Spectrophotometry

Coupled Assay

- Many compounds of biological importance do not have a distinct absorption maximum λ_{max} . Nevertheless, their concentration can be determined if they can be linked to or coupled with a reaction that fulfills the following conditions :
- 1- The reaction they are coupled or linked to should be a reaction that produces or allows the production of a substance that has a characteristic absorption peak .
- 2- The reaction they are coupled or linked to should allow a stoichiometrical production of

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 .

Example : To 2.0ml of a glucose solution 1.0ml 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 at 340nm of NADPH = 0.91, a_m= 6220

The following are the reactions taking place ;

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

Coupled Assay

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

Glucose has no absorbtion at 340nm , but NADPH does , and from reaction 1 mole of glucose produces 1 mole of NADPH , thus each mole of NADPH produced originates from 1 mole of glucose in the original solution .

Absorbance at 340nm is the absorbance of NADPH = 0.91

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

$C_{NADPH} = 0.91 / 6220 = 1.46 \times 10^{-4}$ 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

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Problems

Calculate the absorbance and the transmission at 260nm and 340nm of the following solutions in a 1cm cuvette . a) 2.2×10^{-5} M NADH

b) 7×10^{-6} M NADH plus 4.2×10^{-5} M ATP .

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

a) This solution contains one absorbing substance (NADH).

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

$A = \text{Log } I_o / I$, thus $0.33 = \log 1.0 - \log I$,

$0.33 = -\log I$, $I = \text{antilog} - 0.33 = 0.464$.

Absorbance and transmission at 340nm,

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

$A = \text{Log } I_o / I$, thus $0.1368 = \log 1.0 - \log I$

Problems

$0.1368 = -\log I$, so $I = \text{antilog } -0.1368$

$I = 0.729$.

b) The solution contains two absorbing substances ,

At 260nm

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

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

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

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

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

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

$I = 0.177$.

At 340nm only NADH absorbs .

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

$A = \text{Log } I_o / I = 0.043 = \log 1.0 - \log I$, 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 340nm and 0.9 at 260nm .

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

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

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

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 340nm 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 260nm 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 .