SPECTROPHOTOMETRY

COUPLED ASSAY

Many compounds of biological importance do not have a distinct absorption maximum λ_{max} .

Their concentration can be determined if they can be linked to or coupled with a reaction that fulfills the following condition ..

If the promote the formation of another compound that has a characteristic absorption peak.

In coupled assay reactions the product of the first reaction is the substrate of the following reaction ;

A + B ------------> C + D ------------> E + F

A = substance under study that does not have a distinct λ_{max}

F = has a distinct λ_{max}

Thus A can be estimated by measuring the absorbance of F

COUPLED ASSAY EXAMPLE

To 2.0 ml of a glucose solution; 1.0 ml of a solution containing excess ATP, $NADP^+$, $MgCl_2$, hexokinase and Glucose -6-phosphate dehydrogenase was added. Calculate the concentration of glucose in the original solution. Absorbance of final solution at 340 nm increased to 0.91, a_m= 6220, L = 1 cm.

$$\text{Glucose + ATP} \xrightarrow{\text{Hexokinase. } Mg^{+2}} \text{glucose -6-phosphate + ADP}$$

$$\text{Glucose-6-phosphat} + NADP^+ \xrightarrow{\text{glucose -6-phosphate dehydrogenase}} \text{6-phosphogluconate} + NADPH + H^+$$

COUPLED ASSAY EXAMPLE

Glucose has no absorption at 340 nm, but NADPH does!

From the reaction: 1 mole of glucose produces 1 mole of NADPH, thus each number of NADPH moles produced originates from every mole of glucose in the original solution.

Absorbance at 340 nm is the absorbance of NADPH = 0.91

$A = a_m \times C \times 1 = 0.91 = 6220 \times C \times 1$

$C_{NADPH} = 0.91 / 6220 = 1.46\times10^{-4}$ M

Thus there is 1.46×10^{-4} M of glucose present in the *test* solution

The glucose concentration in the *original* solution = $1.46 \times 10^{-4} \times (3/2) = 2.2 \times 10^{-4}$ M

↑ Dilution factor

PROBLEMS

Calculate the absorbance and the transmission at 260 nm and 340 nm of the following solutions in a 1 cm cuvette. a) 2.2 x10^{-5} M NADH b) 7 x 10^{-6} M NADH plus 4.2 x 10^{-5} M ATP.

a_m		
Compound	260nm	340nm
NADH	15000	6220
ATP	15400	0.0

a) This solution contains one absorbing substance (NADH)

$A_{260} = a_m \times C \times l$

$A_{260} = 15000 \times (2.2 \times 10^{-5}) \times 1 = 0.33$

$A = \text{Log } I_\circ / I$

$0.33 = \log 1.0 - \log I$

$0.33 = - \log I$

$I = \text{antilog} - 0.33 = 0.464$

Absorbance and transmission at 340nm

$A = 6220 \times 2.2 \times 10^{-5} \times 1 = 0.1368$

$A = \text{Log } I_\circ / I$

$0.1368 = \log 1.0 - \log I$

PROBLEMS

$0.1368 = -\log I$

$I = \text{antilog } -0.1368$

$I = 0.729$

b) The solution contains two absorbing substances

At 260nm

$A = A_{NADH} + A_{ATP}$

$A_{NADH} = 15000 \times (7 \times 10^{-6}) \times 1 = 0.105$

$A_{ATP} = 15400 \times (4.2 \times 10^{-5}) \times 1 = 0.646$

$A_{Total} = 0.105 + 0.646 = 0.751$

$A = \text{Log } I_\circ / I = 0.751 = \log 1.0 - \log I$

$0.751 = -\log I$, $I = \text{antilog } - 0.751$, $I = 0.177$.

At 340 nm only NADH absorbs

$A = 6220 \times (7 \times 10^{-6}) \times 1 = 0.043$

$A = \text{Log } I_\circ / I = 0.043 = \log 1.0 - \log I$, so $I = \text{antilog } - 0.043$, $I = 0.905$

PROBLEMS

Calculate the concentration of ATP and NADPH in solutions with absorbance's:

a) 0.15 at 340 nm and 0.9 at 260 nm.

b) Zero at 340 nm and 0.750 at 260 nm.

c) 0.22 at 340 nm and 0.531 at 260 nm.

a_m		
Compound	260nm	340nm
NADPH	15000	6220
ATP	15400	0.0

a) Since this solution contains two absorbing substances , thus we will start with absorbance at 340nm since only NADPH absorbs

$A_{340nm} = A_{NADPH}$ **only**

$A = a_m \times C \times l = 6220 \times C \times 1$

$C = 0.15 / 6220 = 2.4 \times {}^{10-5}$ M

PROBLEMS

$A_{260nm} = A_{ATP} + A_{NADPH}$

$A_{NADPH} = a_m \times C \times l = 15000 \times 2.4 \times ^{10-5} \times 1 = 0.36$

$A_{ATP} = A_{Total} - A_{NADPH} = 0.9 – 0.36 = 0.54$

$A_{ATP} = a_m \times C \times l = 0.54 = 15400 \times C \times 1$

$C = 0.54 / 15400 = 3.5 \times 10^{-5}$ M

b) Since Absorbance at 340 nm is zero , and NADPH is the only absorbing substance at that wavelength thus the concentration of NADPH is zero

Accordingly the absorbance 0.75 at 260 nm is the absorbance of ATP only.

$A_{ATP} = a_m \times C \times l$

$C_{ATP} = 0.751 / 15400 = 4.8 \times 10^{-5}$ M

c) At 340nm only NADPH absorbs

$0.22 = 6220 \times C \times 1$

$C_{NADPH} = 0.22 / 6220 = 3.5 \times 10^{-5}$ M

PROBLEMS

At 260nm both ATP and NADPH absorb

Thus $A = A_{ATP} + A_{NADPH}$

$A_{NADPH} = 15000 \times (3.5 \times 10^{-5}) \times 1 = 0.53$

Since $A_{NADPH} = A_{total}$

Thus ATP concentration must be Zero