RESEARCH TECHNIQUES IN BIQCHEMISTRY AND MOLECULAR BIOLOGY

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Preface

It is one thing to teach students research techniques; it is quite another to teach them *how to do research.* Some will argue that the conduct of research cannot be taught formally, but can only be learned individually through the process of actually doing it. In the final analysis, this is of course true, and most graduate training programs are founded on this principle. The Ph.D. degree is a certification that the student did not sink when he was immersed in the laboratory, but learned to swim reasonably well, and can now make his own way through the seas of scientific research.

While this approach to the training of graduate students ultimately produces independent, self-reliant scientists, most teachers agree that it should not be carried to the extreme case of requiring green, unskilled students to tackle sophisticated research problems. Instead, beginning students are usually required to take a course in which they are taught a variety of common research techniques and procedures. However, implicit in many of these courses is the assumption that since the conduct of research can't be taught, it is therefore not necessary to design a techniques course in the form of a research project. The danger in this point of view is that it may result in a techniques course that is not very relevant to the way in which research is actually done. Thus, in the extreme case, the student may be presented with a sequence of exercises which, although illustrating important research techniques, may be totally unrelated to one another.

This approach suffers on several counts. First, the student does not get a sense of the continuity, relationship, or purpose of the experiments. There is no central or dominant theme. In basic research, this theme is provided by the questions one is trying to answer: Is there an enzyme in a bacterium that performs a certain important chemical reaction? How can it be purified? Once purified, can the enzyme be used to prepare a certain product in large amounts? Can this product then be used to study the mechanism of other enzyme systems? And so forth. In the absence of such guiding questions, the student is

left with no idea *why* the experiment is done, nor whether it works sufficiently well to achieve any particular purpose or goal. A second fault inherent in this approach is that the student spends too little time preparing his own substrates. He is often allowed to use commercial preparations without first determining their purity, concentration, etc. Here again the system deviates from basic research practice, where reagents and enzymes are purified and analyzed before the interesting experiments can be undertaken.

Given these general notions of what should be avoided, we set out to design a research techniques course from scratch. The chief limitations were that a relatively large number of students (12 to 24) had to be accommodated with a small number of teaching assistants and a limited equipment budget. This precluded the possibility of assigning each student an individual research project, and necessitated a fairly formal course structure. Within this framework, then, we sought to develop a series of experiments which were tied together by a single unifying question or theme. It was intended that this would give the student a sense of purpose, and enable him to appreciate the relevance of a given experiment on the basis of how necessary it was to help him answer the big question later on. The exercises were planned so that most of the student's time would be spent in preparing and characterizing the reagents needed to perform the subsequent key experiments. Thus he would be required to judge the success of each operation by determining whether the result was sufficiently good to allow him to perform the next one. Finally, we tried to include as many different research techniques as possible that pertained to the problems at hand.

The result of our labors is the laboratory research course described in the following pages. The major theme is the study of protein biosynthesis *in vitro*, and the goal is to repeat two classic experiments in the field. The first is the proof that synthetic polynucleotides can serve as messenger RNA analogues *in vitro*, in that they direct the polymerization of specific amino acids into polypeptide. This is one of the most important and exciting discoveries in the history of molecular biology and Marshall Nirenberg, a codiscoverer, received a Nobel prize for it in 1968. Although the major goal of the course is to repeat several of these historic experiments, most of the exercises are actually related to preparing and characterizing the synthetic polynucleotides to be used as messengers. This necessitates a partial purification of the enzyme polynucleotide phosphorylase.

The second historic experiment to be repeated is the demonstration by Hoagland and Zamecnik that amino acids are not polymerized directly into polypeptides, but are first activated by being esterified to transfer RNA molecules. The final experimental proof of this point is left to the student to design and carry out by himself.

This course was originally designed for college seniors and first year graduate students in biochemistry who have had little or no previous experience in the laboratory. The only prerequisites are the standard introductory courses in

biochemistry and organic chemistry, although experience in quantitative analysis is helpful.

This book could never have been written without the advice and assistance of many colleagues and friends. In particular, we are indebted to Mr. Fred Clarke for his work on the development of Chapter 6.

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R. E. T.
M. R. N.

Advice to Instructors

This course was designed to be given in one semester, with two four-hour laboratory periods per week. There are twenty-two experiments, each of which can be completed in one period provided that students work in pairs. Sets of closely related experiments are grouped into chapters. The last chapter (13) suggests a short series of experiments the students might design and conduct on their own initiative.

As pointed out in the Preface, this course was organized around one central theme—the study of the mechanism of protein synthesis *in vitro*. Within this area two major goals are set. The first is to repeat the historic experiments of Nirenberg and Matthaei, which not only proved the existence of messenger RNA and laid the groundwork for the deciphering of the genetic code, but also established the "incorporation experiment" as the most important single research tool in the entire field. The second goal is to repeat the experiments of Hoagland, Zamecnik, and others, which showed that amino acids are not polymerized directly by ribosomes but first must be "activated" by attachment to transfer RNA. Virtually all of the individual experiments in this manual are essential to, or bear directly upon, these two general objectives. We have found that an introductory lecture at the beginning of the course, in which this historical background is presented and the goals of the course clearly stated, is very much appreciated by students. They should also be encouraged to read the Preface.

The materials and chemicals required are listed at the beginning of each experiment, and the amounts given are sufficient for one pair of students, unless otherwise indicated. However, inexperienced students generally use far more of any given reagent than they need. Therefore, unless you plan to rely on draconian measures, it is a good idea to prepare 1.5 times as much of a reagent as theoretically should be necessary. Two lists of equipment are included, one containing small items to be provided for each pair of students, and the other consisting of heavy equipment sufficient for eight to ten pairs of students.

Reagent chemicals should be ordered from any reputable company two months or more prior to the date planned for use.

You should realize that not all the experiments described in this manual can be expected to work perfectly the first time they are tried. There are many reasons for this, but the main ones are a) variation in the purity or activity of commercially supplied reagents and materials, b) errors in the preparation of stock solutions, c) ostensibly minor alterations in the protocol which may cause unforeseen difficulties later on, d) unanticipated malfunction of equipment. The only way to avoid these pitfalls, and save yourself much embarrassment and loss of time, is to conduct a trial run of each experiment several months prior to the scheduled starting date of the course. If anything goes wrong in the trial run, you then have plenty of time to straighten things out. Some operations are more accident-prone than others, however, and a list of specific caveats about these follows.

Experiment 1

The purchase of bacterial cells often presents a problem, since there is no guarantee that cells obtained from different sources will behave in the same way. We have always purchased *Micrococcus lysodeikticus* cells from Miles Laboratories, Inc. (P.O. Box 272, Kankakee, Illinois) as the spray-dried powder, and have always verified by telephone that the particular lot number to be shipped was satisfactory for the preparation of polynucleotide phosphorylase. (This latter point is important, as some lots of cells have failed to produce active enzyme in the past). In any case, it is essential that the instructor conduct a trial run of Experiments 1 and 2 to make sure that the particular lot of cells in hand behaves normally. For example, occasionally one finds that more tris base is needed to neutralize the cell suspension, or that a longer time (or higher lysozyme concentration) than is indicated in Experiment 1 is required for complete lysis. Similarly, it is sometimes found in Experiment 2 that an ammonium sulfate concentration of 57% saturation is not quite high enough to precipitate all the polynucleotide phosphorylase activity, and that up to 63% saturation gives better results.

Repeatedly warn students that all crude cell extracts and enzyme preparations must be kept as cold as possible at all times. Any vessel which will be used to receive such a solution must be prechilled. Temperature is also critical to the success of the ammonium sulfate fractionations; keep plenty of ice in the bath surrounding the beaker containing the enzyme solution.

Store all buffers and dialysis tubing in a refrigerator or cold room to minimize bacterial growth. This is especially important for anything that comes into contact with polynucleotides. It is advisable to sterilize by flaming any implement used to withdraw pieces of dialysis tubing from the communal supply. Before it is used, this dialysis tubing should be boiled for 30 minutes in 0.1 M $NaHCO_3$; this process is repeated three times with fresh salt solution; the tubing is washed five times with distilled water, then suspended in 50% ethanol

and allowed to stand overnight at room temperature; the ethanol wash is decanted, the tubing is washed again in 50% ethanol for 30 minutes, and then washed ten times in distilled water. Tubing prepared in this manner should be stored in the cold in distilled water. To prevent bacterial growth, EDTA (1 mM) can be added to the solution.

Before knotting the open end of a filled dialysis bag to seal it, twist the open end tightly so that the contents will be under pressure. This will prevent the sample (dialysand) from being diluted by an influx of dialysate.

Vigorous stirring is essential in order for dialysis to be complete (see Fig. 1-2).

Be sure that an ample source of glass-distilled or deionized water is available. When large volumes of this are needed to make up dialysis buffers, be sure to prechill the amount required in a refrigerator or cold room well in advance.

Assign each pair of students an identification number which should be clearly written on any containers they wish to store in the refrigerator, freezer, etc.

Encourage students to be extremely careful in washing and rinsing all glassware. You should be careful to provide a detergent which does not contain phosphate, since remaining traces of this on glassware can ruin the Pi analyses in Experiment 3, and subsequently.

A small "back-up" quantity of polynucleotide phosphorylase and polynucleotides [either prepared in advance by the instructor or purchased from P-L Biochemicals (1037 W. McKinley Ave., Milwaukee, Wisconsin), Miles Laboratories, etc.] sometimes comes in handy to replace losses or failures.

All the buffers used throughout this manual are made up and adjusted to the proper pH at room temperature.

Experiment 2

Whenever an aqueous sample is to be stored frozen, be sure that it is placed in a container that will not crack when the sample freezes. Plastic scintillation vials are excellent containers for small volumes (up to 20 ml).

Experiment 3

Commercial preparations of nucleoside diphosphates are generally hydrates, and this must be taken into account when calculating the amount needed to make up stock solutions. Also, substantial amounts of orthophosphate are occasionally present, either as a result of spontaneous hydrolysis or as contaminants. Since orthophosphate inhibits the polymerization reaction, it is important to ensure that it is not present to an extent of more than a few percent. If a sample is found to be contaminated, it should be discarded or returned to the supplier.

Solutions of nucleoside diphosphates undergo appreciable spontaneous hydrolysis at acid pH. As the product of the hydrolysis is itself an acid, the reaction is autocatalytic. It is therefore advisable to adjust the pH of the stock solutions to about 9 when making them up. Since they are frequently sold as the free acids

or monosodium salts, there is a great deal more acid present than one might think. Hence, one must plan to add a comparatively large amount of NaOH to neutralize the NDP stock solutions.

When adding the components of a reaction mixture to test tubes for the assay of enzymatic activity, it is best to add the enzyme solution to the mixture last. Also, it is a good idea to mix the components in a tube after each addition.

In reference to Fig. 3-3, if the capillary force is not strong enough to lift the meniscus of the liquid all the way to the top of the pipet, a slight negative pressure must be applied. To do this, squeeze the bulb *very* slightly, cover the hole with your index finger, then slowly lessen the pressure on the bulb. Caution: if this procedure is overdone, liquid will be drawn into the chamber of the pipet holder, which must then be disassembled and cleaned.

Experiment 5

The consistency of the $ZnCl_2$ precipitate is variable. If the particles are very fine, complete precipitation may not be obtained in the desk-top clinical centrifuge. In this case use the refrigerated preparative centrifuge with the angle head rotor and soft rubber adaptors of the appropriate size.

Experiment 6

Since the rates of migration of 3′- and 5′-UMP are variable, and depend on temperature, the number of chromatograms in the tank, etc., it is a good idea to check the resolution of these markers before allowing chromatograms to dry. This can be done by removing a wet chromatogram from the tank and viewing with a UV light source in the dark. If further resolution is desired, the still wet paper can be returned to the tank for further chromatography.

Experiment 8

Due to the large amount of micropipetting involved in this experiment, it may be preferable to have each pair of students do only one set of tubes (Recipe 6-1 *or* 6-2), not both sets.

Experiment 9

Note the money-saving option described in Footnote 4.

Experiment 10

The Dowex 1 resin should be prewashed several times each in 0.5 M NaOH, in 0.5 M HCl, and then in distilled water until the pH of the wash returns to neutral.

Experiment 11

The temperature of the viscometer baths should be constant to ± 0.1°; wider fluctuations impair accuracy of measurements. All solutions to be used in the viscosity experiment should be filtered by the instructor using Millipore HAWP filters which have been presoaked for 20 minutes in distilled water. The purpose of this is to remove dust particles from the solutions. Since it is usually not convenient to filter the poly A solutions, the students should remove particulate matter by centrifuging for 5 minutes in the desk-top centrifuge and carefully pipetting off the supernatant solution.

The method for filling a viscometer described in this experiment is the correct one, although it is rather tedious and difficult. A simpler, although less accurate, method is to fill (and empty) the viscometer by means of a plastic pipet through the large bore arm. (Never use a glass pipet as small chips broken from the tip are likely to plug the capillary.) The sample is then drawn into the capillary arm by suction on a rubber tube placed over the top of this arm.

Viscometers may be purchased from the Cannon Instrument Co. (P.O. Box 16, University Park, Pa.).

Experiment 12

The cells used for the preparation of the S-30 fraction are frozen *E. coli* K12 early log phase cells grown on enriched medium and prewashed. They are purchased from the Grain Processing Corp. (P.O. Box 341, Muscatine, Iowa). These should also be ordered well in advance (six to eight weeks) and pretested.

Experiment 13

The procedure described in this experiment for drying filters and counting is the cheapest, although it is time consuming. Quicker methods are: a) dry the filters for 10 minutes under an infrared lamp before counting in a toluene-base scintillation fluid, or b) count the wet filters directly in Bray's solution (see *Analytical Biochemistry, Vol. 1,* p. 279, 1960), which dissolves them.

Experiments 20-22

The degree of separation of activating enzymes on DEAE-cellulose is variable and depends to some extent on the source of the ion exchange cellulose. The product of the Brown Co. (Berlin, New Hampshire) has been found most satisfactory. It is recommended that each student pair use a different ^{14}C amino acid in the assay, so that the distribution of many different enzymes can be compared.

Equipment List

MINIMUM EQUIPMENT REQUIRED BY ONE PAIR OF STUDENTS

1 Stop-watch (calibrated to 1/10 sec.)
1 15 cm ruler
1 pH paper (pHydrion, wide range)
1 Black "Magic Marker," or other indelible marking pencil
1 Large rubber bulb (for pipetting phenol)
1 Mortar (10 cm outer diameter) and pestel
1 Thermometer (0°-100°)
1 Pr. scissors
3 Boxes matches
1 Ice bucket
1 Wash bottle (plastic)
1 Tripod
1 Asbestos-wire gauze
1 Hair dryer (optional, but useful for drying samples applied to chromatography paper)
1 Plastic-coated magnetized spin bar
1 Stirring motor (magnetic)
1 Bunsen burner, with rubber connecting tube
2 Bottle brushes
1 Test tube brush
1 Ring stand
4 Rubber or asbestos coated clamps (small, with fasteners for attaching to ring stand)
1 250 ml grad. cylinder
2 100 ml grad. cylinder
3 25 ml grad. cylinder
Kimwipes or other tissues
Paper towels

Parafilm
Aluminum foil
1 Buret, 50 ml
1 1000 ml beaker
2 600 ml beaker
2 400 ml beaker
2 250 ml beaker
3 150 ml beaker
3 100 ml beaker
1 Crystallizing dish (150 × 75 mm)
25 Screw cap scintillation vials (glass)
1 250 ml Erlenmeyer
1 250 ml vacuum flask (for Millipore filtration)
100 Millipore filters (AAWP, 25 mm)
1 Sintered glass filtration support (Millipore, 25 mm dia.) with one-hole rubber stopper
1 Funnel (Millipore)
1 Clamp (Millipore)
1 Pr. forceps (Millipore)
1 "DEAE-cellulose" column (see Fig. 12-5)
1 Viscometer (Cannon-Manning, Semi-Micro, Calibrated, size A-100)
1 No. 20 Syringe needle
1 Rubber Serum-Bottle Stopper (3/8 in. by 27/64 in.)
1 Piece rubber tubing (2 ft long, 3/16 in. inner diameter, 1/16 in. wall thickness)
1 Piece Tygon tubing (3 ft long, 1/32 in. inner diameter, 1/16 in. wall thickness)
1 Piece Tygon tubing (2 in. long, 1/8 in. inner diameter, 1/16 in. wall thickness)
1 Piece Tygon tubing (1/2 in. long, 5/16 in. inner diameter, 1/16 in. wall thickness)
100 Pasteur pipets (disposable, 5 3/4 in. long, 7 mm outer diameter)
1 Piece copper wire (3 ft long, 18 gauge)
1 Piece glass tubing (6 in. long, 7 mm outer diameter)
1 Test tube rack for large tubes
1 Test tube rack for medium tubes
1 Test tube rack for small tubes
1 Screw clamp for rubber tubing
2 Small rubber pipetting bulbs (for Pasteur pipets)
Assorted Drummond "Microcaps" (1λ, 2λ, 3λ, 4λ, 5λ, 10λ, 15λ, 20λ, 25λ, 30λ), with holders
5 Pipets (10 ml)
10 Pipets (1 ml, calibrated to 0.01 ml)
10 Pipets (0.2 ml, calibrated to 0.001 ml)

2 Heavy stirring rods (glass)
20 Plastic scintillation vials or reagent bottles (for storing frozen samples)
4 Conical centrifuge tubes (glass, 15 ml, graduated)
20 Conical centrifuge tubes (glass, 12 ml)
15 Conical centrifuge tubes (glass, 5 ml)
52 Medium size test tubes (13 X 100 mm; only 26 medium tubes are needed if each pair of students does only one set in Exp. 8)
20 Small size test tubes (10 X 75 mm)
25 Large size test tubes (16 X 125 mm)
2 Spatulas (nickel)
1 Funnel (glass, 65 mm diameter)
1 Small sewing needle
1 Rubber stopper (No. 0)
1 Centrifuge tube (plastic, for Spinco 40 rotor (or equiv.), with cap)
2-3 Centrifuge tubes (nitrocellulose, for Spinco SW-39 rotor (or equiv.))
1 Centrifuge bottle (plastic, 250 cc)
2 Centrifuge tubes (plastic, 50 cc)

MAJOR EQUIPMENT (SUFFICIENT FOR 8-10 PAIRS OF STUDENTS)

One cold room, or refrigerator fitted to contain three magnetically stirred 3 liter dialysis beakers, plus storage space

One large deep freeze

Two large constant temperature water baths

One refrigerated preparative centrifuge with two rotors, one capable of spinning 250 ml capacity bottles at 13,000 xg, the other of spinning 50 ml tubes at 24,000 xg

Two or three desk-top "clinical" centrifuges, with swinging bucket type heads capable of spinning 15 ml conical tubes

One preparative ultracentrifuge, with one swinging bucket rotor capable of spinning 5 ml tubes at 130,000 xg (or equivalent), and one angle-head rotor capable of spinning 10-12 ml tubes at 105,000 xg. The type of swinging bucket rotor used in Experiment 15 is not critical. The experimental protocol is written for a Spinco SW-39 rotor, although any of several more modern 6-bucket rotors might be more convenient

Two or three small desk-top pH meters, accurate to 0.1 pH unit

One or two spectrophotometers, for use in the UV and visible wavelength ranges

One or two colorimeters, for use in the visible wavelength range

Two large viscometer baths, temperature control within $\pm 0.1^{\circ}$

One analytical semimicro balance

One or two single pan triple beam balances

One double-pan "Harvard Trip" type balance

One or two chromatography tanks, each capable of containing eight chromatograms 25 cm wide and 57 cm long

One liquid scintillation counter (two-channel)

One automatic pipet washer (syphon type)

One drying oven

One deionizer (or source of deionized or glass distilled water)

One lyophilization apparatus (optional, depending upon whether polynucleotide products of Exp. 9 need to be concentrated, as described in Exp. 10, and Footnote 4 of Exp. 9)

One uv light source (for examination of chromatograms; see Chapter 5), and a room which can be made completely dark

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