Large-Scale Oligonucleotide Synthesizers

I. Basic Principles and System Design

NORMAN G. ANDERSON,* N. LEIGH ANDERSON,
JOHN TAYLOR, AND JACK GOODMAN

*Large Scale Biology Corporation,
9620 Medical Center Drive, Rockville, MD 20850*

ABSTRACT

The central problem in scaling up oligonucleotide synthesis is to expose each element of a large bed to the same conditions obtaining in very small ones, for the same intervals of time. Our analysis suggests that scale-up is chiefly limited by fluid path length through the bed. By using annular beds in zonal centrifuge rotors of unique design, with fluid flow controlled by combining centrifugal force with differences in physical density between reagents, reagent fronts may be kept exactly perpendicular to the direction of flow in each bed element. Under these conditions, bed volume may be increased by increasing rotor length and diameter. The rotor is lined with polypropylene or Teflon®, and has a thick tempered glass end window. Transparent rotary valves of a unique design allow any of 47 reagents to be selected and the direction of flow through the rotor to be controlled. A photodiode spectrophotometer provides complete absorption spectra on fluid in the rotor inlet and outlet lines every 4 s, and a large balance weighs effluent from the synthesizer continuously. The entire operation is controlled by a work station, and steps may be programmed by time, absorbance, or reagent mass. Reagents are identified by spectra, and trityls are integrated on line. A detailed time-stamped log file provides a complete record of each synthesis.

Index Entries: Oligonucleotide synthesis; antisense; phosphorothioates; specific sequence drugs; aptamers; ribozymes.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

This article describes a new class of biopolymer synthesizers in which synthesis occurs in an annular bed of solid-phase support in a zonal centrifuge rotor. A combination of centrifugal force and density differences between solutions is used to displace one solution by another quantitatively. The result is an efficient synthesis system, with decreased reagent utilization, which can be scaled up to production levels.

Ordered-sequence biopolymers, including conventional DNA, antisense DNA, RNAs, including ribozymes and nexamers, peptides, so-called peptide DNAs, aptamers, diversomers, polysaccharides, and a variety of new compounds composed of ordered arrays of monomers not normally found in biological systems (diversomers), now hold great promise as pharmaceuticals (1). The alternatives for manufacture of these compounds include stepwise solution-phase synthesis, stepwise solid-phase synthesis, and fragment assembly either in solution or on solid-phase supports. For the near future, it appears that compounds longer than approximately ten monomers (10-mers) will be synthesized using solid-phase methods based on protocols that are currently available. Although this article is concerned with oligonucleotide synthesis, many of the problems considered relate to the synthesis of all types of ordered polymers.

Thus far, no one has reported the development of oligonucleotide synthesizers capable to producing the batch sizes that will be required if any one of the approx 40 compounds now under development were to be approved for production as a pharmaceutical. Estimates of the amounts ultimately required vary from hundreds of grams per year for some topical applications, to metric tons per year for some systemic uses. Requirements will also vary depending on whether the application is for AIDS, tropical parasites, major human cancer, or for a rare disease afflicting only a few individuals, and also on the dose required per patient.

The problem of developing production synthesizers is rendered more complex by the FDA requirement that production systems and methods be in place when a new drug is approved, i.e., it is necessary to show that the drug can indeed be produced. After approval, it is difficult and costly to change methodologies. Although amounts of drug sufficient for animal and some initial clinical trials can be made using present synthesizers, companies aiming to develop and produce new classes of sequence-specific drugs are faced with the problem of concurrently developing both the drugs and new production-scale systems for their synthesis.

It might be thought that synthesis can be scaled up simply and linearly by increasing the size of each component and of reagent volumes. In practice, however, it appears to be a universal experience that the efficiency of oligo synthesis drops off as synthesis is scaled up, for reasons that have not been fully understood. Unfortunately, unsuccessful results are either not reported or not reported in detail. Hence, it is difficult to

assemble and intercompare data and experience on scaling up oligo synthesis from different laboratories, especially when proprietary sequences and methods are involved. We have been unable to find published data on synthesis in beds having volumes in the range of 300 mL to several liters. We are, however, aware of laboratories continuously running ten or more conventional commercial instruments producing a few grams of crude product per run, and pooling these batches for animal and clinical studies. It is difficult to escape the conclusion that a requirement is emerging for much larger-scale synthesizers.

Although considerable advances have been made in solution-phase synthesis of oligonucleotides (2), it appears that for the near term, large-scale oligo synthesis will be done on solid-phase supports (3). We therefore consider here only problems relating to the scale-up of solid-phase synthesis, and demonstrate that synthesis in a zonal centrifuge allows precise control of fluid flow in large beds having a short path length.

SCALE-UP OF SOLID-PHASE OLIGONUCLEOTIDE SYNTHESIS

Scale-up problems in oligonucleotide (oligo) synthesis arise partly from the very high cost of doing extended large-scale optimization studies and, more importantly, from factors that appear to be inherent in the physical chemistry of scale-up itself. Present small- and intermediate-scale protocols have been optimized during thousands of runs in many laboratories and involve many chemical tradeoffs, often between reagent concentration and time. The conventional cycle involves use of a solid-phase support with the first nucleoside, plus protective groups, already attached. After washing the support, the terminal trityl group is removed (deblocked) in acid in dichloromethane (DCM) and the deblocking solution replaced with dry acetonitrile (ACN). The first synthon (usually a phosphoramidite) plus an activator (tetrazole) is added to couple the second protected nucleotide. Coupling time is adjusted to achieve maximum coupling efficiency. If coupling time is prolonged, some detritylation may occur, resulting in double coupling, and may also result in the formation of branched chains, especially at a guanine residue. After additional washing in acetonitrile, the phosphorus is oxidized in a partially aqueous iodine solution if a conventional DNA oligo is being synthesized, or sulfurized (for example, with the Beaucage reagent) if a phosphorothioate is being made. After additional washing, a capping solution is introduced to cap unreacted nucleotides. This step is followed by additional washing in preparation for the next repeating cycle.

Deblocking to remove trityl groups is a reversible reaction that must be carried as close to completion as possible. The acid used must be strong enough to remove these groups and wash them away, but not

strong enough to cleave the N-C1' glycosidic bond in purine deoxy-nucleosides to produce depurination. If the trityl groups are not completely removed or if they are allowed to recombine, the next coupling step will not extend those trityl-protected nucleotides, resulting in a "deletion mutation." Also, we have discovered that as the oligonucleotide being synthesized is lengthened, trityl groups tend to adsorb and be retarded chromatographically in the controlled-pore glass (CPG) support. This effect appears to be owing to the fact that the growing oligo chains impart ion-exchange characteristics to the support. Hence, it may be necessary to change washing conditions through a synthesis.

Coupling of phosphoramidites to the growing chain can only be done in the absence of water. However, water is often included in the oxidation step, and must be removed by extensive and complete washing before the next coupling step.

Final yield is a function of coupling efficiency, and efficiencies of 99% or over are sought. As has been noted (4), "none of these [reactions] can be regarded as totally safe," and, "the essential principle remains that a slight change in reaction conditions...can give a very large change in the amount of side product." Problems in scaling up DNA synthesis recur in RNA scale-up, but with the added difficulty that additional protecting groups are employed.

We conclude that there are two alternative strategies for developing large-scale solid-phase oligonucleotide synthesizers (and later peptide and polysaccharide synthesizers). The first is to use large conventional reactors or columns, and to attempt to alter and improve the chemistry through a series of systematic and very expensive studies (which may not be successful). It will be difficult, however, to escape the basic problem of conventional linear scale-up, which is that it becomes increasingly difficult to produce rapid and complete solvent and reagent changes in the times allotted, and as the size of the systems is increased, to treat all elements of the bed identically. Solid-phase synthesis may appear superficially to be analogous to chromatography. However, chromatography rarely deals with time-dependent chemical transformations, or with the problem of minimizing the usage of expensive solvents and reagents through a long series of repeating cycles.

The second approach is to use the reagents and schedules that are successful on a small scale, and attempt to devise a new reactor that duplicates small-scale conditions for all individual solid-phase support particles in a bed, exposing each to exactly the same conditions for the same intervals of time, and to achieve these conditions by using centrifugal force combined with density differences between solutions to control flow through an annular bed. We have adopted the latter approach.

There is an additional advantage to the second approach, which is that it allows new protocols that have been developed on small- or intermediate-scale systems to be directly translated to production synthesizers.
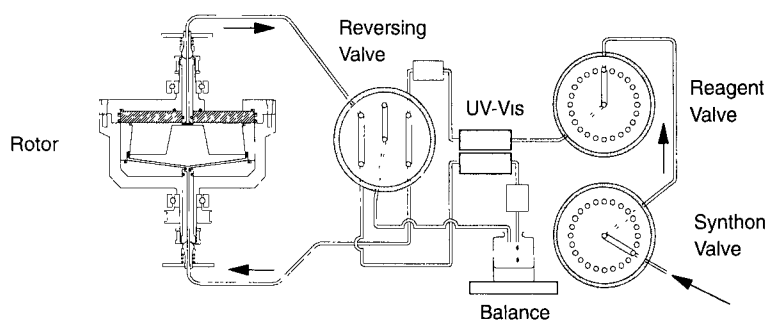
Fig. 1. Schematic diagram of the production centrifugal oligonucleotide synthesizer. Reagent valves shown on the right are connected in series to provide reagents from up to 47 sources (not shown), and direct fluid through a spectrophotometer flow cell to the reversing valve. The reversing valve can vector incoming fluid to either rotor bottom (edge) as shown, to the rotor top (center), or to drain. Fluid exiting the rotor is vectored by the reversing valve through a second spectrophotometer flow cell to the waste vessel on the balance.

Protocols used for one can run on the other, for the simple reason that the bed path length can, as will be shown, remain comparable.

Development of a Monitoring and Reagent Delivery System

A wide variety of configurations have been employed to valve a series of different solutions into a reaction vessel and to monitor some aspects of the synthesis process—all under computer control. The requirement here is for a design that includes:

1. Reagent sequencing valves that can be scaled up both in terms of flowthrough and the number of inlet ports;
2. A reversing, purging, and drain valve that can reverse flow through a centrifugal reactor or can purge all lines to drain;
3. Positive means for producing fluid flow;
4. A spectrophotomoter that continuously monitors the optical absorbance over a wide range of wavelengths of both input and output streams;
5. Means for measuring flow through the system continuously; and
6. A work station that controls the entire system, monitors all variables, displays results rapidly, and provides a complete and detailed record of each synthesis.

In subsequent papers, we will describe the use of this system to evaluate different protocols and different rotor sizes. The system is shown diagrammatically in Fig. 1.

We decided at the onset to make the system, including computer control and programming, literally and figuratively as transparent as possible so that all operations may be either inspected visually, or may be monitored through instruments with the results conveniently displayed in real time. Reagents contact a limited range of resistant materials (Teflon®, glass, polypropylene, Kalrez®, zirconia, quartz, or sapphire), and use of metal (to prevent any possible contamination of the product by heavy metals) has been avoided in initial designs.

Fluid Transport

A versatile system is required for producing a wide range of flow rates against differing resistances. We have examined pumps, gravity flow, and gas pressure. Use of pumps requires that there be a positive method for detecting overpressures and for stopping them if a valve is inadvertently closed, or if a line is clogged. Further, pumps of a wide range of sizes would be required as the system is scaled up. Materials of construction are presently limited to fluorocarbons, including Kalrez®, glass, and some other ceramics, thus restricting the range of available pumps considerably. Gravity flow may ultimately be the best choice, with fluids of different densities arranged at different heights to achieve uniform flow. For 10 psi, the reagent reservoirs need only be an average of 20 ft above the valves—a situation achievable in a production environment. For the present experimental test-bed system, however, gas pressure appears to be the best choice using dry helium because of its low solubility. In the present system, we have used large plastic-coated reagent bottles and closures of our own design.

Valving

An enlarged set of reagents is required for the synthesis of highly modified sequences. These may include, for example, use of new polymer backbones, sulfurization of only a few phosphates on an antisense DNA, use of new and unique monomers, and the addition of end groups, which promote transport into cells or which target specific cell types. In addition, when physical density is used to control flow through a bed, wash solutions of intermediate densities may be required, thereby increasing the number of reagents used. When gas flow cannot be used to purge a system all the way to and through a reaction bed, as is the case with centrifugal systems, it is advantageous to use multiport valves to replace the rows of solenoid valves that are conventionally gas-purged.

The zero-holdup valves we have developed are shown in Figs. 2 and 3. The valves are designed on the theory that all valves leak, even though the leakage may be too small to detect, and that the operation of the valves and any leakage should be visible to the operator. The valves therefore have transparent faces. The reagent valves consist of a station-
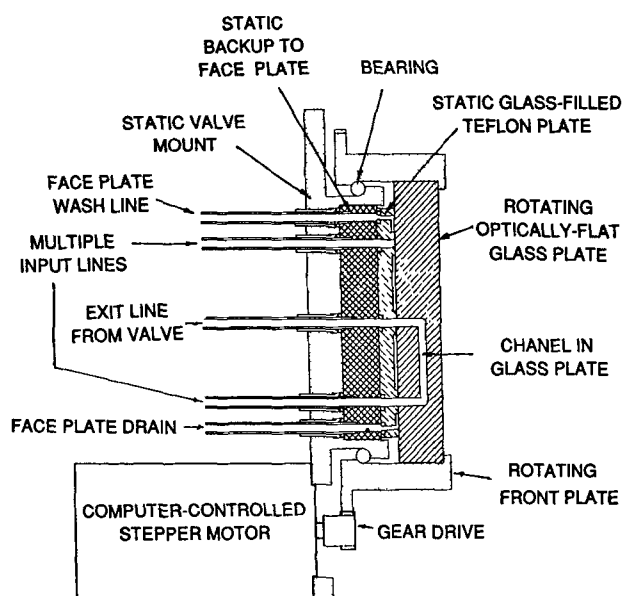
Fig. 2. Diagram of side view of 24-port rotary reagent selection valve. Rotating front plate and attached rotating optically flat glass plate are the moving parts.
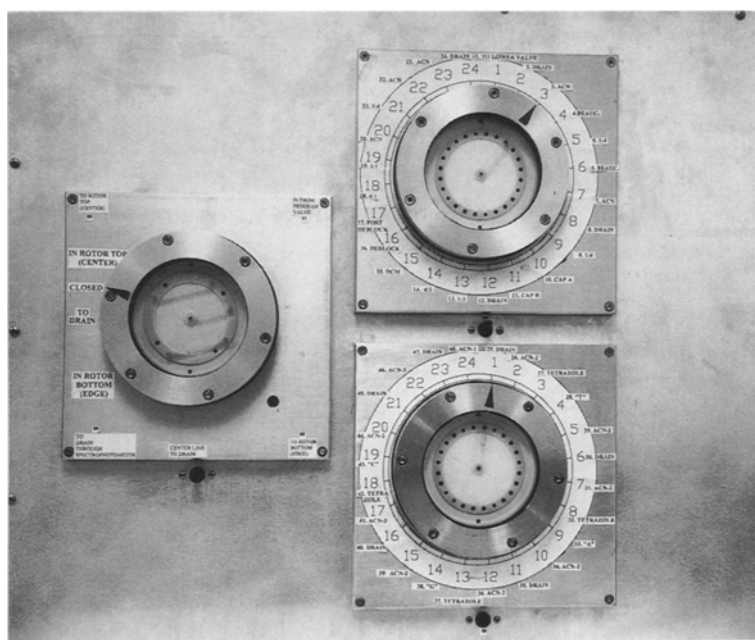

Fig. 3. Photograph of three rotary valves used to control fluid flow. Valve to the left is the reversing valve, and two valves on the right are reagent valves.
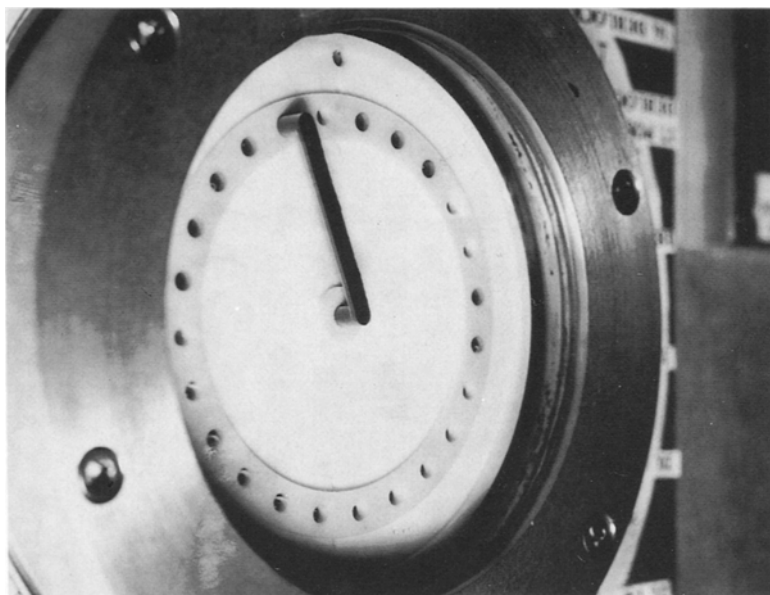
Fig. 4. View of rotating front of reagent valve plate with dye in the line. Channel in glass valve plate is shown connecting one reagent port to center exit line.

ary valve block with 24 input lines arranged in a circle, and one output line at the center. The face of the valve is a thick plate of Pyrex® or quartz, which contains an embedded channel connecting the center line to one of the edge lines, as is shown in Fig. 4. The plate is supported on a precision bearing and is rotated by a computer-programmed stepper motor. The stationary block is fabricated from glass-filled Teflon™. The rotating plate is ground flat to within 1/2 wavelength of light. The stationary valve block is undercut leaving three rings in contact with the face plate. These are the center exit line ring, the ring including the 24 input lines, and an additional ring at the edge so that any leakage is caught and is visible, thus giving two concentric chambers that are additionally connected with small holes so that fluid or air can drain from the inner chamber to the outer one. Two additional lines connecting to the top and bottom of the outer chamber allow acetonitrile or other solvent to be introduced into the bottom of the valve face and out the top, and also to drain back when the input line is connected to drain, thus washing away any solutes that might crystallize at the edges of the contact areas.

The lower valve (valve 2) in Figs. 1 and 3 connects to, and drains into valve 1, giving a maximum of 47 reagent input lines. Additional reagent valves may be added if a larger number of reagents is required.

The input lines are arranged so that all repeating reagents, except activators to be mixed with synthons, are programmed through valve 1,

and the reagents of valve 1 are in the order used. Synthons are introduced through valve 2, and synthons and activator lines are present in pairs interspersed with solvent lines. At the synthon-coupling step, line 1 of valve 1 is connected to the center line draining valve 2. It is evident that as the face plate revolves over each synthon line and each activator line during a full rotation, a very small amount of synthon solution is introduced into the channel in face plate and carried along. The valve is therefore stopped briefly at each solvent line (between the synthon and activator pairs) to wash out the line to drain whenever the valve is moved. Each synthon is mixed with activator by oscillating the valve between the two adjacent positions to give a mixture containing equal volumes of synthon and activator. Tests with colored solvents demonstrated the complete mixing of two components before entering the rotor.

The third valve is a reversing and drain valve that connects the output from valve 1 to either the top or the bottom of the rotor through flow cells in the spectrophotometer, or to drain. A close view of the rotating face plate with dye in the line is shown in Fig. 4.

Absorbance Measurements

Absorption spectra of fluid flowing through the input and the exit lines are recorded from 190 to 800 nm using a Hewlett-Packard Model 8452A photodiode array spectrophotometer. The initial blank is taken using acetonitrile in the case of oligonucleotides, and spectra are recorded from 0 to 3 OD units during an entire run. The double-flow cell with quartz windows are constructed using a Kalrez® gasket to give flow paths of 0.8 and 0.78 mm for the input and exit lines, respectively, as determined using a dye solution of known optical density. The flow cell is moved using a computer-controlled gas-pressure-driven movement at 2-s intervals to give one complete spectrum from each line every 4 s. During deblocking, the input flow cell is not monitored, and the exit flow cell is monitored at 1-s intervals to obtain more data points. Absorbance data can be displayed in real time as discussed below, and all absorbance spectra from an entire run are also stored for subsequent evaluation.

Flow Measurements

In large-scale systems, it is essential to measure flow through the system continually as the final check for possible valve failure and leaks, and to ensure that the desired amount of reagent is delivered at each step. Flow measurement also provides one option for programming, and valve movements may be based on either time or integrated flow for each step. A variety of flow meters are available, and the best of these uses intermittent pulse heating of a stream, with downstream detection of each pulse. However, the most robust means for measuring flow is to weigh the effluent continuously, and multiply the weights by the physical densities of the solutions used. We have incorporated into the present system

an electronic balance capable of measuring weights up to 161 kg with 1-g precision (Sartorius Model F150S-D2).

Physical Density Measurements

The physical densities (g/cc) of the solutions used are measured as prepared using a Mettler DA-110 density/specific gravity meter, which measures to four decimal places. We have also incorporated these into the synthesizer to measure density on-line. However, present versions of these instruments were found to be fragile and unreliable in prolonged use, and further development is needed to produce a routinely useful on-line device. Density measurements can be very useful, however, and provide an additional check on the composition of reagents actually flowing in and out of the rotor.

ALTERNATIVE REACTION VESSEL CONFIGURATIONS

We have described a system that can deliver reagents in a programmed sequence, can monitor the synthesis as it progresses, and can be scaled up using larger versions of the same components, all using the same program and work station. The next question is that of choosing a reaction vessel configuration that can be similarly scaled up.

Five general preparative reaction vessel configurations have been proposed, which are:

1. An enlarged packed column;
2. A fluidized bed with resuspension and mixing using gas;
3. A fluidized bed with suspension produced by mechanical shaking;
4. A zonal centrifuge system in which flow is stabilized by a combination of centrifugal force and density differences between solutions; and
5. A combination system that can combine two or more of the above.

We review these here to illustrate the problems besetting the first three, and then discuss how these are solved in a centrifugal system.

Scaling Up by Increasing Packed Column Volume

Oligo synthesis in small columns works, even though the available solid-phase synthesis supports are heterogeneous in size and give rise to small microdifferences in resistance to flow in the bed. Although these small-scale flow anomalies are inconsequential in small columns, they produce problems in large columns, especially at fast flow rates.
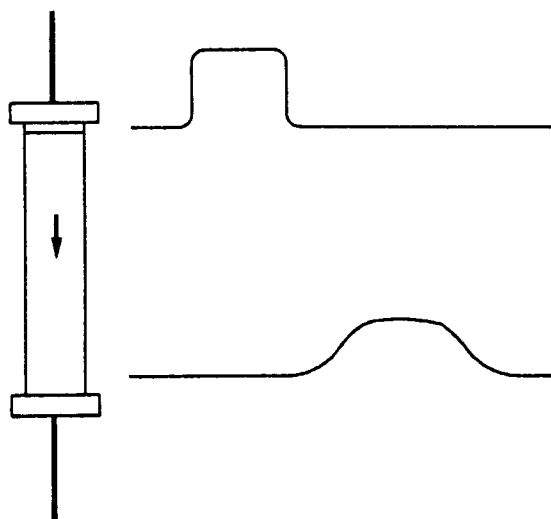
Fig. 5. Diagrammatic presentation of problems associated with increased column length. The interface between successive solvents is degraded, the time required to change solvents is increased, and if the flow rate is increased to compensate for increased bed volume, the effect of local flow anomalies is increased.

In CPG, the included volume is approx 40% of the bed volume (5). Exchange between the included volume and the flowing excluded volume thus produces an additional widening of zones or fronts. The net effect is that particles at the top of a column experience sharp changes in concentration as each new reagent moves into the column and is exposed to the maximum reagent concentration, whereas particles at the bottom experience gradual and asynchronous changes at both the advancing and trailing boundaries. For very fast reactions, for example, when activators that yield coupling times of 30 s or less are used, it will be desirable to run reagents through in narrow concentrated zones so that up to three different solutions may be in the bed at one time. If the zone is a narrow one, then the concentration in the zone center will diminish as it moves down the column, and the longer the column, the greater the effect. Zone or front broadening for any solid support is therefore a direct function of the column length. The effect of scaling up by merely lengthening a column is shown diagrammatically in Fig. 5.

Hence, it appears that the more ideal column is a relatively short one, with capacity obtained by increasing the cross-sectional area of a column, with resultant difficulties in head space and the collecting space below. Directing fluid in such a manner as to flow into the column with a sharp front perpendicular to the direction of flow over a large area is a difficult problem, and becomes more serious as the cross-sectional area of the column is increased and the anomalies shown in Fig. 6 occur. Flow is
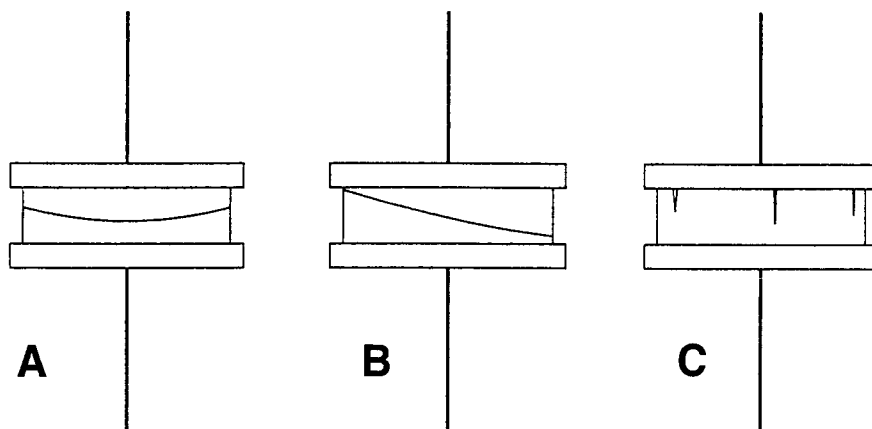
Fig. 6. Problems associated with increasing bed volume by increasing column diameter. **A** shows bowing of a front owing to the longer flow path from the inlet line to the edge and back to the center. **B** illustrates tilting of a front owing to uneven flow, whereas **C** illustrates streaking owing to local flow anomalies.

more even when the bed is composed of homogeneous particles. Unfortunately, no supports presently available are of uniform size and shape. The effects of imperfections in flow through a column may be diminished by repeatedly recirculating reagents through it; however, this prevents the use of highly concentrated reagents (where the desired stoichiometry can be achieved in less than a bed volume), and recirculation does little to improve washing. However, if the actual synthon concentration in a column at one time is not sufficient to drive a coupling reaction to completion, it is useful to recirculate synthon through a column, or to introduce it in a series of steps of increasing concentration so that the final step drives the reaction as far toward completion as possible.

Synthesis Using a Gas-Stirred Fluidized Bed

In a gas-stirred fluidized bed system, the bed is resuspended and stirred using an inert gas, such as argon or helium, in a tapered vessel. The solvent may be removed by draining, with the external volume expelled by gas pressure to produce a semidry bed. Since the included volume is retained, that volume dilutes the next solution added. As a result, washing or any other change of solutions, no matter how many times repeated, approaches a quantitative replacement asymptotically, and many changes are required.

An advantage of this system is that gas pressure may be used to propel solutions, to wash reagents out of lines during solution changes, and to resuspend the bed. CPG is very sensitive to shear, and gas resuspensions appears to be sufficiently gentle not to fragment the glass.

Synthesis Using a Mechanically Stirred Fluidized Bed

The synthesis chamber may also be mechanically shaken in a circular manner to fluidize the bed. The chamber configuration is usually different from that used for gas resuspension, and the shaking movement is controlled to prevent fracturing the support. In practice, mechanical and gas resuspension may be combined. The same disadvantages of included-volume carryover mentioned for gas-stirred vessels apply here also.

ZONAL SYNTHESIS ROTORS

Although column and fluidized bed configurations have been successfully used for intermediate-scale synthesis, for example, in the range of 10–100 mL of bed volume, and could conceivably be scaled up further, but with reduced efficiency, serious problems arise if kilogram and multi-kilogram batches of support are to be considered.

A solution to the fundamental problem is to carry out synthesis in specially designed zonal centrifuge rotors (6). Two problems are solved simultaneously. The first is to provide a configuration with a large bed volume and with a short path length, and the second is to use a combination of centrifugal force and density differences between solutions to provide very positive control of fluid flow through the bed.

The basic principles of zonal centrifuge rotor design and operation are well known (6), and hundreds of them have been built. In such hollow bowl rotors, flow is arranged to be along radial lines, with inflow from either the rotor center or the edge. Flow in the rotor is stabilized by either step or continuous density gradients to produce circumferential isodense zones. These are quite insensitive to local differences in resistance to flow in a bed. Even if large void spaces exist in a bed in such a rotor, flow will be the same through the bed and the void. Flow is here stabilized in a manner unknown in chromatography at $1g$. The basic concept is shown diagrammatically in Fig. 7, which shows a small column of the type used in conventional synthesizers, a sector-shaped vessel of the same capacity, and the enlargement of the sector shape to annular beds of support having the same flow path length, but with very much greater volumes. Flow through the annular bed in the zonal rotor is actually better controlled than in a small synthesis column because very positive and continuing forces are applied to the contained liquids. Hence, the same schedule of solutions and the same time intervals can be made to apply to both. This is an enormous advantage in scaling up synthesis from a bench to a production level.

It is extraordinarily fortunate that the physical densities of the major solvents used are very different, being 1.325 g/mL for dichloromethane
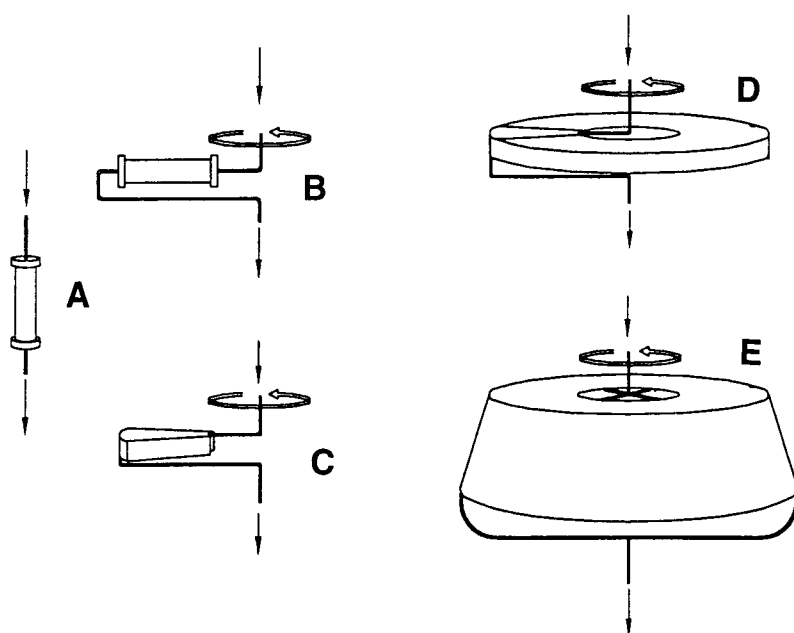
Fig. 7. Schematic illustration of derivation of a centrifugal synthesizer from a short column. (A) Short column used in conventional bench-scale synthesizers. (B) Column of similar dimensions operated in a centrifugal field. (C) Same bed volume as in B, but modified to have a sector shape. (D) Sector-shaped bed enlarged to form an annulus of much larger volume, and with identical flow path length along the radius. (E) Elongation of bed shown in D with a tapered edge to collect fluid and vector it back to the lower axial line.

and 0.786 g/mL for acetonitrile at 20°C. The maximum density difference to be exploited is therefore 0.539 g/mL. In a centrifugal field of only 100g, this translates into an effective density difference of 53.9 g/mL, or three times the difference in density between uranium metal and water. This provides both an extraordinary stabilizing force and a continuing force to move any stray liquid element rapidly to its proper density zone, even in the absence of bulk flow. This is quite different from the situation in a column at 1g, where a wayward volume of fluid is only swept along by mass flow, and where density differences produce movement only very slowly. Actually, the effects of density differences on flow through conventional packed chromatographic beds are rarely even considered in designing separations.

Note that the entire idea of using a centrifugal field is to stabilize zones. Bulk flow through the rotor is not the result of centrifugal force, but of an applied external pressure. The familiar principles of the basket centrifuge, therefore, do not apply here. This is because the rotor is a closed system, and the two fluid lines employed to move fluid through the rotor are both on the axis of rotation.
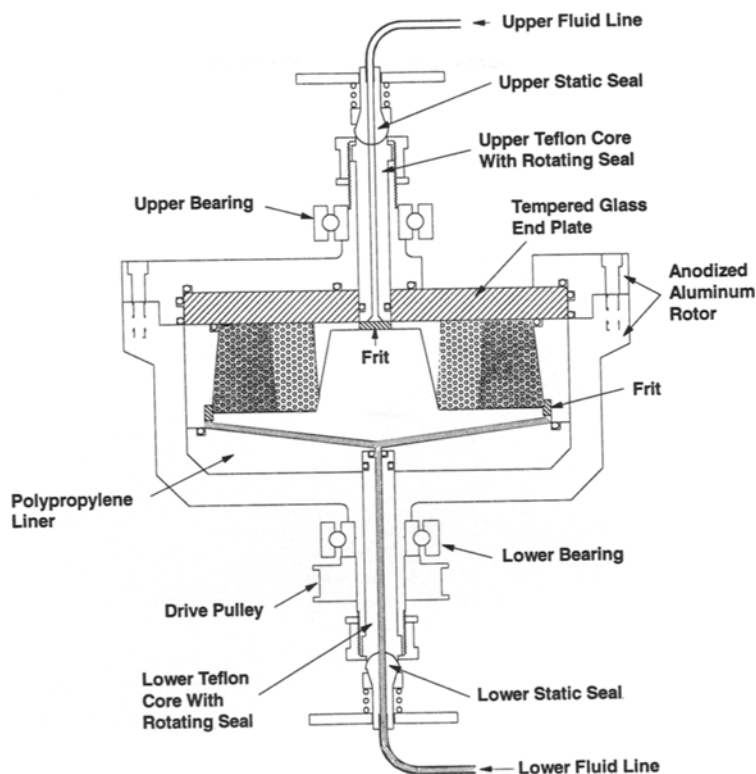
Fig. 8. Diagram of construction and operation of a production centrifugal oligonucleotide synthesize (PCOS) rotor. The anodized aluminum rotor housing contains a polypropylene insert that defines the reaction chamber, which is visible from above through a tempered glass end plate. Upper and lower rotor shafts contain Teflon® cores. The reaction chamber is shown packed with solid-phase support particles. Also shown diagrammatically is the displacement of a light fluid already in the rotor by a denser one flowing in from the edge to form a concentric ring with the inner edge visible in the solid phase support bed. Fluid flowing in through the lower fluid line and seal moves to the edge frit, and then into the edge of the reaction chamber. The upper fluid line connects through a rotating seal and a porous frit with the center of the reaction chamber. The rotor is belt-driven through a drive pulley on the lower shaft.

The design used to spin the bed annulus shown in Fig. 7 is illustrated diagrammatically in Fig. 8, and the components of a synthesis rotor are shown in Fig. 9. In practice, it is desirable to eliminate any contact between reagents and metals. Hence, as shown, the internal spaces are all lined with either glass, Teflon®, glass-filled Teflon®, or polypropylene. In the initial design, the rotating fluid-line seal is of glass-filled Teflon®, whereas the static seal is a polished glass hemisphere. The two fluid lines required are at opposite ends of the rotor to prevent crosscontamination. Porous
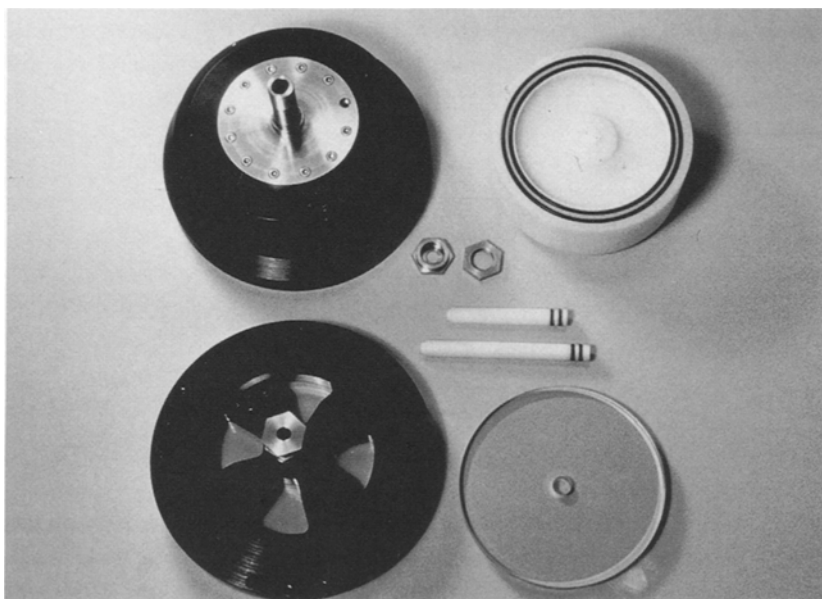
Fig. 9. Components of a zonal synthesis rotor. Upper left: Lower rotor housing: lower left, upper end cap; center, Teflon® cores lining upper and lower rotor shafts; upper right, polypropylene rotor insert; and lower right, tempered glass end window for upper end cap.

sintered polypropylene or Teflon® frits are used to keep the solid-phase support in the rotor. So that packing of the solid-phase support and movement of trityl-containing zones through the rotor can be observed, a tempered glass end window is included in the rotor.

In such a rotor, the fluid path through the bed can be short, with bed volume increased by increasing the average radius of the circumferential bed, by increasing rotor length, or both. Note that we have previously developed continuous-sample-flow-with-banding rotors with an annular volume of 3.6 L (8), and a total volume of approx 8 L, which are now operating at speeds up to 40,000 rpm. Much larger rotors are feasible.

Unlike conventional zonal rotors, no vanes are required in synthesis rotors to prevent circumferential fluid movement owing to Coriolis forces. The packed bed controls such flow. Care in designing the internal surfaces is required, however, to vector the circumferential iso-density zones into and out of the rotor. The methods by which this is done have been previously described in detail (6,7). We originally designed the rotor to hold a 400-mL bed volume, naively thinking that this would be considered a small volume, with subsequent rotors having multiliter volumes. As we soon discovered, 400 mL are beyond the needs (or perhaps financial daring) of most antisense companies at this time. Three rotor cores

with reduced volume were therefore designed that had capacities of approx 18, 68, and 340 mL, the latter for flow studies. In these rotors, the remainder of the volume is occupied by polypropylene.

Note that some organic solid-phase supports swell and shrink during a synthesis cycle, giving rise to cracks in packed beds. These cause severe problems in noncentrifugal synthesis. However, with density-gradient stabilized flow in a centrifugal field, such bed anomalies do not pose a problem, since flow is almost completely immune to local flow restrictions. In addition, the centrifugal field itself contributes to even packing, and to the elimination of cracks in packed beds. Note that in zonal synthesis rotors, the beds are packed through the upper metal shaft during rotation, with a vacuum on the lower edge line. After packing, the upper Teflon® shaft is inserted, and the upper seal assembled.

Organic solid-phase synthesis supports generally float in pure dichloromethane. If excessive packing is a problem, floating the bed against the center frit during each coupling cycle would be advantageous. Regardless, bed floating does not affect density-stabilized fluid flow.

Hybrid Synthesis Systems

Before discussing results obtained in the rotor described, it should be noted that hybrid systems are feasible that combine essential properties of all of the previous four systems. A zonal synthesis rotor may be spun with its axis horizontal, and be as effective as when spun in an axis-vertical position, or it may be arranged so that the axis of rotation may be changed. If the rotor is rotated slowly with its axis horizontal, the bed may be resuspended and kept resuspended. Resuspensions may be by slow rotation, oscillation, or by shaking. If the rotational velocity is then increased, the particulate support is sedimented into a packed bed, and the bed volume can be exchanged by density-controlled zonal flow. The rotor may also be designed so that resuspension occurs with the axis vertical, using flowing gas or by mechanical shaking. These options may prove useful in much larger systems.

WORK STATION CONTROL OF THE SYSTEM

A work station is used to program the entire system, and to acquire, process, and display data. Programming of runs and display of processes in progress are done through the screen shown in Fig. 10, which duplicates valve positions, indicates the parameters for each step, and displays results in progress. The upper right-hand corner buttons control choice among programming mode, simulation of a program, and actual synthesis. In the programming mode, the values for rotor speed are set using
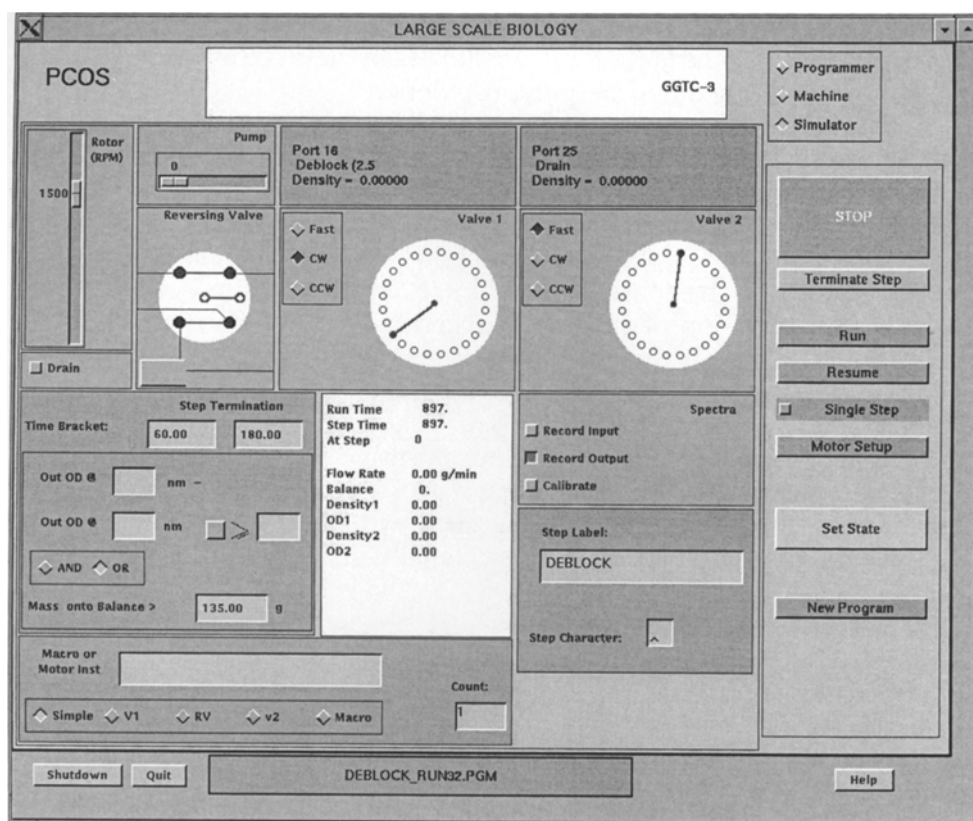
Fig. 10. CRT screen display for PCOS program. Sequence synthesized is shown at top. Program proceeds through a series of discrete "states," which are both set, using keyboard and cursors, and displayed on the screen shown. A sequence of these states constitutes a program. Screen displays include, along right side, controls to stop, terminate run, resume, run single step, motor setup (valve setting calibration), set state, and new program. Upper center section includes, from the left, slider for setting rotor speed, reversing valve setting, reagent 1 valve position and description of connected solution, reagent valve 2 setting and description of connected solution together with indications of direction of rotation and speed of movement of each valve. Lower left block includes step termination parameters, including time bracket (max and min.), control OD settings, and desired mass increment on balance. Center display includes run time, step time, flow rate, balance reading, and (when used) density meter readings. Right center shows spectrophotometer controls, step label, and base being added. Macros are controlled by bottom left panel. Shutdown, quit, program name, and help are along the bottom.

a slider, the positions of each valve set with a cursor (and the composition of the reagent chosen automatically displayed), absorbance measurements are enabled, and the parameters to control the length of each step chosen. These include choice among time, specific absorbance measurements, density measurements, or the weight of effluent from the system.
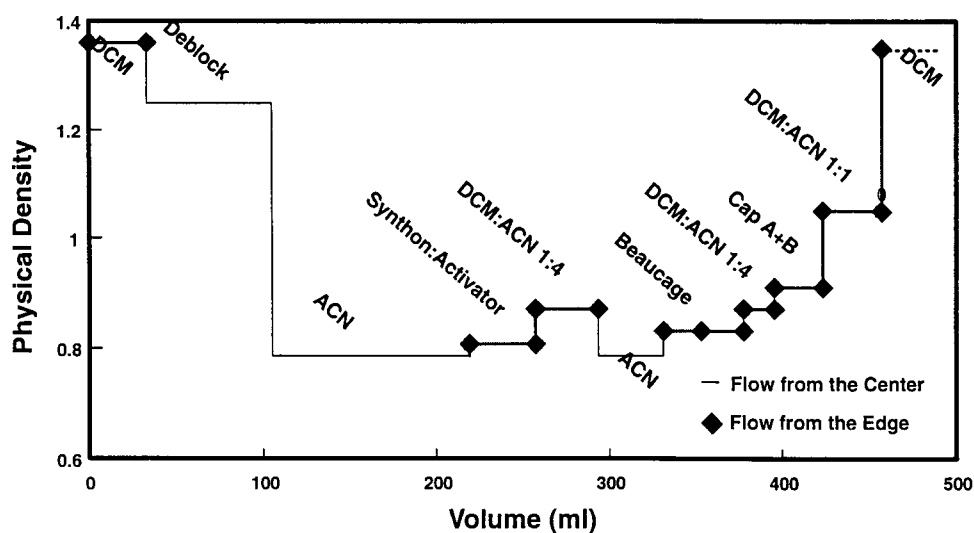
Fig. 11. Reagent density schedule for a single synthesis cycle. Physical density is indicated along the *Y* axis, and reagent volume along the *X* axis. Solutions in decreasing physical density order flow in from the center, whereas those in increasing density order flow in from the edge. Data for rotor core having a bed volume of approx 78 mL.

Once the parameters for a given step are chosen, they are stored, and the operator then continues on to the next step. Once a complete program is written, it may be reviewed either a step at a time or at a preset rate to simulate a run. In the run mode, the system is completely automatic. A typical synthesis cycle plotted in terms of time and density of the reagents used is shown in Fig. 11. When the sequence of reagents is in order of decreasing density, flow is in through the rotor center. When in order of increasing density, it is in from the edge. The complete prototype system is shown in Fig. 12.

Synthesis of Poly T

Using a 68-mL insert, a T12 phosphodiester polymer was synthesized with an average stepwise coupling efficiency of 97.2%, whereas a T11 phosphorothioate was synthesized with an average stepwise coupling efficiency of 99% in collaboration with Henri Sasmore of Isis Pharmaceuticals (the details will be published elsewhere). Over 30 additional syntheses of proprietary compounds have also been made. Our general conclusion is that there is a crossover point below which column, suspended bed techniques, or centrifugal methods are equally as good, whereas above that point, which is probably in the range of a bed volume of 100 mL, centrifugal systems are superior. Although centrifugal systems of higher speed might prove to be even more efficient than small columns, cost considerations probably do not justify the added mechanical complexity for small-scale synthesis.
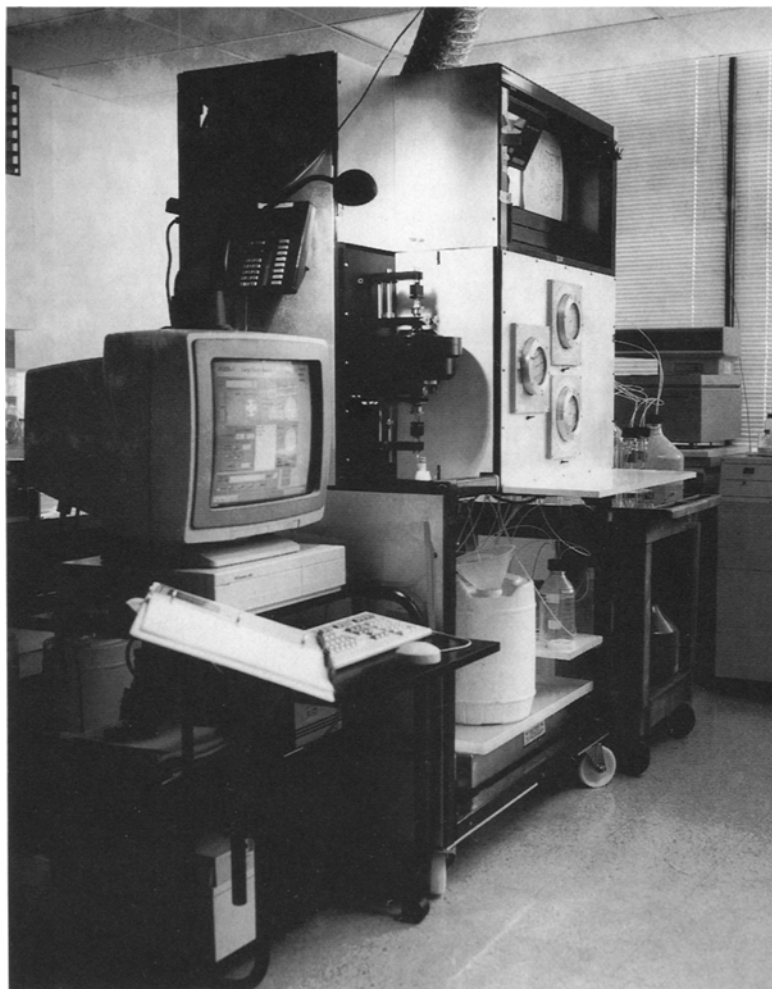
Fig. 12. Photograph of complete prototype production centrifugal oligo-nucleotide synthesizer system. Work station is shown on left, and rotor is in center attached to frame supporting electronic components, valves, and the large balance underneath. Feed reagent bottles, only partially visible, are to the right.

OPTIMIZATION STUDIES

The high cost of doing complete large-scale syntheses precludes extensive ''cut and try'' experiments. Rather, methods must be found for dissecting syntheses and optimizing each step. This requires several types of data we are only now beginning to be able to obtain, some of which are described here.

The efficiency of replacement of one solution by another in a porous support in a centrifugal field depends on the efficiency of centrifugal stabilization, on the diffusion coefficients of the solvents and solutes involved, on the porous nature of the support, on flow rate, on the density difference between succeeding solutions, and on a number of other factors, including any adsorption of solutes to the support or to the growing oligo chains. All of these factors tend to widen the boundaries between reagents and to increase the volume required to change completely one reagent for another.

The densities of reagents may, in many cases, be adjusted by changes in solvents. For example, a small amount of methylene chloride may be added to an acetonitrile solution. In the reagent schedule shown in Fig. 11, the minimum density difference is between acetonitrile and acetonitrile plus synthon, and is approx 0.06 g/mL. It is of interest to know whether this small density difference can stabilize flow, and what volumes are included in the widening fronts. Were there no such broadening, one could run reagents through as narrow zones, greatly decreasing reagent consumption.

Initially we would like to separate the study of solvent flow from that of solutes, since the latter may be retarded because of molecular mass differences or adsorption. Fortunately, DCM and ACN have different absorption spectra in the far UV, and we are able to analyze mixtures by following absorbance at 212 nm. In the experiment shown in Fig. 13, we have filled a 340-mL rotor with spent 500-Å pore size CPG, which has been extensively washed with DCM and then filled with ACN. With the rotor spinning at 2000 rpm, a solution of 5% DCM in ACN (w/w) was run into the rotor through the edge line. The density difference between these solutions is approx 0.016 g/mL, i.e., very much less than the density difference between any two solutions actually used in synthesis. The sharp step function shown (A) is the absorbance as measured in the input line, whereas (B) shows the absorbance in the output line. Note the sharp rise in A and the slow rise in B, the delay being a measure of the liquid volume in the rotor. When the rotor was full of the DCM/ACN mixture, flow was reversed, and the rotor contents pushed out from the center with pure ACN. During this experiment, the output weight was continuously measured. The total absorption difference between the two solvents was measured, and the solvent mass required to produce 98% of that difference was calculated. When flow was from edge to center, 78 g of solvent (approx 100 mL) was required, whereas for flow in the opposite direction, 260 g or approx 320 mL were required. This is a worst-case example, since far larger density differences than those used in this experiment occur between all steps during an actual synthesis. From this, we conclude that less than a third of the rotor volume is required to change
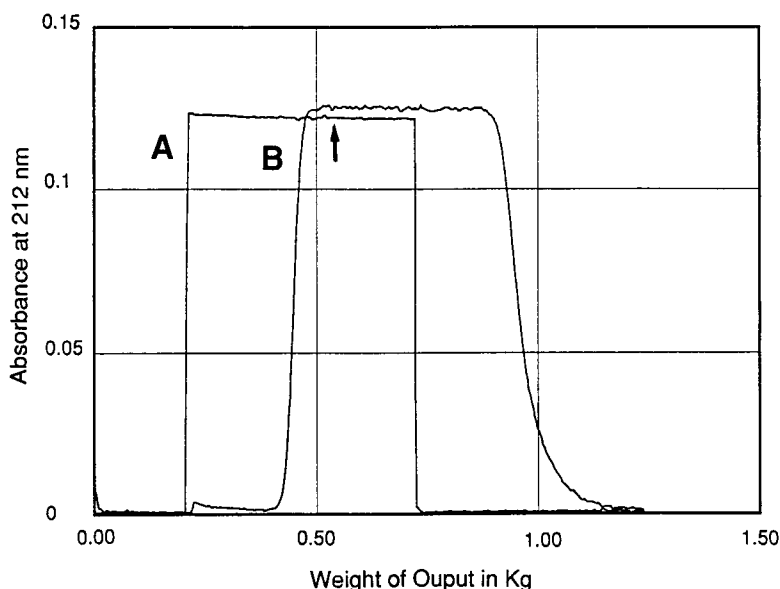
Fig. 13. Stabilization of flow by a minimal density difference in a 340-mL rotor core. Initially, rotor was filled with ACN, which is displaced by 5% DCM in ACN flowing in from the edge. After complete solution replacement, the 5% DCM in ACN is displaced by ACN flowing in from the center. Absorbance at 212 nm along Y axis, mass of liquid delivered along X axis. (A) Absorbance in inflowing line. (B) Absorbance in outflowing line. Arrow indicates points of flow reversal.

solvents during centripetal flow, whereas almost a rotor volume is required for flow in the other direction. For most of our syntheses to date, we have used three-rotor volume for solvent changes—a volume we now know is far in excess of that required. Current synthesis protocols introduce all reactants from the rotor edge.

The differences in replacement volumes with flow direction appear to be the result of inefficient vectoring at the rotor wall, since all of the fluid in a zone or boundary flowing to the edge must flow circumferentially around the rotor edge to reach the exit lines. Although the wall of this rotor is tapered, additional improvements may be made, for example in the form of a cloverleaf configuration. Note that vectoring is improved by higher centrifugal fields.

Since absorbance measurements are made over a wide range (190–800 nm) and stored, it is feasible to follow highly absorbing materials, such as synthons, using off-peak measurements at carefully selected wavelengths. This means that it will be possible to measure synthon utilization by determining how much synthon is lost from an advancing front as compared with a control study using underivatized CPG. The results can be compared with the integrated absorption of the trityl groups released during the deblocking step.

DISCUSSION

A major objective in drug discovery and development is to find agents that react with specific molecular targets. The targets are almost always biopolymers synthesized in vivo from a limited set of monomers, including amino acids, nucleotides, and to a lesser extent, lipids and sugars. The unique properties of these natural biopolymers derive from the order in which these monomers are assembled.

Given the extraordinary interactive specificity achieved in nature by assembling a limited set of monomers, it is natural that pharmacologists should try to mimic nature and explore ordered polymers as drugs in a systematic manner, and to produce new modifications of them that have not previously existed in nature. These include new and modified peptides, antisense oligonucleotides (1) with modified backbones, ribozymes, and a variety of interactive polymers that have been called aptamers, diversomers, and nexamers. In the past, most synthetic drugs have been synthesized as single entities by solution-phase chemistry and have, with the exception of a few peptide hormones, not been assembled in order from a set of monomers.

Although short oligonucleotides and peptides have been synthesized by solution-phase chemistry, and although these may ultimately be combined by fragment assembly for production purposes, it appears that for the near future, commercial large-scale production will be done by solid-phase synthesis. If one or more of the antisense or other sequence-specific drugs now under development come into general use as pharmaceuticals, there will be a requirement for a new class of larger-scale production synthesizers that are programmable, that can control and schedule the flow of a relatively large number of different reagents, that are fully instrumented to facilitate optimization of synthesis protocols, that are based on well-understood principles, but that do not now exist.

The system described here is the first step in the development of a new species of large-scale systems in which all components can be readily scaled up. The requirements of incremental scale-up account for our attention to a new class of valve that can be made at almost any scale, to the use of standard analytical instrumentation and use of balances to measure flow, to the use of centrifugal force to stabilize fluid movement, and to the use of the hollow-bowl zonal centrifuge configuration to provide a large bed volume with a short fluid path length. By using similar or identical materials, components, and/or basic designs at each stage in scale-up and similar chemistry, validation for the FDA is greatly simplified.

Given the high cost of the products to be made and the fact that the value of the product in a single synthesis may exceed the cost of the synthesizer, we have felt it important to instrument the system as completely as possible, to make it easily programmable and highly reliable, and to record as much pertinent data during each run as may be useful. We have

also felt it important to build very flexible instruments that can be adapted to the synthesis of a wide variety of different ordered-sequence drugs based on different chemistries.

Centrifugal synthesis provides a new and unique approach to the large-scale production of biopolymers. The upper limits of this technology remain to be explored both theoretically and experimentally.

ACKNOWLEDGMENT

REFERENCES

1. Riordan, M. L. and Martin, J. C. (1991), *Nature* **350**, 442,443.
2. Bonora, G. M., Biancotti, G., Maffini, M., and Scremin, C. L. (1993), *Nucleic Acid Res.* **21**, 1213–1217.
3. Carruthers, M. H. (1989), in *Oligonucleotides. Antisense Inhibitors of Gene Expression.* Cohen, J. S., ed. CRC, Boca Raton, FL, pp. 7–24.
4. Gait, M. J. (1984), *Oligonucleotide Synthesis.* IRL Press, Oxford.
5. Haller, W. (1983), in *Solid Phase Biochemistry*, Scouten, W. H., ed. John Wiley, New York, pp. 535–597.
6. Anderson, N. G., ed. (1966), *The Development of Zonal Centrifuges.* National Cancer Institute. Monograph No. 21.
7. Anderson, N. G., Waters, D. A., Nunley, C. E., Gibson, R. F., Schilling, R. M., Denny, E. C., Cline, G. B., Babelay, E. F., and Perardi, T. E. (1969), *Anal. Biochem.* **32**, 460–494.