EXPERIMENTAL TECHNIQUES IN BIOCHEMISTRY

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Dedicated to Professor GREGORIO WEBER

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PREFACE

This book is intended for graduate student "techniques" laboratory courses, and it attempts to provide experience in most of the more common techniques used in biochemical research. Most students go from these courses to their own research, and, for this reason, the book contains many hints on what is considered "good technique." Because we have tried to describe the techniques in more depth than is found in most laboratory manuals, we feel this book can also be used by people doing postgraduate research.

Biochemistry students enter graduate school with widely varying backgrounds. Consequently, we have tried to provide relatively detailed experimental procedures without providing experiments (since each department makes up its own). However, it is most important that the student understand what he is doing and why, so every chapter contains an extensive theoretical section.

We have also tried to relate the techniques to each other, stressing similarities and differences as much as possible. This has often meant descriptions of processes on a molecular, physical-chemical level. In order to make our descriptions

easily understood, we have tried to keep them on a qualitative, verbal level, using equations as concise ways of summary, rather than as the descriptive basis. We feel this will help the student who is not strong in physical chemistry, while providing possible additional insight for those who are.

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CONTENTS

1 GENERAL TECHNIQUES: PREPARATION OF MATERIALS

1.1 CLEANING GLASS- AND PLASTICWARE AND CUVETTES

The success of experiments will always depend to some extent on the freedom from contamination of the reagents, and this depends in part on the cleanliness of their containers.

However, we stress that *no* container, be it metal, organic, glass or any plastic, is completely inert. For example, the walls of containers made of plastic such as polyethylene in effect form separate hydrocarbon phases, through which water can slowly diffuse (so that the concentration of aqueous reagents will tend to change with time) or in which nonpolar solutes or solvents can dissolve (1). And anything dissolved in the walls of a plastic container is essentially impossible to remove by washing. Note that some heavy metal cations and inhibitory anions can form nonpolar complexes with some substances, and contaminate a plastic container. Consequently, plastic bottles or other plastic vessels which have come in contact with such compounds should

never be used for anything else. Gases such as ammonia (found in increasing concentrations in commercial floor and window cleaners) can also penetrate plastic containers; these should not be used where contamination with ammonia, carbon dioxide, etc., can be troublesome.

1.1a Washing Plasticware

The procedure for cleaning plasticware depends on the plastic—see the Table of Chemical Resistance of Plastics (Appendix 1). For resistant plastics, we soak the object to be cleaned in 8 *M* urea which was brought to pH 1 with HCl. The acid urea is rinsed off with glass-distilled water, and the object is then washed in 1 *M* KOH, then water, 10^{-3} *M* EDTA, then several more rinses with water. All suitable (resistant) plasticware should receive such an initial wash. Afterwards, a dilute detergent (usually SDS) wash should be sufficient, though occasional acid urea-base washes should be given the objects.

No plastic should be treated with dichromate or any oxidizing agent, as these will tend to produce free carboxyl groups on the walls of the vessel.

1.1b Washing Glassware

Glass behaves like a mixed-bed ion exchange resin, though one of very low capacity. Glass surfaces will bind a variety of anions and cations, and in working with enzymes, especially metal-sensitive ones, this fact can have unfortunate consequences.

Most treatments of glassware, beyond the usual detergent and distilled water washes (0.5% Alconox is preferred), involve exchanging away unwanted adsorbed ions by rinsing the glass with very strong acid or base, or both. We wash glassware first with ethanolic KOH. This removes any greases or oils, and a mono-layer of glass besides, so a rinse should suffice. The ethanolic KOH is rinsed off with distilled water.

Some substances which should not be exposed to this solution are: glass wool, sintered glass, or glass- or quartzware such as cuvettes which have highly polished optical surfaces. The strong base will etch the polished surfaces or weaken the thin glass fibers or partitions.

A soaking in dichromate cleaning solution will remove any organic material remaining, if sufficiently prolonged. Stubborn material may require that the cleaning solution be heated for greater effect. The dichromate rinse will leave a film of adsorbed dichromate on the glass. After rinsing with distilled water, this film is removed, along with any remaining unwanted cations, by a further rinse with concentrated nitric acid (preferred because it is also an oxidizing agent). Do this in a hood! The glass is then rinsed thoroughly with glass-distilled water. If no organic material is present on the glass, only the ethanolic KOH and nitric acid washes need be used.

According to Westhead (2), the nitric acid wash will remove only the super-

ficial cations. Over a period of days, fresh cations, originally in layers beneath the surface, will migrate to the surface, and the glass will have to be re-rinsed. These cations (such as calcium) are of course present throughout the glass, being in the original material used in the manufacture. Westhead has also found that acetate solutions somehow retard this migration.

The glass may be considered clean when water spreads evenly over the surface and does not pull away from the glass to form patches and drops.

It might be expected that freshly cleaned glass surfaces would interact with proteins, and this has been observed in several instances. Lactic dehydrogenase is inactivated by exposure to glass surfaces at low pH. Small amounts of yeast enolase are adsorbed to glass at neutral pH's. However, these interactions are usually noticeable only when working with very dilute protein solutions. For this reason, it is best to work with concentrated solutions, but, when the use of dilute solutions is unavoidable, two tactics can be employed. An excess of some protein such as bovine serum albumin can be added to the solution, or the glass can be coated with dimethyldichlorosilane. The latter is dissolved in benzene (as a 1% solution), heated to 60°C, and then the glass is rinsed with it. The glass is dried in an oven, and then one or two additional coatings of the silane are added in the same way.

Sintered glass and glass wool should be cleaned with dichromate cleaning solution and nitric acid.

1.1c Washing Cuvettes

Manufacturers recommend washing cuvettes with dilute detergent. Scrub gently using a thin stick with a twist of cotton on it. More stubborn cases may require dichromate cleaning solution and nitric acid—but only if the cuvettes are *fused* at the corners and not cemented.

Your work may require cuvettes with a very low level of fluorescence. For this use fused silica or quartz cuvettes. To clean these you should soak them overnight or heat them to 60°C for 30 minutes in 50% nitric acid and 50% sulfuric acid (simply mixing the acids—with care—will give a solution that is warm enough). The cuvettes are then rinsed in doubly glass-distilled water and soaked briefly in ammonium hydroxide. Then they are allowed to dry under an inverted glass beaker on a clean glass petri dish.

1.2 PROCEDURE FOR CLEANING DIALYSIS TUBING

The tubing should be soaked about an hour in 1% acetic acid. Then it is allowed to stand, with gentle stirring, for a few minutes in glass-distilled water, which is replaced by 1% sodium carbonate, 10^{-3} *M* EDTA. After a few more minutes of gentle stirring, the alkaline-EDTA solution is replaced by fresh solution and heated, with stirring, to about 75°C. The hot liquid is poured off, replaced by more alkaline-EDTA, and heated again to 75°C or so. The

solution is again replaced, this time with glass-distilled water. This is poured off after a few minutes stirring, and more water added. This is heated to about 75°C, poured off, and more water added. After a few minutes, the tubing is placed in glass-distilled water in a plastic bottle and stored in a refrigerator with a few drops of chloroform added to discourage bacteria. Acid-washed glassware should be used for the soaking and heating steps.

N.B. Nucleases and other enzymic contamination in dialysis tubing must be removed by *boiling* the tubing once for 30 minutes (no more) in alkaline EDTA. For dialyzing substances with sensitive sulfhydryl groups, the tubing should be heated once in 0.1 *M* sodium ascorbate +0.001 *M* EDTA.

1.3 PREPARATION OF CHROMATOGRAPHIC MATERIALS

1.3a Procedure for Washing Substituted Celluloses

The dry cellulose powder is poured into 1 *M* sodium acetate and thoroughly suspended with a magnetic bar and magnetic stirrer. About 3 liters of solution are used for each 100 g of cellulose.

The suspension is allowed to settle until a distinct zone of settled material has formed (about 10 minutes). A considerable amount of fine cellulose particles may be still in suspension. If so, a pipette attached to an aspirator is used to remove as much of this fine suspension as possible. The remaining sedimented cellulose and solution is poured into a Buchner funnel and filtered nearly dry.

If the cellulose has not been used before, it is then resuspended in the same volume of 0.5 *M* NaOH, allowed to settle for about 20 minutes, and the "fines" aspirated off, as before. This is filtered, and the cellulose cake is resuspended and washed twice more with fresh 0.5 *M* NaOH. Do not allow the cellulose to stand for excessively long periods (e.g., overnight) in the strong base or acid. These will hydrolyze the cellulose, producing more "fines," or can actually remove the chemical substituents.[1]

After the third wash with NaOH, the cake is suspended in water (3 liters/100 g) and thoroughly dispersed with stirring. Then HCl is added slowly with stirring to a final concentration of 0.5 *M*. After stirring for five minutes, the suspension is allowed to settle as before, and the aspiration and filtering are repeated. The cellulose is washed again with 0.5 *M* HCl, once with 0.1 *M* HCl in 95% ethanol, methanol or acetone, and then washed with water. It is finally resuspended in 0.1 *M* sodium acetate and 3×10^{-3} *M* EDTA, and the pH of the cellulose suspension adjusted to the pH of use or preference. The EDTA is added because celluloses tend to pick up heavy metal ions. The cellulose is allowed to stand overnight, and the pH is checked again and readjusted if necessary. The final washings should be with 2-3 changes of

[1]This is especially true for phosphorylated cellulose.

water. The cellulose is filtered nearly dry and stored with a couple of milliliters of chloroform added.

Glass-distilled water and acid-washed glassware (for vessels in actual contact with the cellulose) should be used throughout.

Once the celluloses have been given this preliminary treatment, further cleanings (for example, after using the cellulose in a protein purification step) should be done in the same way, except 0.1 *M* acid and base solutions are used. If contaminants are adsorbed which resist this treatment and 0.5 *M* washes as well, discard the cellulose.

1.3b Washing of Molecular Sizing Materials

Sephadex (plain or substituted) should be cleaned by two successive washes in 0.1 *N* NaOH, a wash with water, a wash in 0.1 *N* HCl, then (substituted Sephadexes *only*) with 0.1 *N* HCl in 95% ethanol. The material is rinsed with more water and 10^{-3} *M* EDTA. The EDTA is washed out with more water before pH adjustment or storage. Chloroform should *not* be added to these substances or to Bio-Gels. Sephadexes may be autoclaved or stored in 0.9% NaCl and 0.2% NaN_3. Sephadexes lower than G-50 may be sterilized with 1% NaOH, followed by a sterile water rinse.

Bio-Gels cannot be treated to pH's above 9.5 without some hydrolysis of the amide groups. So Bio-Gels should be washed as with Sephadexes *except* that 1% $NaHCO_3$ should be substituted for the 0.1 *N* NaOH. Bio-Gels should *not* be sterilized with heat—neurotoxins may be formed.

1.3c Preliminary Preparation of Dowex Resins

The Dowex 1, 2, or 50 should first be suspended in one liter/lb of ethanol (95% or absolute): acetone, 1:1. Then the resin is filtered nearly dry and resuspended in water with a magnetic stirrer and magnetic bar, and solid sodium acetate added to a final concentration of 1 *M*. The Dowex is collected by filtration in a Buchner funnel, resuspended in 0.5 *M* NaOH, and filtered again. It is washed again by suspension in 0.5 *M* NaOH and filtration; then a third time with 0.5 *M* NaOH by pouring one liter/lb through the Dowex cake in the Buchner funnel. The Dowex will have changed color from yellow to a reddish brown because of the methyl orange, an indicator present in all Dowexes. The methyl orange cannot be removed completely. Indeed, Dowex should be rewashed if used after long storage.

After the sodium hydroxide washes, the Dowex is again suspended in water, and HCl added to a concentration of 3 *M* (about a 1:4 dilution of the concentrated acid) and filtered. An additional 0.5 *M* HCl wash follows and finally the resin is washed with water until the wash liquid is neutral; that is, until all HCl is removed.

Dowex 1, 2, and 50 are now in the chloride or acid forms, and these can

be used immediately if desired. If you want to convert any of the Dowexes to other salts, say Dowex-1 formate, they must undergo further washing.

1.3d Cleaning Other Chromatographic Materials

A wide variety of materials is used for chromatography, and the cleaning procedure depends on the material. DEAE-cellulose for thin layer chromatography, for example, is cleaned like regular DEAE. However, whatever the material used, it should be washed beforehand with the solvent used in the planned chromatography. This is true whether the material is DEAE in a column, paper, or Sephadex. After sufficient solvent has dripped off or percolated through, the material can be washed with water (to remove salts) and dried if the chromatography is done on the dry material.

1.4 PREPARATION OF REAGENTS

1.4a Water and Other Solvents

Most reagents you will make up are aqueous ones, and it pays to critically consider the quality of the water you use. While tap water may serve for a few reagents, most will require at least deionized or distilled water. Deionized water has been passed through a mixed-bed ion exchange resin and is consequently very low in concentration of ionic substances. On the other hand, the resins used tend to give off ultraviolet-absorbing substances, and, of course, nonionic substances are not removed. Therefore in work where a very low ultraviolet "background" is required, deionized water should not be used without further treatment. Since some plastic containers also tend to leach ultraviolet-absorbing substances and glass tends to give off (exchange) ions, the storage vessel should be chosen depending on the intended use of the water.

Distilled water tends to be low in ions and other nonvolatile substances. Volatile substances, like carbon dioxide, HCl and acetic acid, will not be easily removed by distillation. Aromatic compounds, which tend to be fluorescent, may also come over, but in general, water distilled in a glass still will be adequate for most experiments.

If really high quality water is desired, it must be distilled from acid permanganate: 1 g of potassium permanganate plus 1 ml of phosphoric acid per liter of water is distilled in a clean glass still. The hot permanganate oxidizes all organic substances, and the resulting distilled water has a very low ultraviolet absorption and fluorescence.

1.4b Chemicals

One must add clean chemicals to clean water for best results. Most inorganic chemicals can be purchased with an analysis that states what major contaminants occur and roughly in what amount. However, many chemicals will not

come with any analysis, and in these cases a healthy skepticism as to their purity is the best attitude. Some of these compounds, such as Tris or acrylamide, can and should be recrystallized at least once before using. Others, such as DPNH, can be purified by chromatography (see Chapter 3). Generally, reasonable results can be obtained in most experiments with commercial supplies.

1.4c Accurate Reagent Preparation

In weighing out material for a reagent, it is sometimes convenient to weigh out a relatively large amount of the material and make up a stock solution, from which small amounts may be withdrawn at convenience. Then too, some chemicals are more stable in more concentrated solutions. Stock solutions can also be used to cut down on the amount of pipetting needed and at the same time reduce variability between a number of similar incubation mixtures, assay solutions, etc.

Generally, molar solutions are used—solutions made up to a given volume. Molal solutions are prepared by mixing a certain number of gram formula weights with 1000 grams of water or other solvent. Molal solutions are seldom used in biochemistry. "Normality" refers to the molar concentration in equivalents, especially of acid or base. A frequently used term of concentration is the "percent of" some compound. This is either: weight percent—grams of compound in 100 grams of solution (not solvent); or volume percent —milliliters per 100 milliliters of solution.

For best accuracy, the student should learn to differentiate between TC ("to contain") and TD ("to deliver") containers. The former container will *contain* say 100 ml if the liquid is at the 100 ml mark. Such vessels must be rinsed out with the solvent to get all the reagent. TD containers will *deliver* 100 ml of liquid into another container when tipped, but contain slightly more than 100 ml.

Temperature effects on the volume of solutions are not large in the case of water, but can be significant with other solvents. Effects of temperature on the pH of buffers vary with the buffer. The pH of Tris buffers (and probably all primary amine buffers as well) is markedly affected by temperature, decreasing almost one pH unit with an increase of 30°C in temperature. The pH of phosphate buffers shows less temperature dependence.

The pH of a buffer can change with dilution as well. Generally, large dilutions of buffers should not be made without checking the final pH. Excessively dilute (say 10^{-4} *M*) buffers will probably be ineffective, due to residual ions in the water, especially ammonium and bicarbonate ions.

Various methods are used to avoid contamination of reagents. The most obvious has been mentioned: storing them in properly cleaned containers. Keeping fingers away from contact with reagents and surfaces in contact with reagents is absolutely necessary, since fingerprints contain not only fats

and oils, but metal ions, phosphate, amino acids and at least one enzyme, a nuclease. Sealing the container against vapors such as carbon dioxide, ammonia or hydrochloric acid can be done temporarily with Parafilm. Parafilm itself, however, is a wax and is susceptible to attack by toluene or benzene vapors.

Bacterial contamination is not confined to reagents containing organic chemicals. We have observed bacteria growing on the inside of polyethylene glass-distilled water containers and in glass jars of magnesium chloride solutions. Presumably, they live off ammonia and carbon dioxide in the water. This may be retarded by storage in a freezer or refrigerator, by adding a drop of chloroform to the reagent before sealing, or by filtration through a Millipore filter (the 0.45 micron pore size is sufficient). Growth also seems inhibited in more concentrated solutions.

One minor precaution against contamination is not to pipette excess reagent back into the bottle. Of course, this is only if the reagent is not expensive or hard to replace.

1.5 PIPETTES AND PIPETTING

Graduate students in biochemistry should have learned how to pipette reagents before entering graduate school, but in fact some have not. Several types of pipette are commonly in use: volumetric (not graduated), serological (graduated to the tip), measuring (not graduated to the tip), long tip (these may be serological or measuring), and microliter ("lambda").

For adding large volumes of solution with the greatest accuracy, volumetric pipettes are used. These are filled to above the mark ("fill line") while held vertically, the outside is carefully wiped off, and the excess solution released to the fill line. While still held vertically, the specified volume of solution is released into the desired receptacle until the flow stops. Wait about ten seconds for solution on the walls of the pipette to collect in the tip and withdraw the pipette with a twisting motion, holding the tip against the inside of the receiving vessel. Do not blow out the remaining liquid.

For adding intermediate volumes, use long tip pipettes (or measuring pipettes, if long tip pipettes are not available). Use the appropriate size of pipette; do not try to measure out say 0.2 ml samples with a 5 ml pipette. Draw the reagent up past the "0" mark, wipe off the outside of the pipette, release the reagent to the "0" mark while holding the pipette vertically, and touch off the tip end. Then move the pipette to the receiving vessel and allow the liquid in the pipette to drop to the desired level. Keep the pipette vertical and the flow slow—take twenty seconds or so.[2] Then touch the tip again to the inside of the receiving vessel and remove the pipette.

[2]Longer for viscous solutions.