# Bioluminescence

Reference

Biochemical Luminescence-*S.Brolin and G.Wettermark*

# Outline

- Introduction
- Principle
- Case Study
- Summary

# Fluorescence

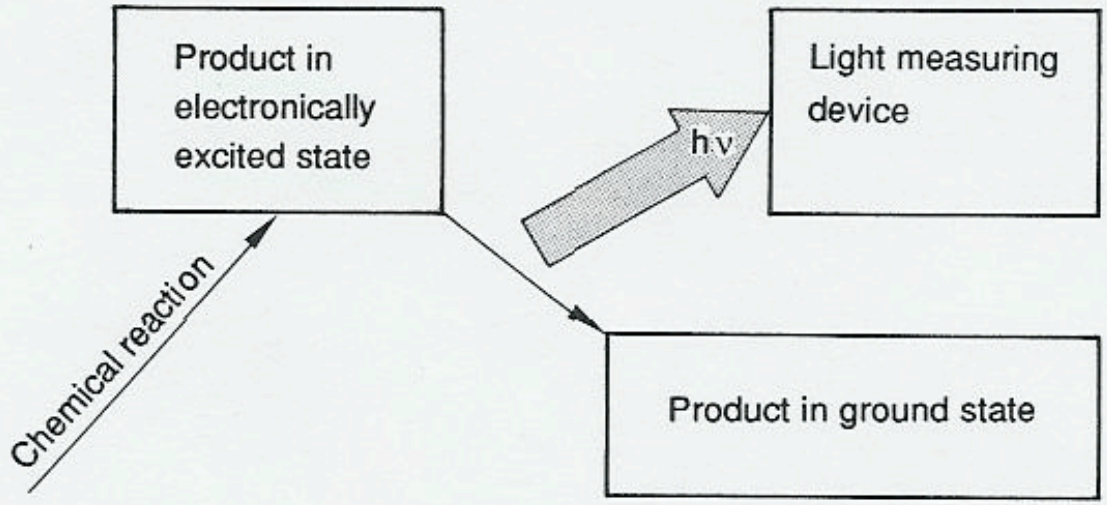



**Figure 1-1.** Reaction scheme of chemiluminescence analysis. Reactants which give rise to a product in an electronically excited state can be determined, if the return to the ground state is associated with the emission of light. The analysis applies to a great variety of molecules, including synthetic compounds and species obtained from biological extracts [2].

# Jablonski diagram

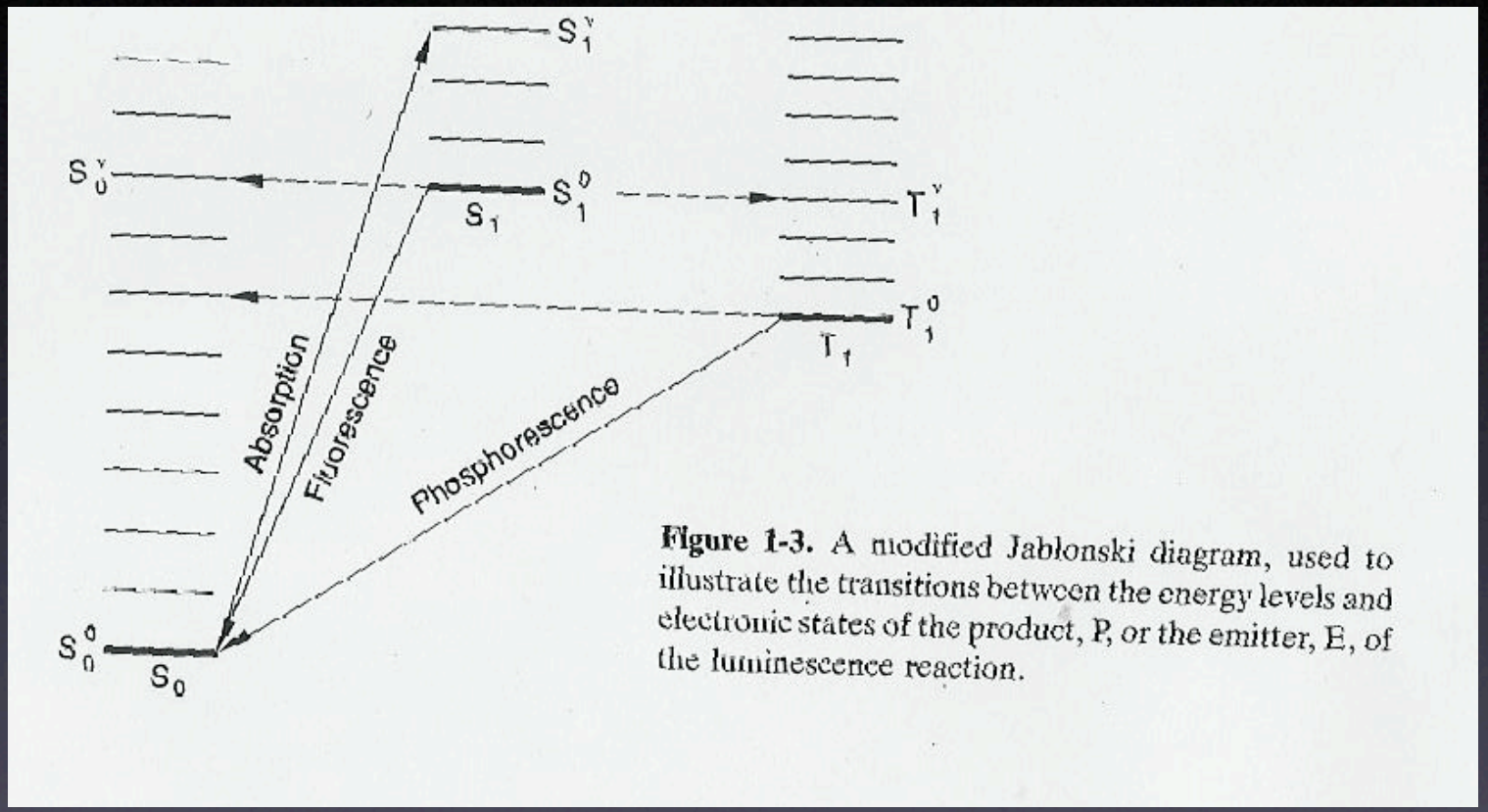



**Figure 1-3.** A modified Jablonski diagram, used to illustrate the transitions between the energy levels and electronic states of the product, P, or the emitter, E, of the luminescence reaction.

# Difference between Luminescence and Fluorescence

| | |
|---|---|
| In luminescence, the light intensity is a direct measure of the reaction rate.<br><br>The excitation is chemical( reaction), where a reactant is consumed. | In Fluorescence or Absorption photometry, the intensity of the outcoming beam measures the concentration of a reactant or a product.<br><br>The excitation is physical |

# Comparison between Luminescence and Fluorescence

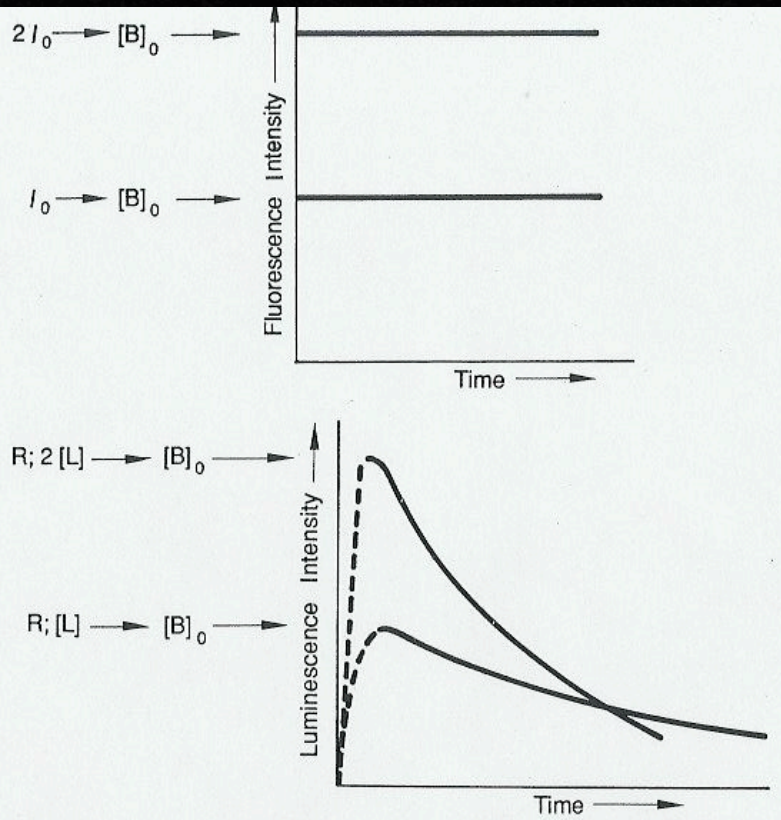



**Figure 2-1.** Schematic presentation of fluorescence analysis as compared with luminescence analysis of a compound B. In fluorescence analysis, a doubled intensity of the exciting light doubles the intensity of the ensuing fluorescence, which remains steady with a constant exciting beam. In fluorometry, the intensity is a measure of the *concentration*.
In luminescence analysis, B is consumed, and it is shown that doubling the luciferase concentration results in a twofold increase in initial maximal output and also in an increased fading rate, implying a crossing of the curves. There is usually a time lag before the maximal intensity is reached, which is indicated by the dotted initial parts of the curves. The intensity is a measure of the *rate* of the luminescence reaction, cf. equation (2-2).

# Salient features of Luminometry

- Luminometry: 10,000 times more sensitive than absorption spectroscopy, 1000 times more sensitive than fluorometry.
- Can detect as little as 0.6 picograms of ATP or .1 femtograms of luciferase.

# Bioluminescence

- Chemiluminescence - Studying light emitted by a chemical reaction
- Bioluminescence - type of chemiluminescence in which the chemical reaction is catalyzed by enzymes

# Advantages

- light output proportional to the amount of material present.
- Extraordinary sensitivity, wide range and Inexpensive instrumentation

# Applications

- Microbial Contaminations
- Biomass Estimation
- Clinical Research

# Microbial Estimation

- Luminometric assay of ATP measures all the micro-organisms present in a sample.

| | |
|---|---|
| Application | #Drinking water Contamination #Microbiological control of pharmaceuticals, cosmetics,milk, and other food products |

# Biomass Estimation

- Total live Biomass estimation in soil and sediments
- Effect of pollutants in marine micro-organisms

# Clinical Research

- Assessing the effect of antibiotics on microbial growth
- Effect of various drugs on mamallian cells

# Principles of Analytical Applications

**Table 2-1.** Principal schemes in bioluminescence analysis. – Determinable substances are: B Inducer of Bioluminescence, C Energy Carrier, D Energy Donor, E Coupling Enzyme, L Luciferase.

| Reaction type | Substance analyzed (example) | | |
|---|---|---|---|
| | B | | L |
| (1) $(R +) B \xrightarrow{L}$ light | ATP | | Firefly luciferase |
| | $FMNH_2$, NADH | | Bacterial luciferase |
| Analysis of light inducer B (R symbolizes necessary reactants) | | | |
| | C | D | E |
| (2) $C + D \xrightarrow{E} B$ | ADP | PEP | Pyruvate kinase |
| | $NADP^+$ | G-6-P | G-6-P dehydrogenase |
| Analysis of nucleotides, substrates, and enzymes employing a reaction producing B (and analysis of B) | | | |

$$-d[B]/dt = k_2 [B]$$ ⟶ A

Light intensity is proportional to the rate

$$I = -b\ d[B]/dt,$$ ⟶ B

b is some constant, combining A and B, we get

$$I = b\ k_2\ [B].$$

Integrating eqn A

$$[B] = [B]_0 \exp\{-k_2 t\}.$$

$$I = b\ k_2\ [B]_0 \exp\{-k_2 t\}.$$

$$I_t = constant \cdot [B]_0.$$

# Reaction types

| Type | | Example |
|---|---|---|
| Without cycling: | | |
| $C + D \rightarrow B$ | (R 4-1) | ADP + PEP → ATP |
| $(R +) B \rightarrow$ light | (R 4-2) | (R +) ATP → light (no ADP recovery) |
| With cycling: | | |
| $C + D \rightarrow B$ | (R 4-1) | $NADP^+$ + (G-6-P) → NADPH |
| $(R +) B \rightarrow$ light + C | (R 4-2′) | (R +) NADPH → light + $NADP^+$ (recycled) |

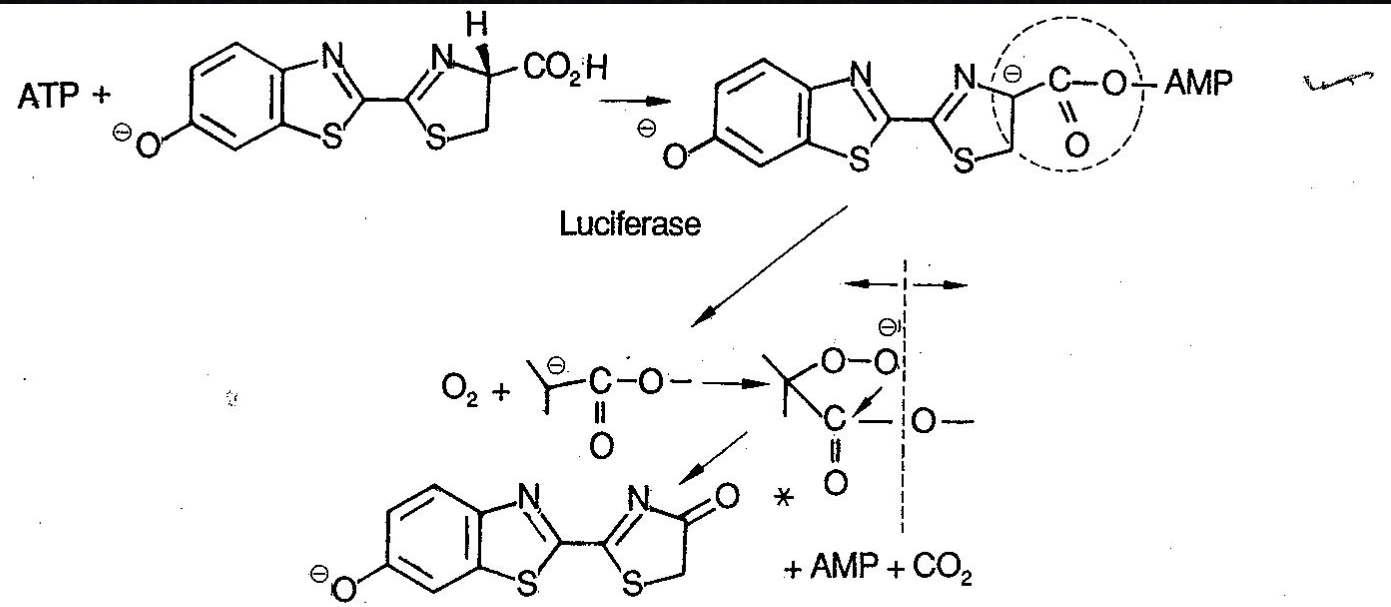



**Figure 1-9.** Some details in the mechanism of firefly luminescence. Luciferin is the molecule responsible for light formation.

# Reaction Scheme

1. A rapid reversible reaction: Esterification of luciferin producing luciferyl adenylate and liberating inorganic pyrophosphate

$$LH_2\text{-}COOH + ATP + E \rightleftharpoons E\text{-}LH_2\text{-}COO\text{-}AMP + PP_i \quad \text{(R 5-2)}$$

2. A rate determining step [1], with a 25 ms time lag before light emission is initiated, and 300 ms before the peak intensity is reached, detachment of a proton and formation of a carbanion, which reacts with molecular oxygen to form excited oxyluciferin, which emits a photon.

$$E\text{-}LH_2\text{-}COO\text{-}AMP + O_2 \rightarrow E\text{-}oxyL\text{-}AMP + CO_2 + light \quad \text{(R 5-3)}$$

3. The slow dissociation of the enzyme product complex

$$E\text{-}oxyl\text{-}AMP \rightarrow E\text{-}oxyL + AMP \quad \text{(R 5-4)}$$

$$D\text{-}Luciferin + O_2 + ATP \xrightarrow[Mg^{2+}]{\text{Luciferase (L)}} Oxyluciferin + PP_i + AMP + CO_2 + light$$

# Extraction Methodology

- Extract ATP using Trichloroacetic acid
- TCA releases ATP from cells and inactivates ATP-degrading enzymes.
- TCA also inhibits luciferase reaction, it is important to determine the minimum amount of TCA necessary.

# Extraction of ATP

- ADP and adenylate kinase presence results in a background emission, because of the production of ATP.
- ADP is removed by the treatment with aphyrase

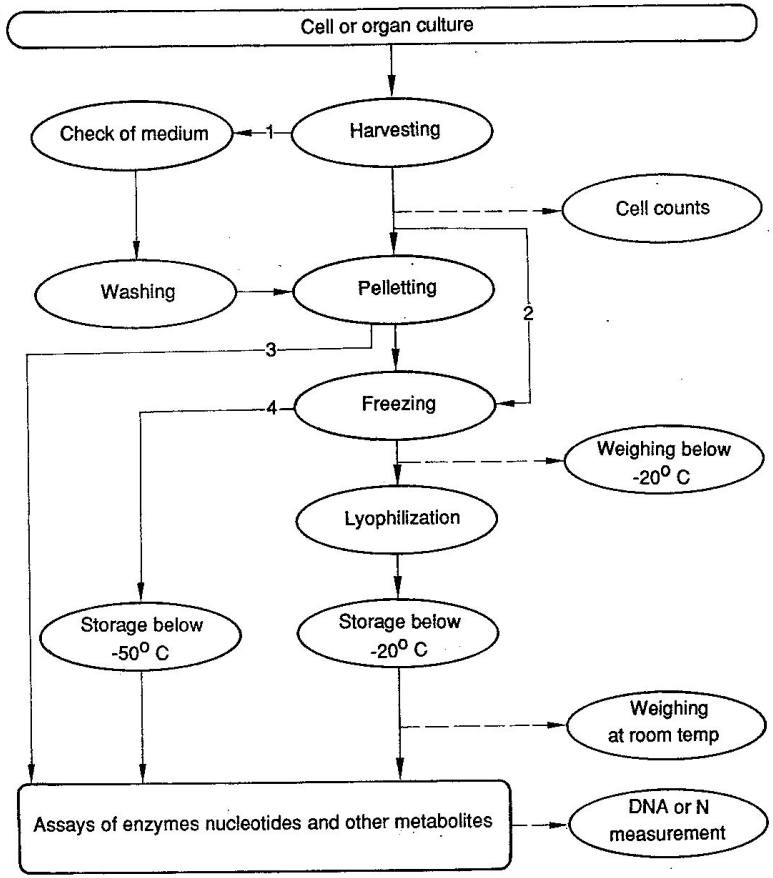



**Figure 6-5.** Scheme for planning the preparation of cells or small organs kept in culture. In the center, the main route is indicated by coarse arrows and surrounded by alternative routes (1–4). To the right, suggestions are given for the quantifications.

Harvesting is often a simple procedure, but disturbances caused by leakage from the cells into the medium may call for processing and checking according to route 1. Normally, the material is enriched by centrifugation, but the material may sometimes be collected without specific measures (route 2).

Freezing and lyophilization disrupt the cellular structure and make the substances to be analyzed readily accessible. Routes 3 and 4 point to simplifications. Following homogenization, the determination of enzyme activity can usually be accomplished. For analyses of nucleotides and other metabolites, route 3 is not advisable without checking the extraction yield and excluding major losses through degradation or conversion of the compound to be determined.

Extraction procedures and final processing for analysis are shown in Figures 6-7, 6-9 and 6-10.

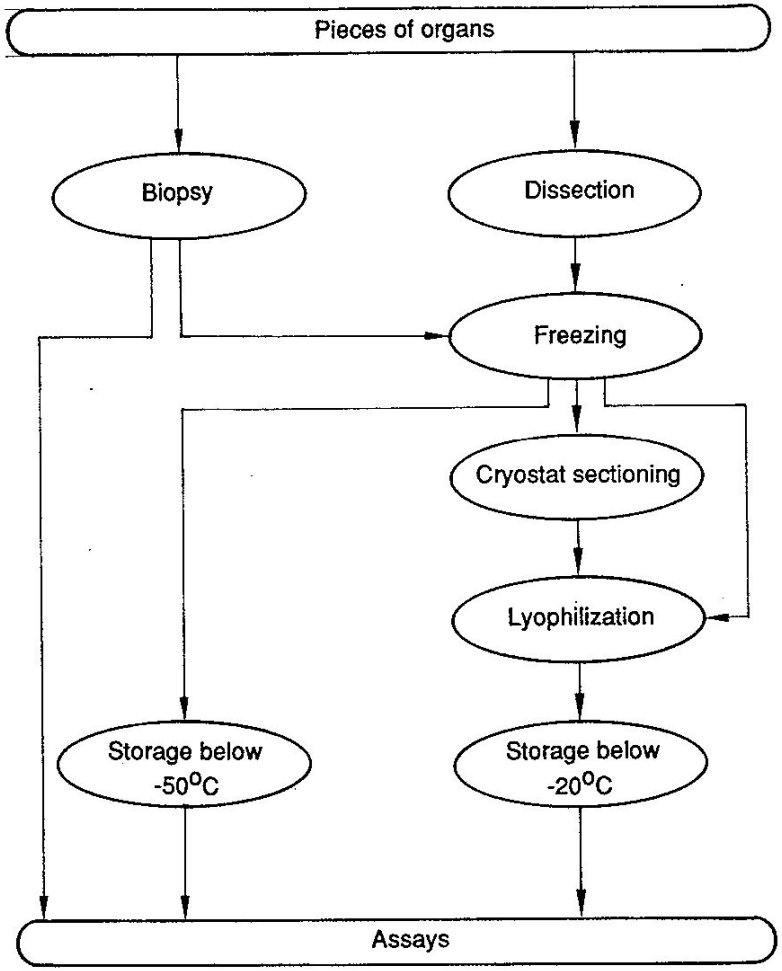



**Figure 6-6.** Planning the preparation of organ specimens. Samples may be obtained from human biopsies or from experimental animals. Testing with animals can decide whether instantaneous freezing is needed for a satisfactory metabolic arrest. Except for cell counts which are irrelevant in this context, the requirements for quantification are indicated in Figure 6-5.
It is sometimes possible to do without the cryostat technique as indicated on the right of the scheme. The simplifications shown on the left are well practicable in enzyme assay, but applications for metabolites require testing for essentially maintained concentrations, preferably by comparison with samples processed according to the main preparative route.

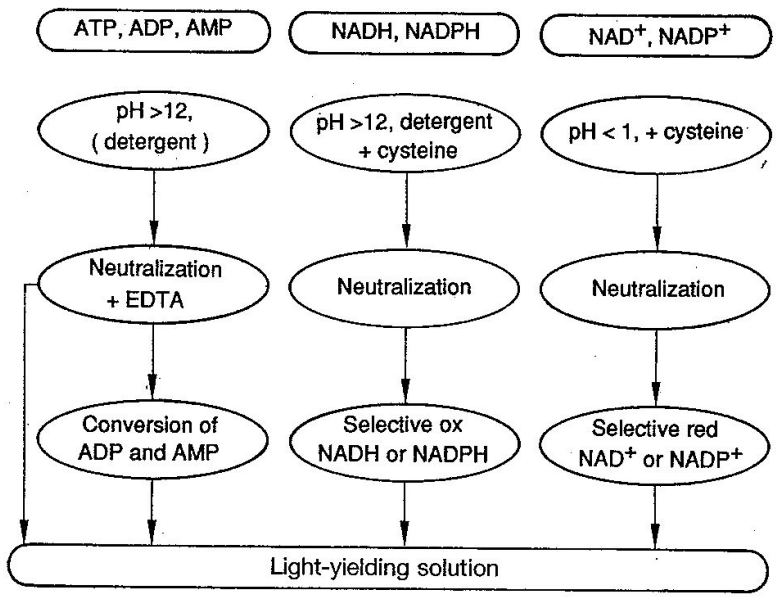
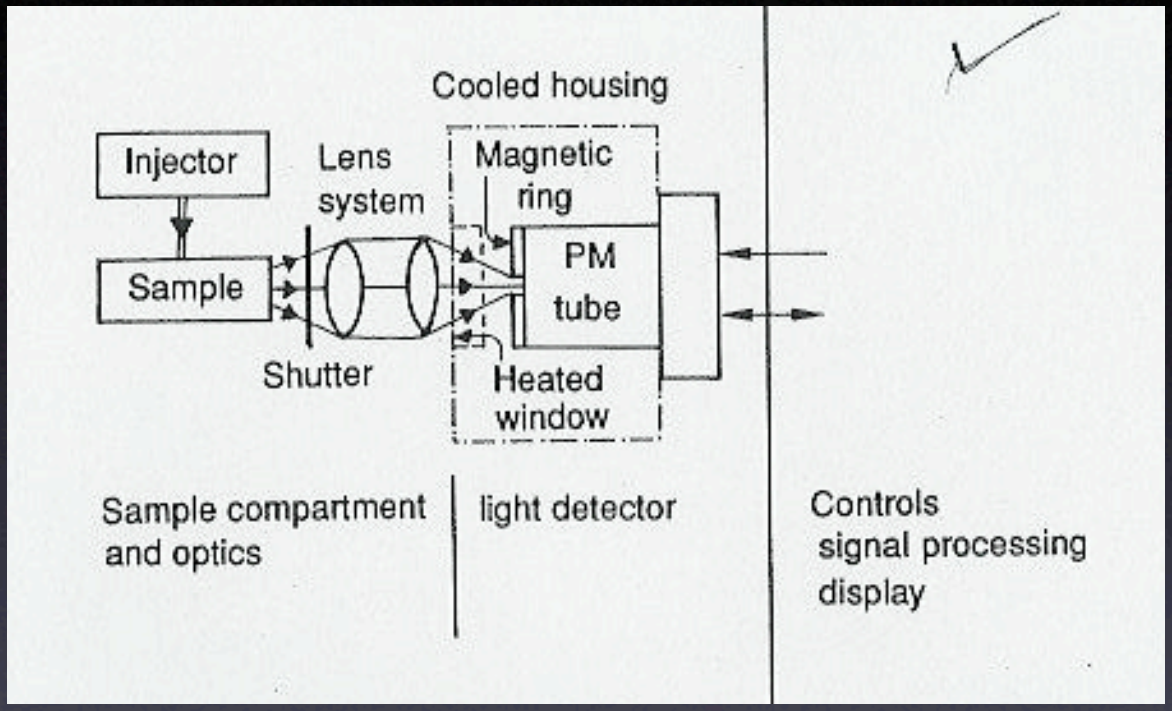



**Figure 6-7.** Completion of the sample processing for analyses of nucleotides.

**Figure 6-7.** Completion of the sample processing for analyses of nucleotides.
Extraction at either high or low pH values prevents disturbing conversions in enzyme reaction
After preceding lyophilization (cf. Figures 6-5 and 6-6), the yield of nucleotides is general satisfactory but may be too low when this measure is omitted. In this case a detergent may be adde to enhance the extraction. This applies likewise to cells with resistant membranes.
Chelating $Mg^{2+}$ with EDTA prevents undesired interconversions in the adenylate pool. Cystein protects against undue oxidation, but not at low pH. Neutralization involves suitable dilution order to minimize interference by disturbing sample components (see Figure 6-3).
The alternatives of either determining ATP alone or the adenylate sum are indicated on th left.
The possibilities of destroying either the oxidized or the reduced forms of pyridine nucleotides a discussed in Figure 6-8. The four forms can be determined after selective oxidation (ox) reduction (red), as shown at the bottom of the diagram. Using enzymatic cycles, continuou reduction in the light-producing solution provides an other alternative to specific determinatio (cf. Figure 7-1). Except for the final nucleotide conversions, the processing is carried out o ice.

# Luminometry Set-up




The sample compartment is equipped for rapid mixing and joined to the optics for focusing the emitted light on the photocathode, which is protected from undue light exposure by a shutter. The light detector has a photomultiplier tube, which is connected to a pulse height discriminator. The sensitivity is enhanced by decreasing the electronic noise, which can be achieved by cooling the tube and shielding off the photocatode, except for a small central area. Fogging of the optical surfaces should be avoided when cooling. Connections for high voltage supply and controls are indicated.

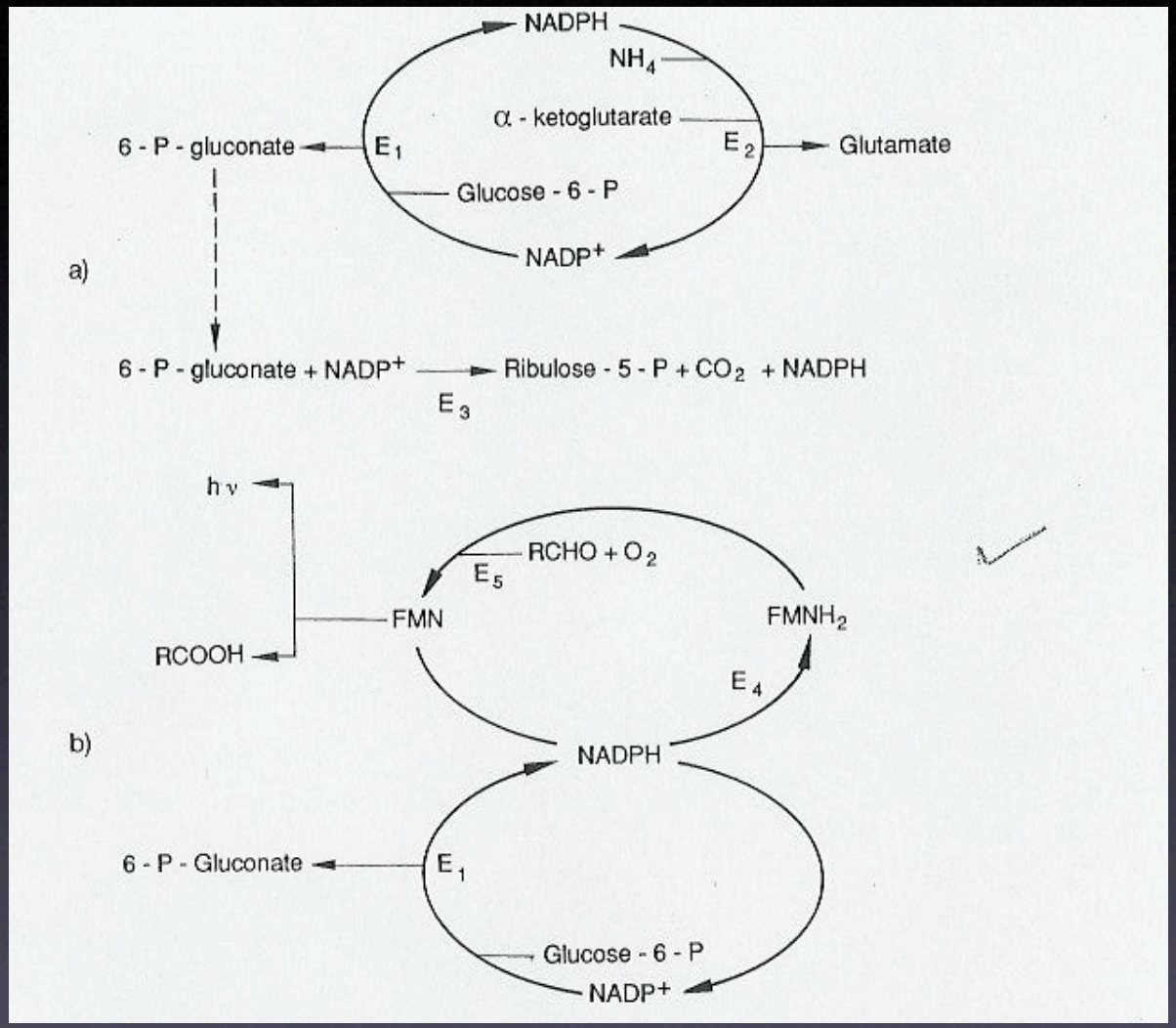
# How it works

- ATP is the limiting reagent
- light reaching the photomultiplier tube is proportional to the amount of ATP in the sample.

# Features of ATP reaction

- ATP dependent oxidation of luciferin by luciferase produces light
- When ATP is the limiting factor of the oxidation, the amount of light produced is proportional to the ATP concentration of the sample.

# Studying Coupling reactions



a) $NADP^+$ is reduced in the glucose-6-phosphate dehydrogenase ($E_1$) reaction and reconverted to $NADP^+$ in the glutamate dehydrogenase ($E_2$) reaction. Either the oxidized or the reduced nucleotide can start the cycling and be analyzed. Amplification is obtained through product accumulation by excess substrates and letting the reaction proceed through many cycles. After stopping the cycling a final separate reaction is arranged for determining the accumulated 6-P-gluconate. Hence, 6-P-gluconate dehydrogenase ($E_3$) and $NADP^+$ are added, as shown below the cycle.

b) The reduction in the cycle occurs as above, but the oxidation is directly linked to the bacterial luciferase system. $E_4$ = oxidoreductase and $E_5$ = luciferase. In this system product accumulation (of 6-P-gluconate) can be replaced by photon storage (using some electronic memory device). Measures for stopping the cycling or additional reactions are not needed, as the assay is finished by setting the time for photon collection.

$$C + D \xrightarrow{E} B \qquad \text{rate constant } k_1$$

$$(R +)\ B \xrightarrow{L} \text{light} + C \quad \text{rate constant } k_2$$

$$I = [C]_0\, b\, k_1\, k_2 (k_1 + k_2)^{-1} [1 - \exp\{-(k_1 + k_2)t\}].$$

$$I_\infty = [C]_0\, b\, k_1\, k_2/(k_1 + k_2).$$

when $k_2 >> k_1$, $I = [C]_0\, b\, k_1 [1 - \exp\{-k_2 t\}]$

$$I_\infty = [C]_0\, b\, k_1,$$

# Other Applications

Substrate Analysis

Enzyme activity

luciferase reaction [2, 4]. Compounds which can

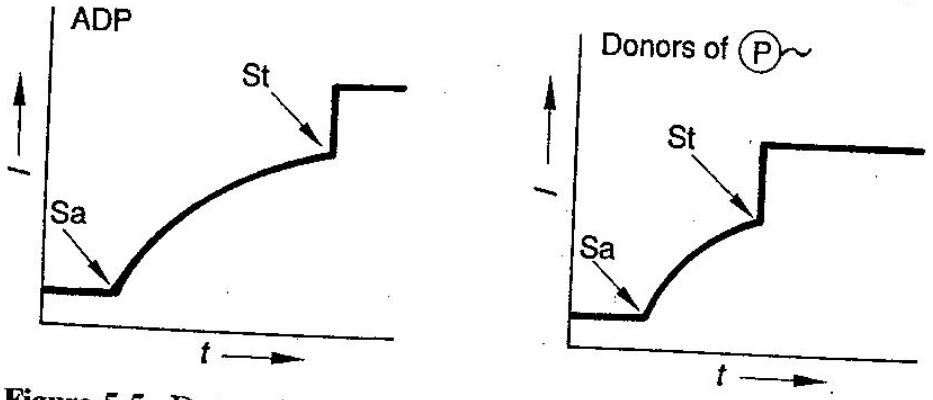



**Figure 5-5.** Determination of ADP (left) and phosphate donors (right) using a reaction directly coupled to a slowly reacting firefly luciferase system. Addition of sample (Sa) results in relaxation at higher intensity. When this level is sufficiently closely approached, an ATP standard (St) is added. The two intensity increments are compared to estimate the amount of ATP formed. ADP and PEP (phosphoenolpyruvate) may, for instance, be analyzed conveniently using the pyruvate kinase reaction.

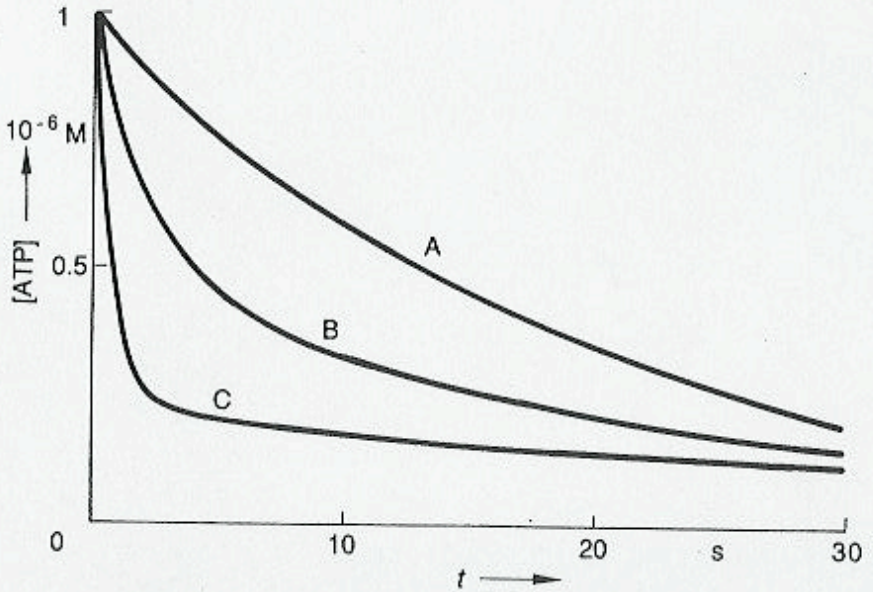



**Figure 5-3.** Calculated effects of the presence of adenylate kinase (AK) and adenosinemonophosphate (AMP) when analyzing $1.0 \times 10^{-6}$ M ATP using the firefly luciferase reaction (R 5-1). It is assumed that:

$$ATP + AMP \rightleftharpoons 2\ ADP \qquad \text{(R 5-5)}$$

occurs parallel to the luciferase reaction, which is assumed to proceed with $k_1 = 0.05\ s^{-1}$. Three cases are illustrated:

A: No AK present

B: $10^{-6}$ M AMP present at the start and sufficient AK present to let the forward reaction (R 5-5) proceed with a rate constant of $0.2 \times 10^6\ s^{-1}\ M^{-1}$, yielding $0.088 \times 10^6\ s^{-1}\ M^{-1}$ for the reverse reaction, using the listed value of 2.26 [52] for the equilibrium constant of reaction (R 5-5).

C: Same conditions as B, expect for an AMP concentration of $5 \times 10^{-6}$ M at the start of the reaction.