Automated Sequential Degradation of Ribonucleic Acids


An instrument has been constructed that can automatically and selectively remove the terminal nucleotide residue from a ribonucleic acid. The instrument is designed to repeat the degradation cycle. This process involves two steps: (1) The base and sugar fragments are removed by the combined action of periodate and amine. (2) The phosphate residue, attached to the originally penultimate residue, is removed by enzymatic hydrolysis. Various techniques have been studied to establish the optimal methods for separating the residual polynucleotide chain from the various reagents and reaction products. In the most efficient and reproducible system, a membrane is used to separate the residual polynucleotide from the reaction products. The enzyme is bound in an active state to a plastic bead, so that simple filtration separates it from the dephosphorylated polynucleotide. All of the chemical and separation steps are quantitative (greater than 95% yield). The analyses are performed automatically by sampling the diffusate, and they are complete before the enzyme hydrolysis begins. The overall efficiency (number of cycles successfully completed) depends upon the stability of the instrument. We have performed up to six cycles with an average yield of 98%.

Additional Keyphrases oligonucleotide analysis • bead-adsorbed enzyme • dialysis • ultraviolet analyzer • alkaline phosphatase separation based on size

One of the products of interdisciplinary research is the development of tools that allow sophisticated measurements to become part of the routine analytical work of a laboratory. As knowledge of the structure and function of nucleic acids increases, there will be a need for nucleic acid sequencing in genetic, environmental, and even clinical laboratories.

There are two general approaches to sequencing ribonucleic acids. The technique most widely used is the specific fragmentation of a pure nucleic acid by a variety of chemical or enzymatic means, the separation and sequencing of the individual fragments, and finally an intellectual reconstitution of these fragments into what must have been the original sequence; this last step requires some sophisticated interpretation of results by the research scientist. The second approach (1, 2) involves a sequential removal of the terminal nucleoside unit by a series of chemical and enzymatic steps and the recycling of these steps, followed by analysis of the released base (see Figure 1).

The latter approach is more amenable to automation, and we describe here the principles of an instrument designed to degrade RNA as well as oligonucleotides. In addition, we present data showing that the instrument can sequentially degrade ribonucleic acids. Two other laboratories have used this approach for routine sequencing (3, 4) and Wieth and Gilham have recently described an apparatus with which they can sequence oligonucleotides.1

Materials and Methods

The details of the construction of the apparatus will be presented elsewhere. A modification of the oxidation technique described by Yu and Zamecnik (6) is used in our apparatus. The tRNA is prepared by phenol extraction of E. coli B according

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to Zubay (6), except DEA adsorption and elution are used as the last step, in place of isopropanol precipitation. The rRNA is dialyzed before use in the sequencer. No special treatments are used for any of the other reagents, except that the amine is decolorized with acid-washed charcoal.

The alkaline phosphatase (orthophosphoric monooester phosphohydrolase, EC 3.1.3.1) is prepared according to Zingaro and Uziel (7) and modified by adsorption to "Plaskon" beads (Allied Chemical Co.) coated with 0.1% "Adogen 464" (General Mills Corp.) previously dissolved in ethylene chloride.

The instrument control console contains a program capable of regulating the seven functions described in Table 1. The fixed program itself is illustrated in Table 2. The organization of the four reactors is diagramed in Figure 2, and the automatic analyzer is connected to the sequencer through a sampling device connected to the diffusate of the dialyzer. Two fluid-dispensing systems are used. The fluid is metered directly from the bottle through a single valve into the enzyme vessel, or the solutions are metered through a common dispensing valve into the oxidation vessel. A diagram of the latter system (Figure 3) illustrates the three timed events required to dispense volumes as small as 0.05 ml or as large as 15 ml or more, with a 2% precision. (See the legend for a description of the operation.)

### Table 1. Instrument Capabilities

1. Control reaction time, temperature, and solution contents in three separate vessels.
2. Dispense fluids (0.05 to more than 15 ml).
3. Transfer fluids between the four reactors.
4. Filter the contents of each reaction vessel.
5. Separate products by dialysis.
6. Analyze released base automatically and collect the remaining sample.
7. Concentrate retentate by ultrafiltration.

### Table 2. Program for Sequencer (Time, ~128 min)

<table>
<thead>
<tr>
<th>Steps</th>
<th>Time</th>
</tr>
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<tbody>
<tr>
<td>1, 2</td>
<td>Reset stops for single or multiple cycle operation.</td>
</tr>
<tr>
<td>3-5a</td>
<td>Add $\text{IO}_3^-$, RNH$_2$. Incubate.</td>
</tr>
<tr>
<td>6a</td>
<td>Stop reaction with glycol.</td>
</tr>
<tr>
<td>7-10a</td>
<td>Transfer to dialyzer and wash vessel (2x). Collect dialysate.</td>
</tr>
<tr>
<td>11, 12</td>
<td>Advance dialysate to sample injector and begin analysis. Collect dialysate.</td>
</tr>
<tr>
<td>13, 14</td>
<td>Dialyze and collect dialysate.</td>
</tr>
<tr>
<td>15</td>
<td>Concentrate and transfer RNA to enzyme vessel. Incubate.</td>
</tr>
<tr>
<td>16-18</td>
<td>Concentrate, wash, and transfer to oxidation vessel.</td>
</tr>
<tr>
<td>19</td>
<td>Return to 1 or 2.</td>
</tr>
</tbody>
</table>

*a The sample volume was 0.7 ml containing 40 to 400 units of E. coli tRNA. 0.15 ml of 0.1 M NaIO$_3$ was added and this was followed by 0.15 ml of the amine solution (1 M cyclohexylammonium acetate, 10% v/v N,N,N',N'-tetramethyl ethylenediamine). The reaction was stopped with 0.4 ml of 0.5 M ethylene glycol.

The dialyzer was a commercially available unit (D-1 size, Biomed Instruments).

A peristaltic pump is used to transfer the reaction solutions between the various reactors. Figure 4 shows a diagram of the four reactors, the pumping system, and the analytical system. (See the legend for a description of the flow path and operation.)

The bases of the oxidation vessel and enzyme vessel are fitted with filters. The oxidation vessel
Fig. 3. The dispensing of small volumes into the oxidation vessel (three steps)

A bottle valve (A) and the waste valve (C) are opened to allow a flushing of the line from the bottle, through the dispense valve (B) and out the waste valve (C). Valve B is then closed, permitting fluid to flow from the bottle to the reaction chamber. After the exact volume has passed through valve B the valve is returned to the original state and the fluid is transferred to the reaction vessel by pressure from the regulated N₂ supply (D). This last valve timer can thus be used to time several 2-min reaction intervals, since the programmer will advance when this timer resets but not when the bottle valve (A), or dispense valve (B), or waste valve (C) resets

has 400-mesh nylon net and the enzyme vessel has a 1-mm thick pad of sintered Teflon with a nominal pore size of 10 μm. (The pad was cut from a 1/4- inch sheet of sintered Teflon bought from Chemplast Inc.) The driving force for filtration is provided by the peristaltic pump.

The diagram in Figure 5 shows the separation by dialysis of the modified RNA from the released base and other reaction products, and the analysis by coupling the diffusate to an automatic ultraviolet analyzer system. The diagram shows two modes of ultraviolet monitoring: by automatic ion-exchange analysis and by continuously monitoring the diffusate. In the first mode a 26-μl sample is removed from the diffusate for analysis, as shown in the insert. The chromatographic solvent is continuously pumped through the column to provide reproducible analytical conditions. The base analysis system is a modification of the procedure of Uziel et al. (8). Figure 6 illustrates the effect of pH on the movement of the bases. Increasing the pH to 5.2 decreases the retention volume and decreases the peak dispersion. In this way the resolution is maintained, and the analysis can be completed in 25 min at moderate column pressure 1378 × 10⁶ N/m² (200 lb/in²). This will permit eventual feedback control on the oxidation process to ensure complete oxidation at each cycle.

Alternatively, the analyzer can be operated so as to by-pass the sampling device and column and monitor the total diffusate. Any diffusate not used for analysis may be collected in a fraction collector or discarded. The dialysand (nondialyzable portion) is concentrated in an ultrafiltration device and passed on to the enzyme reaction vessel.

The ultrafiltration is repeated on transferring the dephosphorylated RNA to the oxidation vessel. This transfer step is expedited by modification of the insoluble alkaline phosphatase as described by Zingaro and Uziel (7). The anionic polymer-enzyme compound is adsorbed to Plaskon beads coated with 0.1 g of Adogen 464 per 100 g of beads. The amounts of cationic Plaskon needed to coagulate the colloidal bound enzyme must be determined empirically because of the variability in the initial binding of protein to the anionic polymer. One needs about 50 μl of cationic Plaskon for each milligram of bound enzyme. The program we have used generates a cycle time that has been as little as 128 min.

Fig. 4. Location and order of operation of the various valves when all the peristaltic pump lines are operated from a single pumping motor

After oxidation valves A (2-way), B (3-way), C (3-way), and D (3-way) open to permit fluid flow into the dialyzer (II), the second wash (Table 2) is left in the oxidation vessel (I) during dialysis, and valves C and D isolate the block from the pulsations of the peristaltic pump. Valve B is returned to normal position so no negative pressure builds up on valve A. After dialysis, A, B, C, D, E, and G are activated to allow the dialyzed reaction solution to pass from the dialyzer through the concentrator (III) and into the enzyme vessel (IV). The vacuum is obtained through valve G. We have used the peristaltic pump to generate the vacuum. The enzyme vessel is heated to 50°C during this time, and the time of hydrolysis is set by the specific activity and quantity of the added enzyme. After hydrolysis, all valves return to their normal position except valves E, F, G, H, and J. The flow is thus directed from vessel IV through the concentrator (III) and into vessel I. Excess wash fluid can be discarded through J in its normal operating position. Circulating water (temperature thermostatically controlled) is used to heat the reaction vessels.
Results

The coordination of all parts of the system has been accomplished. Three consecutive runs have been made with insoluble enzyme present in vessel IV and the third run maintained an average yield of 98% through six cycles of the nine cycles (Table 3). The yield dropped after cycle six. Separate control samples of tRNA have been cycled through 15 cycles of separation and ultrafiltration without enzyme in vessel IV and we recovered tRNA within the range of error of measurement (±3%).

<table>
<thead>
<tr>
<th>Table 3. Automated Sequential Degradation of tRNA</th>
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<tr>
<td>Run</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

Discussion

There are several routes to automation of the chemistry of sequential degradation originally described by Whitfeld (9). The need for the oxidative elimination step and enzyme step is fixed. However, reaction products can be separated from the residual tRNA by any of several procedures, the most commonly used one being essentially a salting out or precipitation procedure. We have successfully used such a process in the manual degradation of a pure tRNA (1, 2). This was in fact the stimulus for this attempt to automate the process. We designed and constructed an apparatus to perform the various manual operations. However, the precipitating and dissolution reagents used—cetyltrimethyl ammonium ion and 1 M salt, respectively—produced several serious problems. The repeated filtration of the precipitate was difficult because a gel-like precipitate occasionally formed, rather than a reasonably flocculent one, largely depending on the rate at which the tRNA solution and the flocculating agent were mixed. An extra step is required for analysis of the concentrated salt solutions used to dissolve the tRNA. This latter problem was not as serious as the former. We turned to using insoluble anion exchangers (the cetyltrimethylammonium compound acted as a soluble anion exchanger). Commercial preparations of Dowex 1X2 and several diethylaminoethyl cellulose derivatives were studied as adsorbing agents. Three considerations prompted us to stop using these agents in the sequencer: the volume needed to elute the tRNA depends on composition as well as chain length (unpublished results of M. Uziel), the molar capacity of the exchanger is low, and a desalting step is needed before analysis.

Separation based on size avoids all of the above complications and simplifies the overall operation by decreasing the number of steps and allowing us to continuously monitor the rate and extent of dialysis. Because effective size controls the separation process, oligonucleotides of any molecular weight may be used, as long as the dialysis membrane can distinguish the residual oligonucleotide from the other reaction products.

Fig. 6. Effect of increasing pH while maintaining the salt concentration and temperature constant

The effect of increasing pH while maintaining the salt concentration and temperature constant

Fig. 5. Main figure: Ultraviolet absorbance (250 nm) of the total diffusate as it passes out of the dialyzer

The half-life of escape of the ultraviolet-absorbing compounds is calculated from the descending slope of the absorbance curve. At room temperature this value is 4.5-5 min for all components, and at about 40°C it drops to 2.5-3 min. We use a total dialysis time of 25 min, which should decrease the concentration of dialyzable components to less than 0.5% of their original concentration. The two peaks are, respectively, the diffusate from the first oxidation cycle (I) of 10 mg of mixed E. coli B tRNA and the second peak (II) is a reagent blank (i.e., no tRNA added). The difference in curve area is due to released adenine

Inset: Oxidation of tRNA

The diffusate was sampled at the time that the peak absorbance was passing through the sample injection device. The first peak is iodate, and adenine (4) is the released base.
The automated analysis is easily incorporated into the dialysis separation scheme and is completed before the phosphate removal step. This permits the inclusion of feedback control devices (computer-controlled) to repeat the oxidation step if the yield is found to be low, or to repeat the enzyme step if that yield is found to be low. Through suitable control of the chemistry of the base separation, it is possible to perform these rapid analyses with high resolution at relatively low pressures (1378 × 10³ N/m²).

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References


