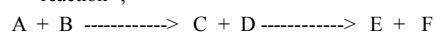


## Spectrophotometry

### Coupled Assay

- Many compounds of biological importance do not have a distinct absorption maximum  $\lambda_{\max}$ . 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 ;



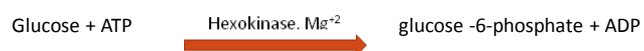
A = substance under study that does not have a distinct  $\lambda_{\max}$  .

F = has a distinct  $\lambda_{\max}$  .

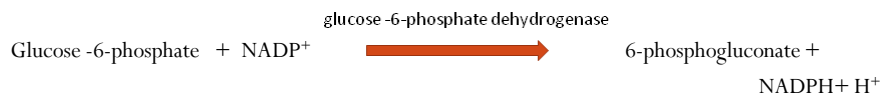
Thus A can be estimated by measuring the absorbance of F .

Example : To 2.0ml of a glucose solution 1.0ml of a solution containing excess ATP , NADP<sup>+</sup> , MgCl<sub>2</sub> , 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 ;



## Coupled Assay



Glucose has no absorbance 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

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

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

Thus there is  $1.46 \times 10^{-4}$  M of glucose present in the test solution.

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

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## Problems

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

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

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

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

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

$$A = \text{Log } I_0 / I, \text{ thus } 0.33 = \log 1.0 - \log I,$$

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

Absorbance and transmission at 340nm,

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

$$A = \text{Log } I_0 / I, \text{ thus } 0.1368 = \log 1.0 - \log I$$

## Problems

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

$$I = 0.729.$$

b) The solution contains two absorbing substances ,

At 260nm

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

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

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

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

$$A = \text{Log } I_0 / I = 0.751 = \log 1.0 - \log I$$

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

$$I = 0.177.$$

At 340nm only NADH absorbs .

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

$$A = \text{Log } I_0 / I = 0.043 = \log 1.0 - \log I, \text{ so } I = \text{antilog } -0.043$$

$$I = 0.905.$$

## 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 .

$$A_{340nm} = A_{NADPH} \text{ only .}$$

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

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

## Problems

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

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

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

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

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

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 .

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

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

c) At 340nm only NADPH absorbs .

$$0.22 = 6220 \times C \times 1$$

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

## Problems

At 260nm both ATP and NADPH absorb ,

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

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

Since  $A_{\text{NADPH}} = A_{\text{Total}}$

Thus ATP concentration must be Zero .