Experimental Section

The following experiments were obtained from the **Workshop on Biochemical Techniques in Proteins and Enzymes,** *held at the Institute of Chemistry, University of the Philippines at Los Banos, Laguna, on May 10-24, 1989. The workshop was sponsored by the International Development Programme of Australian Universities and Colleges and the Institute. These experiments were designed with simplicity in mind, in the hope that they will be of use in the various teaching programmes, especially in institutions where biochemical equipment is minimal.*

PROTEIN MEASUREMENT BY COLORIMETRIC REACTIONS AND BY SPECTROPHOTOMETRY

THE BIURET METHOD

This has low sensitivity, so is most suited to situations when a large amount of sample is available. Several milligrams protein are needed, but it gives a good value that is not much affected by the type of protein. It is based on the fact that in alkaline solution, the peptide chains of proteins bind copper ions, to form a purple color. Ammonium ions and some buffers at high concentration may give an interfering color.

The color produced by the reaction of an unknown protein solution with the biuret reagent must be compared with a standard protein. A standard curve using 1 to 10 mg bovine serum albumin (BSA) should be used, and sufficient unknown added to give a color within that range.

Experiment: Make 1 mL of solutions containing between 1 and 10 mg of BSA, and some unknown samples of appropriate dilutions. To these, add 4 mL of biuret reagent, mix well, allow to stand for 20-30 min. Read the absorbance at 540 nm in the spectrophotometer. Calculate the concentration of protein in the unknown samples.

Biuret reagent: Dissolve 1.5 g copper sulfate ($CuSO_4 \cdot H_2O$) and 6 g sodium potassium tartrate in 500 mL water. To this, add 300 mL of 10% NaOH, stirring well. Dilute to 1 liter. (Note: This reagent is caustic!)

Standard BSA (10 mg/mL): Dissolve 1 g bovine serum albumin in 100 mL of water. Excess solution can be kept frozen, or refrigerated if sodium azide (10 mM) is added.

DYE BINDING

This method is one of the most widely used. It has a high sensitivity (5-30 μg), and is quick and easy to carry out. In strongly acid solution, protein binding to the dye Coomassie Blue shifts its color from pink-brown to blue, which is read in the spec-

trophotometer at 595 nm. Few compounds interfere, but if the protein sample is in strong acid or alkali, it may not give the correct color.

A standard curve should be set up using 5-25 μg BSA in 10 mM phosphate buffer, to a total volume of 1.5 mL buffer. An amount of suitably diluted unknown should also be made to 1.5 mL with phosphate buffer. 1.5 mL of Coomassie Blue reagent is then added to each (mix well!), and the color at 595 nm read after 2-30 min. Calculate the concentration of protein in the unknown solutions, and compare the results with those obtained using the biuret reagent.

SEPARATION OF MYOGLOBIN AND ALKALINE PHOSPHATASE BY ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography was one of the first techniques utilized by biochemists for the purification of proteins. It relies on the fact that proteins possess different ratios of charged amino acids and exhibit isoelectric points (pI) which reflect that composition. The net charge on a particular protein will therefore be a function of the pI of that protein and the pH of the solution. At a pH above its pI a protein will have a net negative charge and therefore will absorb to an ion exchanger with a positive charge (anion exchanger). Conversely, at a pH below its pI the protein will have a net positive charge and will bind to an ion exchanger with a negative charge (cation exchanger). The two most common functional groups used are diethylaminoethyl [$OCH_2CH_2\overset{+}{N}H(CH_2CH_3)_2$, DEAE] in anion exchangers and carboxymethyl [OCH_2COO^-, CM] in cation exchangers. These two are both weak exchangers, weak referring to the fact that functional groups are not charged at extremes of pH (DEAE above pH 9 and CM below pH 4). The pH range of 4 to 9 covers most applications that you are likely to encounter. The insoluble supports that have been used to attach functional groups to must have no affinity for the protein and must not be charged. Cellulose is probably the most widely used support although other polysaccharide supports have also proved useful.

This experiment uses an artificial mixture of two proteins, myoglobin and alkaline phosphatase from calf intestine, to illustrate the use of an anion exchanger (DE-52, which has a cellulose matrix). Myoglobin binds weakly at the equilibration pH of 8.2 and alkaline phosphatase binds more strongly. The two proteins are eluted by the stepwise addition of two NaCl solutions in the equilibration buffer.

Experiment: Clamp a column to a stand so that it is vertical, and pour into it sufficient slurry of equilibrated DE-52 until it reaches the 2 mL mark on the column. (Note: A microcolumn would be most convenient.) Pass 4 mL of 10 mM Tris buffer (pH 8.2) through the column before applying 1 mL of protein solution to it. Wash the sample into the column with equilibration buffer. Start collecting 2 mL samples at this stage. Then elute the column with buffer containing 10 mL of 50 mM NaCl. Myoglobin will be eluted from the column and this will be clearly observed as a red-brown band migrating down the gel. Myoglobin is a haem-containing protein which absorbs strongly at 415 nm. When the elution