the synthesis of phosphodiesters in oligodeoxyribonucleotides. As a new approach to phosphodiester synthesis, Borden and Smith (*4*) described the synthesis of nucleoside-3′,5′ phosphates in strong anhydrous base. In this method the phosphate in the 3′ or 5′ position of a nucleotide bearing a suitable leaving group (*p*-nitrophenoxide) undergoes nucleophilic attack by the ionized 5′- or 3′- hydroxyl group of the sugar to form the intranucleotide bond. We now have used this principle to form the internucleotide bond. The reaction of suitably protected nucleosides and nucleotides is carried out with anhydrous potassium tertiary butoxide as the base and fluoride as the leaving group on the phosphate (Fig. 1). A stepwise synthesis is possible with the use of protecting groups of different stability in acid (Fig. 1, R and R′).

The preparation of TpT by this method was as follows. DMTr-Tp_F (*6*) (20.4 μmole) and T-MMTr (10.7 μmole) in dimethylformamide (1 ml) were reacted with molar potassium tertiary butoxide in hexamethylphosphoramide (0.6 ml) for 15 minutes at room temperature. The reaction was stopped by addition of cation exchanger (Bio-Rad AG 50 W-X2, pyridinium form) (2 ml). The resin was filtered off and washed with 50 percent ethanol, and the combined filtrate was reduced to a gum by rotary evaporation. The methoxytrityl groups were removed by hydrolysis in 80 percent acetic acid (4 hours, 30°C) and subsequent ether extraction from a water solution. TpT was then isolated by DEAE-cellulose chromatography or by a combination of ion-exchange chromatography and paper chromatography (*7*). Based on the amount of T-MMTr used, the yield of TpT isolated was 90.5 percent.

Yields of TpT obtained under various reaction conditions are shown in Table 1. These indicate the following. (i) Dimethylformamide is a better solvent for this reaction than dimethylsulfoxide; (ii) the yields are relatively independent of the excess of potassium tertiary butoxide used in the reaction; (iii) high yields of TpT are obtained with only a twofold excess of the phosphorofluoridate over the nucleoside component; and (iv) the reaction is fast and probably complete in 10 minutes.

In these reactions, thymine was always one of the by-products, indicating breakdown of the glycosidic linkage. In a 15-minute reaction with a tenfold excess of base 2.3 percent of all glyco-

Table 1. Preparation of thymidylyl-(3′,5′)-thymidine. The starting compounds for these reactions were dissolved in 1 to 2 ml of the indicated solvent and reacted with potassium *t*-butoxide at room temperature. The products were isolated by ion-exchange chromatography and paper chromatography after the protecting groups had been removed by hydrolysis in acetic acid.

T-MMTr reacted with	Nucleotide/ T-MMTr	Potassium *t*-buotoxide/ ionizing group	Solvent	Reaction time (min)	TpT formed (%)
DMTr-Tp_F	1.9	10	DMF	15	87
DMT-Tp_F	2.1	10	DMSO	15	17
MMTr-Tp_F	4.4	6	DMF	10	87
DMTr-Tp_F	1.8	1.5	DMF	15	80
DMTr-Tp_F	1.9	1.5	DMF	2	49

sidic linkages were broken, and this was not significantly reduced when less base (1.5-fold excess was used). But glycosidic cleavage was reduced to 1 percent or less by replacing potassium tertiary butoxide with secondary butoxide in the reaction. Then the yield of TpT was 58 and 76 percent respectively when the phosphorofluoridate was present in twofold or threefold excess over the nucleoside component.

The internucleotide bond was also formed when the secondary hydroxyl (3′ OH) attacked the 5′ phosphorofluoridate in the presence of potassium tertiary butoxide (Table 2). The products were isolated by ion-exchange chromatography after the protecting groups were removed, and their identity was confirmed by paper chromatography before and after digestion with snake venom diesterase. Since initial studies had shown that deoxyadenosine was the least stable deoxynucleoside in anhydrous base, it was encouraging to obtain dApT in 50 percent yield. This indicated that the breakdown of the glycosidic link may not be a serious problem when the reaction is carried out for a short time.

The usefulness of leaving groups other than fluoride was investigated by reacting T-MMTr with DMTr-Tp-nitrophenyl or MMTr-Tp-phenyl in anhydrous potassium tertiary butoxide. Although *p*-nitrophenoxide is a good leaving group for the preparation of nucleoside-3′,5′ phosphates (*4*), it was not suitable for the internucleotide bond synthesis, since only low yields of TpT were obtained and thymidine-3′ phosphate was the major product in this reaction. The phenoxide group is displaced too slowly. This was indicated by the low yield of TpT formed and by recovery of most of the thymidine-3′ phenylphosphate.

To develop a stepwise method for the synthesis of oligodeoxyribonucleotides of defined sequence, we studied the reaction whereby the 5′ OH of the nucleoside or oligonucleotide attacks the 3′ phosphorofluoridate of the incoming nucleotide. We also made use of the differences in stability of the methoxytrityl protecting groups in acid, especially when they are located on the 5′- and 3′- position, respectively. Almost complete selectivity was obtained for the removal of the 5′-*O*-dimethoxytrityl group relative to the 3′-*O*-monomethoxytrityl group after 3 hours hydrolysis in 50 percent ethanol containing 2 percent acetic acid (*p*H 3.15) at 25°C. This selective hydrolysis and the isolation of an oligonucleotide bearing a monomethoxytrityl group in the 3′ position which can be used as the starting material for the next reaction, is the basis for the stepwise synthesis. The major steps for the preparation of TpTpT-MMTr are outlined in Table 3. DMTr-TpTpT-MMTr was prepared in 59 percent yield (steps 5 and 6) and from this TpTpT-MMTr was obtained in 83 percent yield (steps 7 and 8). The tetranucleotide DMTr-TpTpTpT-MMTr was formed only in low yield (19 percent) owing to insolubility of the potassium salt of TpTpT-MMTr in dimethylformamide.

Table 2. Preparation of various oligodeoxyribonucleotides. The starting components for these reactions were dissolved in 2 ml of dimethylformamide and reacted with five- to tenfold excess of potassium *t*-butoxide at room temperature. The products were isolated by ion-exchange and paper chromatography after the protecting groups had been removed by hydrolysis in acetic acid.

p_FT-MMTr reacted with	Reaction time (min)	Product isolated	Yield (%)
DMTr-T	15	TpT	81
MMTr-TpT	30	TpTpT	46
DMTr-dA	15	dApT	50

With regard to the purification procedures used, it should be noted that separation of the methoxytrityl intermediates on Sephadex is probably due to hydrophobic interaction since, for example, TpT was eluted before TpT-MMTr. As the chain of the oligonucleotide increases in length, separation on Sephadex becomes impractical. Some results indicated that better separation of these compounds can be obtained on naphthoyl cellulose (*8*) with the use of an ethanol gradient.

Although our results have been promising, two problems have to be overcome to make this method applicable for the synthesis of longer oligodeoxyribonucleotides. One is the cleavage of the glycosidic linkage in strong anhydrous base; the other is the limited solubility of the potassium salts of oligonucleotides in dimethylformamide. The breakage of the glycosidic bond is partially eliminated by the short reaction times used and can be reduced further by replacing potassium tertiary butoxide with secondary butoxide. The low solubility of the potassium salts of oligodeoxyribonucleotides limits the synthesis to a tetranucleotide under present conditions. The replacement of

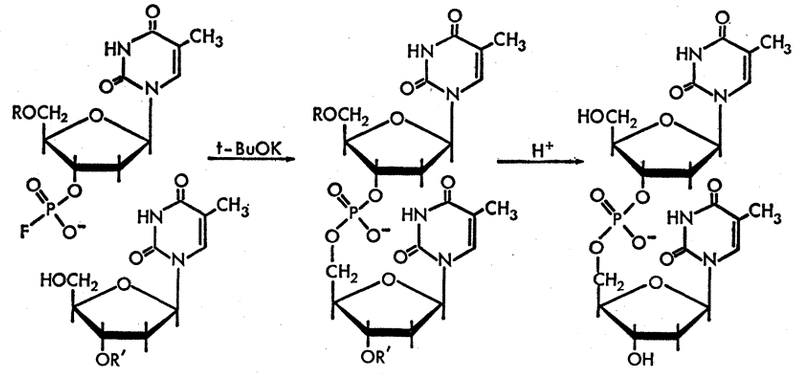

Fig. 1. Synthesis of thymidylyl-(3′,5′)-thymidine (TpT); R is the monomethoxytrityl or the dimethoxytrityl group and R′ is the monomethyoxytrityl (MMTr) group. For the stepwise synthesis the 5′-*O*-DMTr group is selectively hydrolyzed in dilute acetic acid.

Table 3. Stepwise synthesis of oligodeoxyribonucleotides.

Step	Procedure
1	$DMTrTp_F$ + T-MMTr ⟶ DMTr-TpT-MMTr + by-products
2	Purification of DMTr-TpT-MMTr on DEAE cellulose with NH_4HCO_3 gradient in 50 percent ethanol (*p*H 8.2) (*7*)
3	Hydrolysis of DMTr-TpT-MMTr in 50 percent ethanol, 2 percent acetic acid at 25°C for 4 hours
4	Isolation of TpT-MMTr on Sephadex G-25 with $10^{-4}M$ NH_4HCO_3 as eluant (*7*)
5	$DMTrTp_F$ + TpT-MMTr ⟶ DMTr-TpTpT-MMTr + by-products
6	Isolation of DMTr-TpTpT-MMTr on DEAE cellulose using NH_4HCO_3 gradient in 50 percent ethanol (*p*H 9.7) (*7*)
7	Hydrolysis of DMTr-TpTpT-MMTr (step 3)
8	Isolation of TpTpT-MMTr on Sephadex G-25 (step 4)

potassium by another cation, possibly in combination with a different solvent, may overcome this problem.

The synthesis of the internucleotide bond by this new method is fast and specific. Pyrophosphate formation under these conditions has not been detected, and the absence of protecting groups for the amino groups in the three bases will simplify purification of intermediates in a stepwise synthesis. Starting components for the synthesis of oligodeoxyribonucleotides can be prepared by existing procedures or modifications of these. We hope that the synthesis of oligodeoxyribonucleotides in strong anhydrous base will be a good alternative for the existing methods in nucleic acid chemistry.

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5. Abbreviations used are as follows: TpT, thymidylyl-(3′,5′)-thymidine; DMTr-Tp_F, 5′-*O*-dimethoxytritylthymidine-3′ phosphorofluoridate; T-MMTr, 3′-*O*-monomethoxytritylthymidine; DEAE, diethylaminoethyl; MMTr-Tp_F, 5′-*O*-monomethoxytritylthymidine-3′ phosphorofluoridate; p_FT-MMTr, 3′-*O*-monomethoxytritylthymidine-5′ phosphorofluoridate; DMTr-T, 5′-*O*-dimethoxytritylthymidine; MMTr-TpT, 5′-*O*-monomethoxytritylthymidylyl - (3′,5′)-thymidine; DMTr-dA, 5′-*O*-dimethoxytrityldeoxyadenosine; TpTpT, thymidylyl - (3′,5′) - thymidylyl - (3′,5′)-thymidine; dApT, deoxyadenylyl-(3′,5′)-thymidine; DMTr-Tp-nitrophenyl, 5′-*O*-dimethoxytritylthymidine-3′ *p*-nitrophenylphosphate; MMTr-Tp-phenyl, 5′-*O*-monomethoxytritylthymidine-3′ phenylphosphate; TpTpT-MMTr, 3′-*O*-monomethoxytritylthymidylyl - (3′,5′) - thymidylyl-(3′,5′)-thymidine; DMTr-TpTpT-MMTr, 5′-*O*-dimethoxytrityl-3′-*O*-monomethoxytritylthymidylyl-(3′,5′)-thymidylyl-(3′,5′)-thymidine; DMTr-TpTpTpT-MMTr, 5′-*O*-dimethoxytrityl-3′-*O*-monomethoxytritylthymidylyl-(3′,5′)-thymidylyl′-(3′,5′)-thymidylyl-(3′-5′)-thymidine.
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9. Supported by a grant from the Medical Research Council of Canada.

28 October 1969

Fabry's Disease: Alpha-Galactosidase Deficiency

Abstract. *The leukocytes of male patients with Fabry's disease are deficient in α-galactosidase. The α-galactosidase activity in the leukocytes of female carriers of the disease is 15 to 40 percent of the amount present in normal leukocytes. The activities of β-galactosidase, β-acetylgalactosaminidase, and β-acetylglucosaminidase in the leukocytes of affected individuals are normal.*

The metabolic defect in Fabry's disease (*1*) is a deficiency of the enzyme ceramide trihexosidase, which catalyzes the cleavage of galactose from ceramide trihexoside. Consequently, the latter substance accumulates in various tissues. Generalized G_{M1} gangliosidosis is due to a β-galactosidase (E.C. 3.2.1.23) deficiency (*2*, *3*) which results in the accumulation of a ganglioside with a terminal β-D-galactose moiety. While in generalized gangliosidosis the deficiency can be demonstrated with a chromogenic aryl substrate, no such substrate appeared suitable for the detection of the enzyme defect in Fabry's disease. Indeed, it has been suggested (*4*) that the only substrate that can be hydrolyzed by this enzyme is the ceramide trihexoside itself. Many related β-galactosides have no inhibitory effect on the ceramide trihexosidase assay (*4*), and the trihexoside did not influence the brain methylumbelliferyl-β-galactosidase activity (*5*). My data indicate that leukocytes from patients with Fabry's disease are totally deficient in the nonspecific α-galactosidase (E.C. 3.2.1.22) activity. The enzymic defect can now be revealed with chromogenic aryl substrates in almost every other sphingolipidosis, especially in Gaucher's disease (*6*), in generalized gangliosidosis (*2*, *3*), in the usual (*7*) and the exceptional (*8*) form of Tay-Sachs disease, in metachromatic leucodystrophy (*9*), and in fucosidosis (*3*).

The finding (*10*) that a ceramide trihexoside with a terminal α-D-galactose occurs in cancer tissue led to the idea that this substance might also be present in normal tissues and that the glycolipid accumulation in Fabry's disease might be due to the absence of a nonspecific α-galactosidase. Because human leukocytes contain a number of hydrolytic enzymes it was of interest to determine the activity of acid-α-galactosidase in the leukocytes of normal individuals and of patients with Fabry's disease.

Homogenates of purified leukocytes were prepared by the method of Kampine *et al.* (*11*). The activity of β-galactosidase, β-acetylgalactosaminidase, and β-acetylglucosaminidase (E.C. 3.2.1.30) were assayed with fluorogenic methylumbelliferyl substrates; α-galactosidase activity was measured colorimetrically with *p*-nitrophenyl-α-galactoside and fluorimetrically with methylumbelliferyl-α-galactoside as substrate. Both methods gave similar results. The incubation mixtures for the different enzyme assays included: (i) for β-galactosidase, 0.2 μmole of methylumbelliferyl-β-D-galactoside, 6 μmole of acetate buffer (*p*H 4.0), and 20 μl of leukocyte extract in a final volume of 200 μl; (ii) for β-galactosaminidase, 0.6 μmole of methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactoside, 15 μmole of acetate buffer (*p*H 4.0), and 20 μl of leukocyte extract in a final volume of 200 μl; (iii) for β-glucosaminidase, 0.5 μmole of methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucoside, 15 μmole of acetate buffer (*p*H 5.0), and 10 μl of leukocyte extract

Table 1. Enzyme activities in leukocytes of control subjects and of patients with Fabry's disease. The values expressed are nanomoles of substrate hydrolyzed per hour per 10^6 cells. Numbers in parentheses indicate the range of values.

Subjects	Kinship	Age (yr)	β-Galactosidase (mean)	β-Acetylglucosaminidase (mean)	β-Acetylgalactosaminidase (mean)	α-Galactosidase	
						Colorimetric (mean)	Fluorimetric (mean)
		Normal males (n = 15)					
		15–33	76 (27–153)	295 (139–446)	71 (45–128)	13 (6–25)	12 (7–17)
		Fabry's disease, affected males					
L. G.		31	110	433	105	< 0.5	< 0.2
Mo. A.		16	71	485	114	< 0.5	< 0.2
Mu. A.	Brother of Mo. A	9	122	1220	173	< 0.5	< 0.2
		Fabry's disease, female carriers					
L. D.	Sister of L. G.	26	60	346	50	2.4	1.8
R. C.	Mother of L. G.	53	68	378	54	5.6	5.0
Fa. A.	Sister of Mo. A.	11	48	526	96	4.2	3.9
Na. A.	Sister of Mo. A.	6	56	1000	112	3.8	4.9