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

of myoglobin from the column is complete, apply 10 mL of buffer containing 0.5 M NaCl. This will elute the alkaline phosphatase.

Activity measurement. (1) **Protein.** Use the Coomassie blue dye binding method for the measurement of protein in each sample. You will have to dilute the sample with equilibration buffer to bring it within the calibration range.

(2) **Myoglobin.** Measure the absorbance at 415 nm in each sample to determine the elution of myoglobin.

(3) **Alkaline phosphatase.**

$$\text{p-nitrophenyl phosphate} + H_2O \longrightarrow \text{p-nitrophenol} + \text{phosphate}$$

To 1.5 mL of p-nitrophenol solution add 1.5 mL of assay buffer. Place the cuvette in the spectrophotometer, adjust the wavelength to 400 nm and zero the instrument. Add 1.0 mL of enzyme fraction, mix well, and measure the rate of nitrophenol release at 400 nm. The millimolar extinction coefficient of the nitrophenolate ion at pH 8 and 400 nm is 18.2. Calculate the alkaline phosphatase activity in units per mL, where 1 unit of alkaline phosphatase catalyses the release of 1 μmol of phosphate per min.

Spot test. The enzyme can be quickly located by adding 50 μL of sample to 100 μL of substrate and observing the color change. The presence of alkaline phosphatase is indicated by the production of a yellow color.

Reagents: **Chromatography buffers:** (a) Tris buffer: 10 mN Tris-Cl, pH 8.2, (b) 10 mM Tris-Cl pH 8.2 containing 50 mM NaCl, (c) 10 mM Tris-Cl pH 8.2 containing 500 mM NaCl

Assay solutions: 20 mM p-nitrophenyl phosphate in water (keep on ice and in the dark) in 0.1 M Tris-Cl buffer, pH 8.0.

ENZYMATIC ANALYSIS OF SUGARS

This experiment demonstrates the use of enzymes in analysing a mixture of sugars, in the presence of other sugars and substances which might interfere with conventional chemical methods.

Using enzymes that are specific for the particular sugars, we can measure glucose, fructose and sucrose successively in one cuvette in a spectrophotometer. The principle is illustrated below:

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NAD}^+ \longrightarrow \text{6-phosphogluconolactone} + \text{NADH}$$

The reaction mixture contains an excess of ATP to react with the glucose in the unknown sample, the enzyme glucokinase to catalyze the reaction, and the enzyme glucose-6-phosphate dehydrogenase to remove the product, glucose-6-phosphate, and make NADH which is observable at 340 nm. One molecule of NADH is produced for every molecule of glucose present in the original sample. The reaction trace should look as below, and if the amounts of enzymes used are sufficient, the reaction should be over in 2-3 minutes.