SOME RECENT DEVELOPMENTS IN SOLID PHASE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES

K.N. GANESH
Centre for Cellular and Molecular Biology, Hyderabad 500 007, India.

ABSTRACT

The solid phase methodology has evolved to be the method of choice for chemical synthesis of short DNA fragments which have diverse applications in the field of molecular biology and biophysical structural studies. This is the result of constant improvements in the chemistry of internucleotide bond formation, nature of supports and the use of purification methods involving high performance liquid chromatography. By employing continuous-flow methods, the synthesis can be partially or completely automated leading to speed, savings in labour and economy of materials. Some of the developments responsible for this state of art are reviewed.

INTRODUCTION

SYNTHETIC oligodeoxyribonucleotides have numerous practical applications in molecular biology[1]. They serve as primers and templates in enzymatic synthesis, as building blocks for *in vitro* enzymatic synthesis of genes, as probes for specific sequences in DNA and RNA and as model compounds for physico-chemical investigations. Until recently, the methods of chemical synthesis of these fragments depended on solution chemistry which is not only skilled, laborious and expensive but also inefficient for making them routinely available to many needy scientific groups. The development of solid phase synthesis of oligonucleotides analogous to the well-known Merrifield peptide synthesis has now simplified several problems associated with solution methods and is now almost the method of choice for preparation of microgram to milligram quantities of short DNA fragments. This progress originates from constant efforts in various laboratories to improve several aspects of solid phase synthesis such as the search for a good support, efficiency of condensing agents, choice of compatible protecting groups and finally the advent of high performance liquid chromatographic (HPLC) techniques for purification. In addition, the amenability of the solid phase methodology to mechanisation and automation has paved way for commercial availability of synthesisers ranging from completely manual to fully automated "gene machines" which are becoming essential components of most modern biological laboratories. This article reviews in brief some of the recent developments and the state of art in this area which has led to this renaissance. The review is not exhaustive and there is a bias towards methods used in our own laboratory, with the natural consequence of omission of some of the contributions in this area by well-known groups. Two excellent books[2,3] published on this topic discusses the finer details of experimental methodologies and are strongly recommended to the practitioners.

The solid phase methodology consists of the assembly of oligomers in a stepwise manner by sequential addition of monomeric units onto a chain suitably anchored to a resin support. For oligonucleotides, this assembly can either be in 3′→ 5′ direction or in the reverse and the monomeric units required for each vary accordingly. The former is the preferred method since a combination of compatible protecting groups (for hydroxyls at 3′ and 5′, and the exocyclic NH_2 of base) has been well established for this approach. The method involves the attachment of a 5′-O-protected deoxyribonucleoside to a solid support through a succinoyl linkage (figure 1) and the chain assembly by alternation of essentially two reactions: (i) 5′-deprotection and (ii) coupling. At the end of the assembly, the

oligonucleotide is cleaved from the support, protecting groups are removed and the deprotected oligonucleotide is purified by HPLC. The chemistry of the coupling itself can either be the phosphotriester route or phosphite triester/phosphoramidite method (figure 1).

THE SOLID SUPPORTS

The important features characterising the choice of a support for solid phase synthesis are (i) inertness to various chemical reagents used in the assembly (ii) functionalisability/loading capacity and (iii) mechanical strength. Based on these considerations there are five resins which are being currently used for solid phase DNA synthesis (table 1). All of these can be used for phosphotriester method whereas only three are stable to phosphite chemistry. One of the most efficient ways of performing reactions on solid supports is to suspend the support in a column and flow the solvents and reagents through it in a controlled fashion. In such continuous flow systems, removal of excess reagents is faster, efficient and economical with the possibility of on-line monitoring, automation and feed back control. However, packing of the resin occurs under flow conditions leading to back pressure and disintegration of support. This is a serious drawback for high swelling organic resins such as polystyrene and polyamide, though the large loading capacities of these are an asset for macroscale synthesis. The inorganic supports such as silica and glass beads[4] are chemically inert, mechanically rigid to flow conditions and porous enough for penetration of reagents and solvents. This together with their stability to both chemistries and the reasonable loading capacities make them ideal for use in manual and mechanised operation systems. The Kiesulguhr/Polyamide resin is a composite of polydimethylacrylamide gel embedded in macroporous Kielsulguhr and engineered to combine the best advantages of both the inorganic and organic resins[5]. This performs well under flow

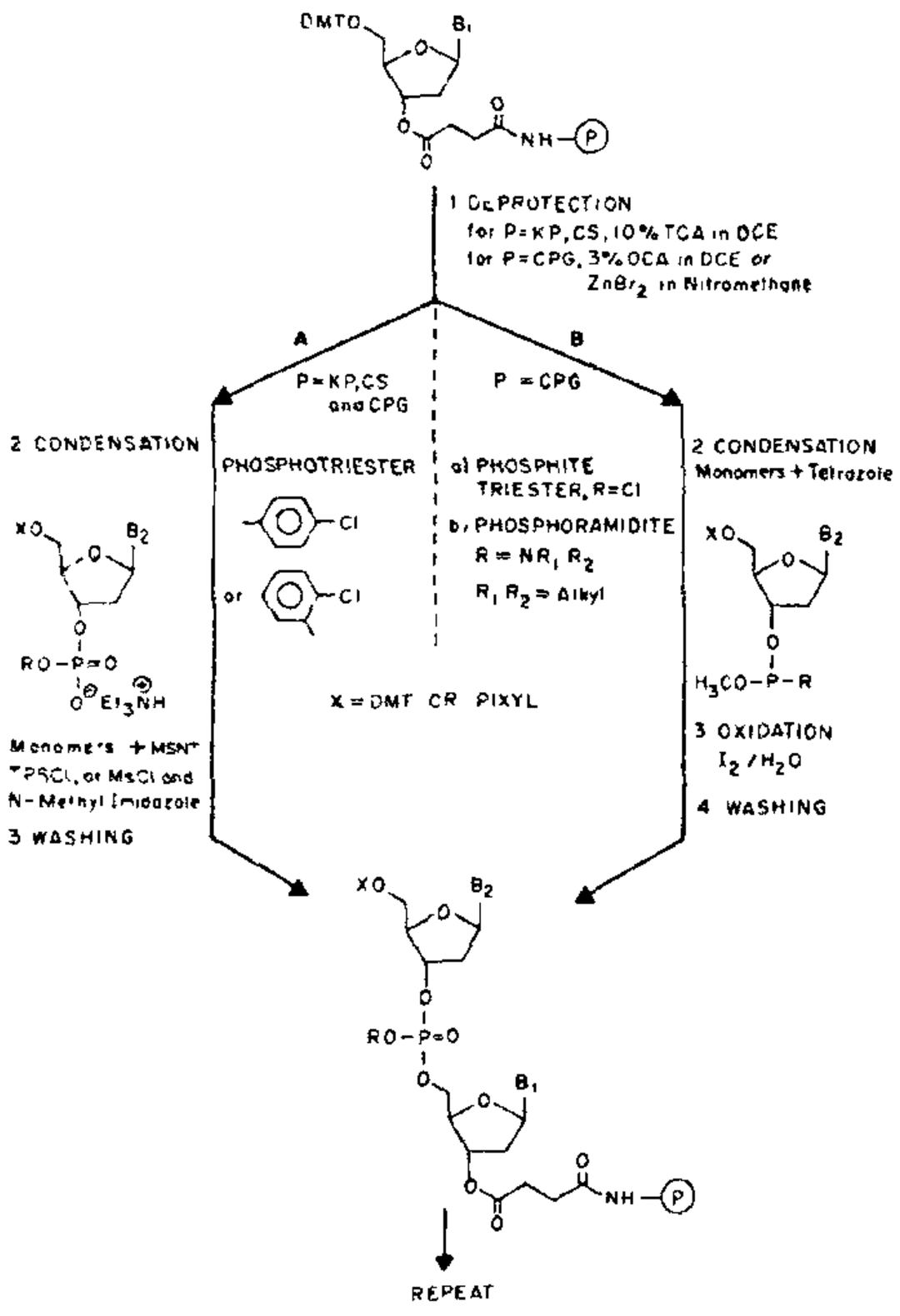


Figure 1. Scheme for oligonucleotide synthesis by solid phase methods. KP = Kiesulguhr/polydimethylacrylamide, CS = Cellulose, CPG = Control pore glass, TCA = trichloroacetic acid, DCA = dichloroacetic acid, DCE = dichloroethane, TPSCl = 2,4,6-triisopropyl benzene sulphonyl chloride, MsCl = Mesitylene sulphonyl chloride, MSNT = mesitylene sulphonyl 3-nitro triazole

Table 1 *Characteristics of five commonly used polymer supports for oligodeoxyribonucleotide synthesis*[3]

Rigidity	Accessibility at low loading < 0.1 mmol/g	Accessibility at high loading > 0.1 mmol/g	Use for phosphotriester	Use for phosphite triester
Polystyrene (1% DVB)	+	++	+	++	+
Silica gel	++	++	+/−	+	++
Glass beads (CPG)	++	++	+/−	++	++
Polyamide/ Kieselguhr	+	++	+	++	−
Cellulose paper	++	+	−	+	−

++ Excellent; + good; +/− moderate; − poor.

conditions but stable only to phosphotriester chemistry. Cellulose in the form of paper discs offers unique advantage for simultaneous syntheses of a large number of oligonucleotides by phosphotriester route in small quantities and is ideal for the preparation of all overlapping fragments required in total synthesis of genes[6,7].

THE CHEMISTRY

The failure of the initial attempts of solid phase synthesis was mostly due to the use of Khorana's classical phosphodiester approach in which the increased polarity of the products with the chain length was incompatible with the non-polar nature of the supports[8]. The advent of phosphotriester and phosphite triester methods, in which the products are non-polar due to the protection of phosphate anionic sites, gave an impetus to the solid phase methodology[2,3]. The progress in the chemistry that has led to the present state of art are (i) development of efficient methods for quantitative deprotection of 5′-hydroxyls with minimum side reactions, (ii) the use of a combination of suitable condensing and activating agents resulting in the reduced coupling times and increased yields for internucleotide bond formation and (iii) the development of reagents to cleave the oligonucleotide chains from the support and deprotection of other functionalities with minimum chain scission. Since the reactions on solid supports are slower than those in solutions, the concentration of solution reactants used are about 5 to 10 equivalents over the resin loading in order to drive the reaction to the product side. Consequently, the complete removal of unreacted reagents at each step is important and this is achieved by washing with solvent volumes of at least 10 times the resin bed volume. These factors amplify the effect of even minor reactive impurities in both the monomers and solvents. So, the most critical and non-compromisable requirement for the success of solid phase synthesis is the use of ultrapure monomers and solvents. We have synthesized all the required monomers (I, figure 2) in a high degree of purity starting from deoxynucleosides using one-pot

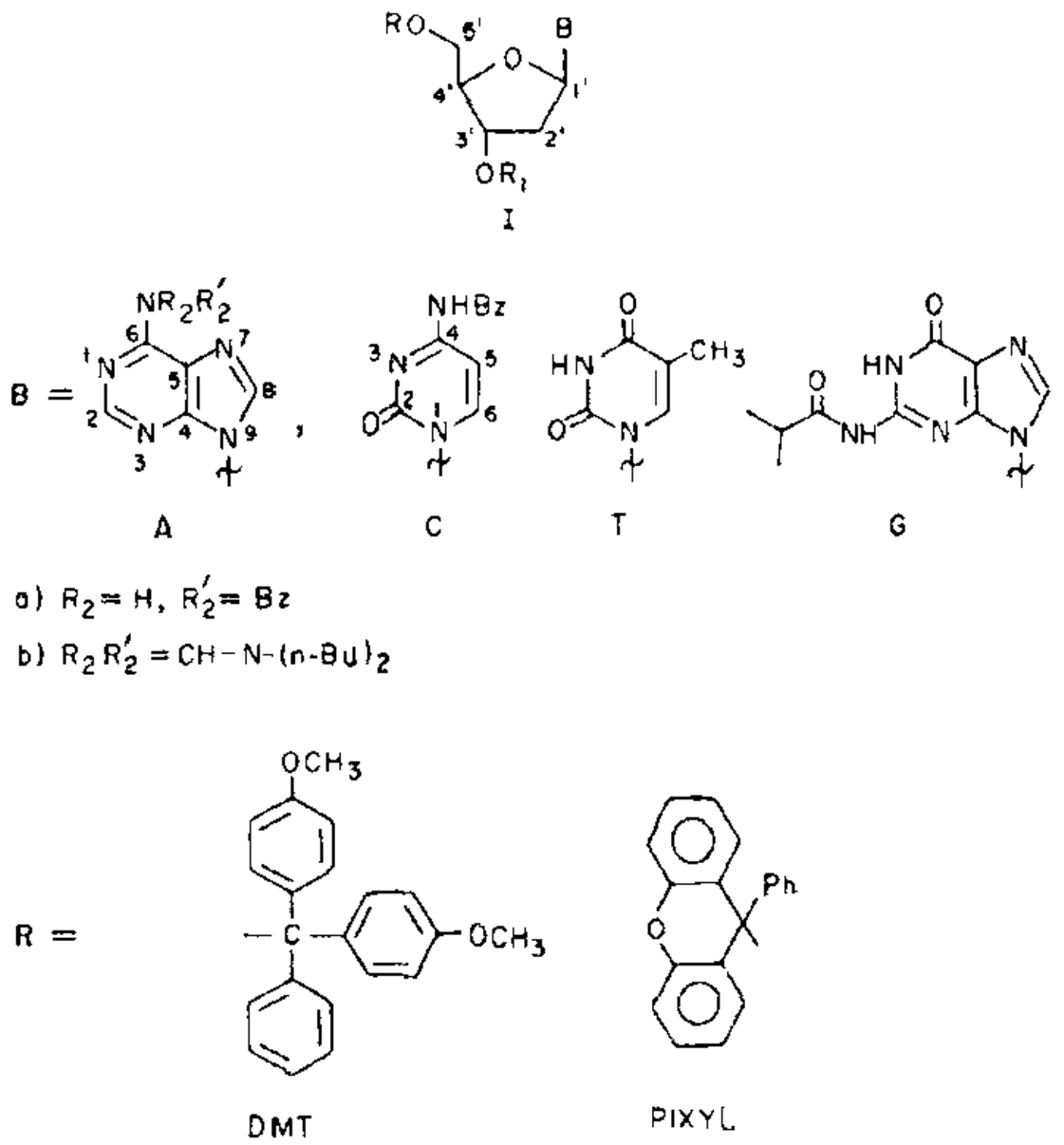


Figure 2. Structures of protected monomers used in oligonucleotide synthesis.

transient protection procedure and indigeneous solvents and reagents to obtain successful synthesis of oligonucleotides by phosphotriester route[9,10].

(i) *5′-terminal deprotection*

This step is common to both the routes (figure 1). It must be quantitative and completed in short times under mild conditions to avoid undesirable depurination of N^6-benzoyl protected adenosines. Weak protic acids such as trichloro and dichloroacetic acids effect removal of trityl group at rates independent of chain length but highly dependent on support and solvent[11] (figure 1). A 3% solution of dichloroacetic acid in dichloromethane causes complete detritylation in about 40 seconds for 5′-A and G and 75 secs for 5′-C and T on glass resin whereas on polyamide, the timings are about 3–4 times

longer. By employing 5′-O-pixyl groups[12] (figure 2) deprotections can be hastened up by about 30%. The Lewis acids (such as $ZnBr_2$) effect deprotection without significant depurination but the efficiency depends considerably on chain length and support. The dimethoxytrityl and pixyl cations are intrinsically chromophoric and can be used as probes for internal assays of synthetic efficiency. The next step in the assembly procedure yields phosphotriester products which differ only in their permanent phosphate protecting groups though the monomers used are different.

(ii) *Phosphotriester approach*

The effectiveness of this approach has been proved in the chemical synthesis of various biologically active DNA fragments. The coupling agents used normally are arene sulphonyl chlorides having bulky *ortho* substituents such as 2,4,6-triisopropyl benzene sulphonyl chloride and mesitylene sulphonyl chloride in combination with nucleophilic catalysts such as tri- and tetrazoles (figure 1)[13]. The active species are actually sulphonyl tri- or tetrazoles which being unstable are generated *in situ* and in addition to improving yields, they decrease the side reactions such as 5′-O-sulphonylation and reduce the reaction times. A good balance in stability and reactivity is achieved by the use of mesitylene sulphonyl-3-nitro triazole which is being widely used at present[11a]. Sulphonyl chlorides or MSNT in combination with N-methyl imidazole have proved to be very effective coupling agents with the advantage that reactions can be carried out in neutral polar solvents like acetonitrile and dichloromethane[9, 14]. The phosphate protecting groups used are either -o- or p-chlorophenyl. The efficiency of condensation depends on the nature of support, the reaction being slightly faster on glass compared to polyamide, but mostly complete within 15 minutes on all supports. A typical assembly cycle is indicated in table 2.

Table 2 *Typical assembly cycle for synthesis of oligonucleotides on OMNIFIT manual DNA synthesiser*

Support	:	T functionalised KP, 25 mg (5.3 μmole)	
Linkage	:	'SUCCINAMIDO'	
Cycle	:	*Solvent*	*Time (min) (flow rate 2 ml min⁻¹)*
		Pyridine	4
		1,2 Dichloroethane	2
		10% TCA in 1,2-dichloroethane	3
		DMF	2
		Pyridine	3
		Coupling (stop flow)	15
Coupling Solution	:	Monomer	40 μ mole (35 mg)
		MSNT	133 μ mole (40 mg)
		N-Methyl imidazole	375 μ mole (20 μl)
		Pyridine	0.3 ml

This cycle was used for the synthesis of a 23 mer (figure 3b)

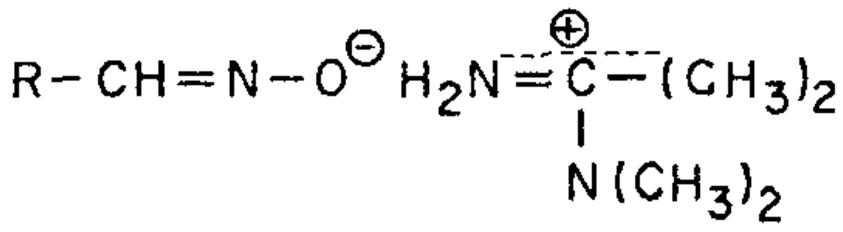
(iii) *Phosphite ester and phosphoramidite methods*

These methods exploit the high reactivity of trivalent phosphorus compounds and involve one step more than the phosphotriester route[15–17]. Because of the speed and efficiency of both these steps, this method is the fastest among the currently available routes. The reactivity of the phosphorylating agents (figure 1, route B) depends on the P–O protecting group and several substitutions have been used ranging from alkyl (R = Me, Et) to 2-trihalo alkyl. One serious side reaction during the condensation is the formation of unwanted 3′-3′ dinucleoside phosphates and this can be suppressed by using hindered protecting groups. An ingenious way to control the reactivity of the active phosphites is by the use of nucleoside phosphoramidites (figure 1, route B) which are stable to water. This stability can be altered by the nature of amido substituents, the increased steric effect about P–N bond leading to increased resistance to water[11(b)]. A number of hindered alkylamine including morpholine derivatives have been used. During the coupling, active phosphitilating agent is generated by the addition of a weak acid such as tetrazole. The resulting phosphite esters (figure 1, route B) are then oxidized to phosphotriesters by aqueous iodine and the combined reaction times are only about 10 minutes. This method which is suitable for only glass and silica

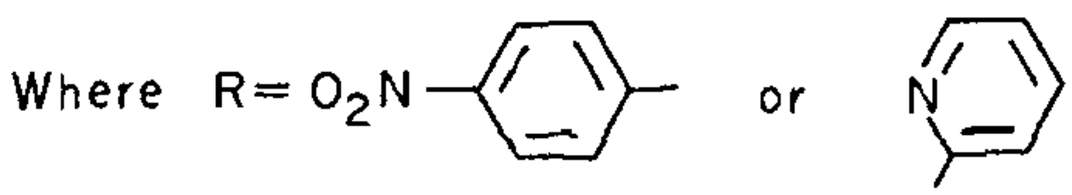
resin is being currently utilized in 'gene machines'.

(iv) *Deprotection*

At the end of the assembly, it is essential to remove all the protecting groups but in the right order. First the phosphate protecting groups are removed to obtain the phosphodiester and subsequently the oligonucleotide chains are cleaved from the support. This is followed by treatment with ammonia which removes the N-acyl groups and finally the acetic acid treatment takes off the DMTr or pixyl group. The product from phosphotriester route is treated with oximate reagent (VII) (scheme 1), (equimolar amounts of 1,1,3,3-



(VII)



tetramethyl guanidine and 2-nitrobenzaldoxime or pyridine 2-carbaldoxime) which deprotects phosphate groups and simultaneously cleaves the oligonucleotide chains from the support. This reagent is also known to reverse base modifications such as sulfonylation at O^6 of guanosine by condensing agents and phosphorylation by activated nucleotides. In the phosphite triester/phosphoramidite procedures where the phosphate protecting groups are different, the reagents used are thiophenol/triethylamine in pyridine, tributyl phosphine, aromatic radical anions or zinc depending on the protecting group. These reagents do not cleave the product from support and this is achieved during the later ammonia treatment.

Since there are no chromatographic purifications during the chain assembly, oligonucleotide impurities which accumulate progressively as a function of chain length must be resolved from the desired product at the end. The likely impurities include those arising from truncated and deletion sequences, base modified chains and those resulting from fragmentations during deprotections.

(v) *Purifications*

High performance liquid chromatography (HPLC) is almost the only method of choice for purification of synthetic oligodeoxynucleotides[19]. Since these are polyanions with liphophilic bases, both anion-exchange and reverse-phase methods are effective for their purification. The excellent resolutions achieved are primarily the result of development of bonded phase columns using silica microparticulates and purifications can be done on analytical (~ 1 A_{260}), semipreparative (1–50 A_{260}) or preparative (>50 A_{260}) scales using appropriate columns.

The initial purifications are best done on ion-exchange HPLC which separates oligonucleotides principally on the basis of their length. Since the desired product is always the longest sequence, its identification is easy as it elutes as a large peak at the end (figure 3). Location of the desired peak is rather unobvious if the crude synthetic mixture is injected directly on reverse phase column. The use of formamide in the ion-exchange elution buffer prevents the aggregation of oligonucleotides and this is particularly important for longer and self-complementary sequences; otherwise resolution is not observed as a function of chain length. The use of radial compression cartridge system instead of conventional column has considerable advantage. With this, separations can be carried out at higher flow rates, with larger amounts of material load, still maintaining reasonable elution profiles and ensuing longer column life times. The desired peak after initial elution from ion-exchange run is desalted over Bio-gel P2 and then injected on a C_{18} reverse phase HPLC column to either assess the homogeneity of the product or even as a second purification step. We have used the recent technique of fast protein liquid chromatography (FPLC) to purify the oligonucleotide mixtures[19]. Compared to HPLC, this offers high resolution in shorter times with reproducibility of elution profiles over many injections. Preparative runs are also possible with sample loads of a few milligrams.

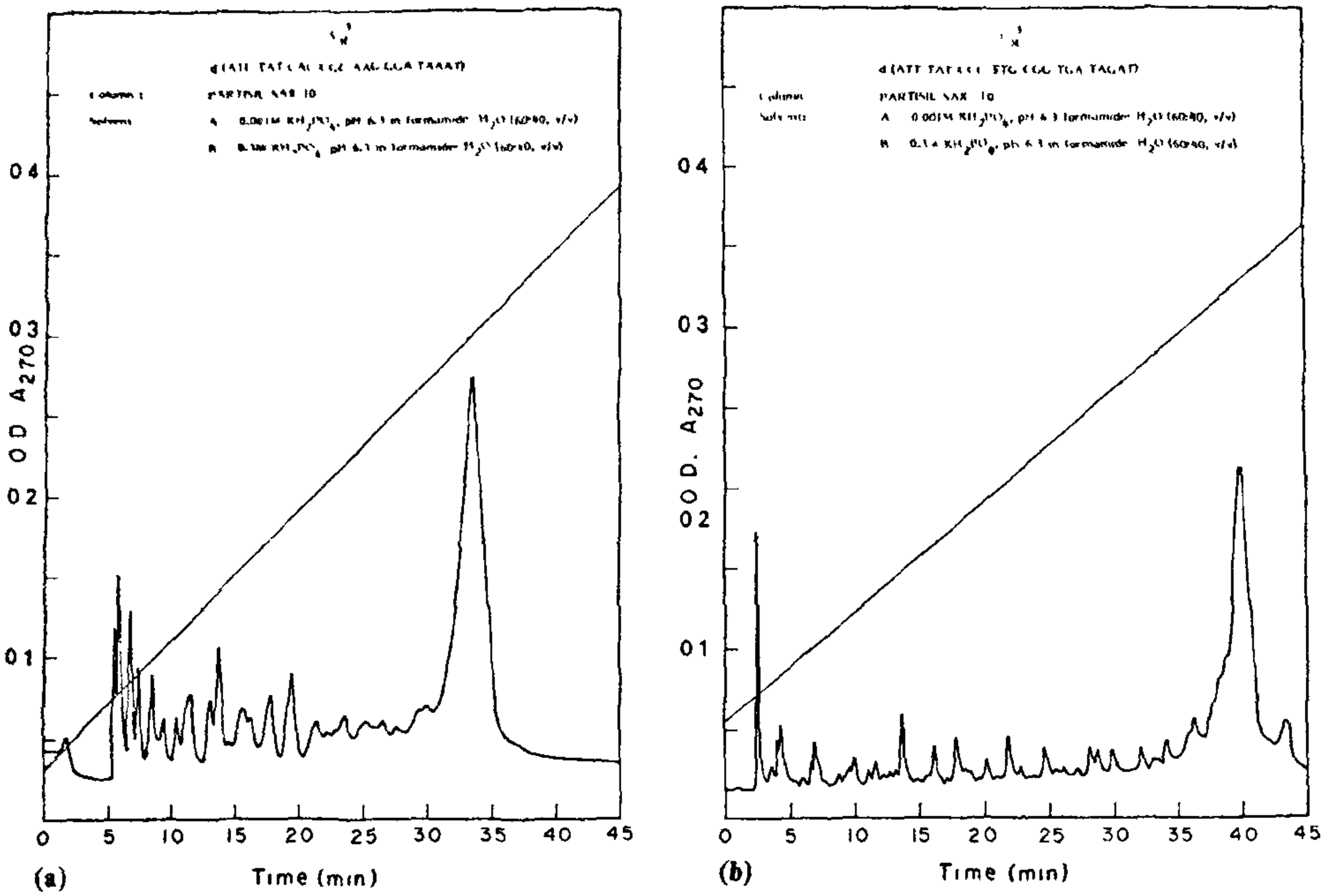


Figure 3. HPLC profiles of oligonucleotide mixtures from solid phase phosphotriester synthesis. (**a**) Assembly on CPG resin and (**b**) Assembly on KP resin.

(vi) *The equipment*

All the advantages of solid phase methodology can best be realised by using a continuous-flow apparatus having a configuration shown in figure 4. This can conveniently be assembled from a kit supplied by OMNIFIT[2,3] which consists of reagent bottles, gas pressure regulator, all-teflon six way rotary valve and glass columns fitted with an adjustable plunger and a septum injector. The mechanical rotary valve allows the selection of solvents and reagents flowing through the column and driven by dry nitrogen or argon at about 1.5 to 2.0 p.s.i. resulting in a flow rate of 1–2 ml/min. At the appropriate point during the synthesis cycle (table 2), the mixture of monomer, coupling agent and catalyst is injected through the septum on top of the column. The acid washings can be collected separately during

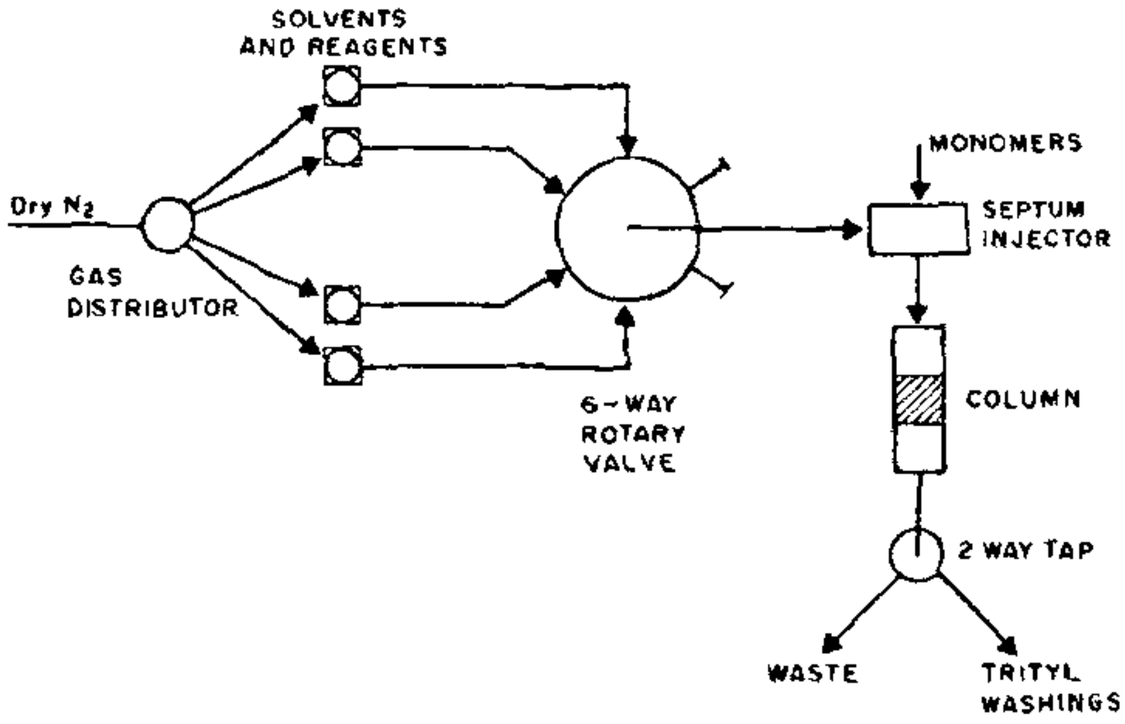


Figure 4. Flow diagram of OMNIFIT manual DNA synthesiser

every cycle for assaying the reactions. One problem commonly encountered is the spontaneous drying of the resin bed due to the dissolved inert gas in volatile solvents, leading to

incomplete reactions on the support. This is overcome by degassing through a reversed-flow of solvent which is achieved by reassembly of the set-up incorporating a two-way stopcock[20]. Using multiway solvent distributors, several columns can be run simultaneously with this set-up and this is helpful for making large amounts of the same chain or simultaneous synthesis of all the required sequences for total synthesis of a gene using cellulose discs as supports[7]. We are at present updating the OMNIFIT apparatus for semi-automatic mode by inclusion of an electronic timer which automatically rotates the valve sequentially after pre-set intervals. The automated gene machines work on a similar principle but in addition has the capability of auto injection of monomers according to a predesired sequence and the whole operations controlled through microprocessors[2].

SCOPE FOR IMPROVEMENTS

Further improvements in the overall yields are possible by reducing the potential for side reactions. The yield per coupling (Y_c) and the overall yield (Y_0) per n cycles are related by the equation:

$$Y_c = 100\,(Y_0/100)^{1/n}$$

and so even a few percent improvement in Y_c contributes to a significant increase in Y_0, for example, increase of Y_c from 95 to 99 % means a hike in overall yield from 35 % to 81 % for a 20 mer. Three serious problems that require to be effectively tackled are (i) minimisation of depurination, particularly during acidic deprotection step (ii) O^6-G and O^4-T-modifications during coupling and (iii) loss of product through cleavage of resin-nucleotide linkage during synthesis. The depurination is minimized by using pixyl group instead of DMTr for 5′-O-protection and by using phtaloyl or di(n-butylamino) methylene gps (Ib) for N^6-adenine protection causing it to be more acid-resistant[21]. The protection of O^6 of G residues by phenyl[22], 2-nitrophenyl[22] or p-nitrophenyl ethyl[23] groups are recommended for synthesis of G-rich sequences. The succinoyl ester linkage replaced by the more base-stable urethan linkage has given much better results in terms of overall yield[24]. Also, the scaling up of the synthetic methods for preparing large amounts of oligonucleotides required for structural work such as x-ray and NMR presents a few problems in terms of choice of support, stochiometry of reaction mixture, resin-handling in continuous flow systems and purifications. Some of these difficulties have been recently overcome[20,25]. Many of these improved monomers and resins are not yet commercially available but nevertheless they emphasize as to how much chemistry is yet to be done to reach perfection in this field. The present preoccupation of chemists with the synthesis of DNA fragments has also hindered the development of methods for RNA synthesis for which the properly protected monomer building blocks are less easily available. One hopes to see more progress in the area of RNA synthesis in future.

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ANNOUNCEMENT

REPUBLIC DAY AWARDS

On the eve of the 37th Anniversary of the Republic of India, the President of India awarded the following titles to eminent scientists of this country:

Padma Vibhushan:– Prof. C. N. R. Rao, Bangalore, Prof. M. G. K. Menon, New Delhi.

Padma Bhushan:– Prof. S. Ramaseshan, Bangalore, Dr S. Varadarajan, New Delhi, Dr Amarjit Singh, Pilani, Prof. B. Peters, Denmark, Prof. B. M. Udgaonkar, Bombay, Prof. E. V. Chitnis, Ahmedabad, Prof. G. Singh, Delhi, Prof. Rais Ahmed, New Delhi, Prof. V. L. Chopra, New Delhi.

Padma Shri:– Dr Biswa Ranjan Chatterjee, Calcutta, Shri Chandra Mohan, Punjab, Prof. D. S. Kamat, Ahmedabad, Dr M. S. Valliathan, Trivandrum, Dr S. S. Sriramacharyulu, New Delhi.

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