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

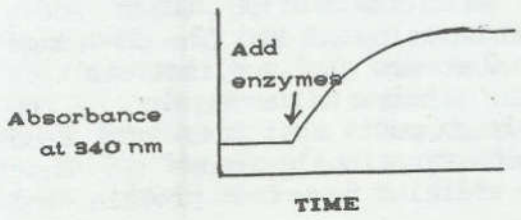
This experiment demonstrates the use of enzymes in analysing a mixture of sugars, in the presence of other sugars and substances which might interfere with conventional chemical methods.

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.



Then a small amount of a solution containing the enzymes fructokinase and phosphoglucose isomerase is added. Fructose now reacts:

Fructose + ATP ⟶ Fructose-6-phosphate + ADP

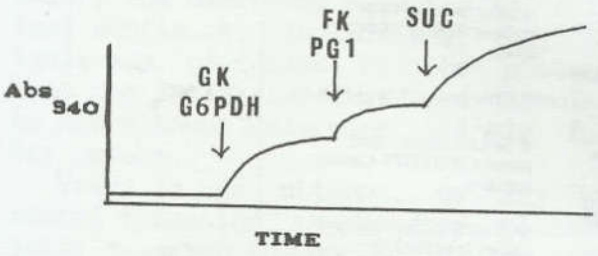
Fructose-6-phosphate ⟶ Glucose-6-phosphate

The glucose-6-phosphate is then acted on by glucose-6-phosphate dehydrogenase, and once more one molecule of NADH is produced from each molecule of fructose.

Finally, a small amount of solution containing the enzyme sucrase, more commonly known as invertase, is added:

Sucrose ⟶ Glucose + Fructose

Both the glucose and fructose molecules are then acted on by the enzymes already present, and *two* molecules of NADH are produced from every molecule of sucrose. In terms of mass, the yield of NADH is approximately the same for sucrose as for the monomer sugars. The total spectrophotometric trace could look like below: however, this depends on the relative amounts of the three sugars.



Experiment: Fruit samples are weighed, and to every gram, 5 mL of 4% perchloric acid (or 20 mL water) is added, and the sample homogenised thoroughly. Insoluble material should be centrifuged, or filtered away, and a measured volume of the perchloric acid extract is taken and titrated with potasium hydroxde (containing some potassium carbonate) to a green colour using bromothymol blue as indicator, or any other acid-base indicator that changes in the pH range 4 to 9. Note the volume after titration, so that you know the volume of this neutralized extract that represents 1 g of fruit. (Note: This neutralization step would be unnecessary if the extracting medium was only water.) Allow the neutralized extract to stand in ice, if possible, for a few minutes, then decant clear extract from potassium perchlorate crystals.

Place 2.5 mL of "sugar buffer" in a cuvette, and add 0.5 mL of suitable diluted fruit extract, or 0.5 mL of prepared standard. Read the absorbance at 340 nm against a control cuvette. Then add 0.05 mL of "GK/G6PDH" enzyme mixture, and observe the change in absorbance, until it stops increasing. Note this value, then add 0.05 mL of "FK/PGI" mixture, and once more follow the absorbance at 340 nm.

Finally add "sucrase" enzyme, and follow the final absorbance change. If the absorbance exceeds 1.0, the experiment should be repeated using a more diluted extract. On the other hand, if the first step indicates a very low level of glucose, a more concentrated sample may be used.

Given that the millimolar extinction coefficient of NADH is 6.22, calculate the concentrations of sugars in the extracts, and the percent, by weight, of the sugars in the original fruit.

Reagents:

(1) 0.5 M KOH + 20 mM K_2CO_3 (or Na_2CO_3)

(2) 4% perchloric acid (v/v)

(3) "sugar buffer": 30 mM K-phosphate, pH 7.5, containing 5 mM $MgCl_2$, 1 mM NAD^+ and 1 mM ATP

(4) GK/G6PDH: 100 units/mL each of glucokinase and glucose-6-phosphate dehydrogenase from *Zymomonas mobilis*.

Alternatively, enzymes hexokinase from yeast, and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, which are commercially available, may be used.

(5) FK/PGI: 100 units/mL each of fructokinase (*Zymomonas mobilis*) and phosphoglucose isomerase (rabbit muscle). Alternatively, if hexokinase was used above, you need only phosphoglucose isomerase, either rabbit muscle or yeast enzyme.

(6) sucrase: 100 units/mL of yeast invertase. (Note: "units" are μmol/min.) ■