Adenosine-5′-triphosphate and Creatine Phosphate

Determination with Luciferase

Bernard L. Strehler

Bioluminescence was first used as an indicator of metabolic activity by *E. N. Harvey*[1]. He used luminous bacteria (*Achromobacter fischeri*) to detect the photosynthetic oxygen production of *Elodea* leaves by immersing the leaves in a thick suspension of the bacteria. *Harvey* and his collaborators also used the inhibition or stimulation of luminescence to study the effect of narcotics, respiratory inhibitors and pressure on enzymes and enzyme systems[2,3]. The first application of bioluminescent reactions *in vitro* to determine important metabolic intermediates was described by *Strehler* and *Totter*[4] in 1952. They used extracts of the luminous organ of the firefly, *Photinus pyralis*, to assay adenosine triphosphate (ATP) and a number of metabolically related substrates and enzymes. The method was based upon the earlier discovery by *McElroy*[5] that aqueous extracts of firefly lanterns which were no longer luminous emitted light once again on the addition of ATP. *McElroy* and *Strehler*[6] then showed that, in addition to the enzyme, ATP and oxygen, two other components were necessary for the emission of light: a divalent cation (*e.g.* Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}, Zn^{++}) and a fluorescent compound called luciferin. During the intervening years, *McElroy et al.*[7–9] have contributed much to the understanding of the mechanism of firefly bioluminescence. *Strehler* and *Totter*[10] and *Strehler* and *McElroy*[11] have published reviews of this method of analysis and its applications. The determination of ATP with luciferase, because of its specificity and sensitivity, has been used for studies on photosynthesis[12], neural function[13], radiation effects[14], oxidative phosphorylation[4] and muscle biochemistry[15,16]. Luciferase preparations are available commercially. The quantitative determination of ATP and creatine phosphate is described here. Assay methods for related substrates (*e.g.* mixtures of ATP, ADP, AMP and creatine phosphate) and enzymes (myokinase, hexokinase, apyrase, creatine kinase) can be found in the original papers of *Strehler* and *Totter*[4,10].

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1) *E. N. Harvey*, Plant Physiol. *3*, 85 [1928].

2) *E. N. Harvey*, Biol. Bull. Wood's Hole *29*, 308 [1915].

3) *F. H. Johnson, H. Eyring* and *R. W. Williams*, J. cellular comp. Physiol. *20*, 247 [1942].

4) *B. L. Strehler* and *J. R. Totter*, Arch. Biochem. Biophysics *40*, 28 [1952].

5) *W. D. McElroy*, Proc. nat. Acad. Sci. USA *33*, 342 [1947].

6) *W. D. McElroy* and *B. L. Strehler*, Arch. Biochem. Biophysics *22*, 420 [1949].

7) *W. D. McElroy* and *J. Coulombre*, J. cellular comp. Physiol. *39*, 475 [1951].

8) *J. W. Hastings* and *W. D. McElroy* in *F. H. Johnson:* The Luminescence of Biological Systems. Amer. Assoc. Adv. Sci., Washington, D. C. 1955, p. 257.

9) *W. D. McElroy* and *A. Green* in *O. H. Gaebler:* Enzymes, Units of Biological Structure and Function. Academic Press, New York 1956, p. 369.

10) *B. L. Strehler* and *J. R. Totter* in *D. Glick:* Methods of Biochemical Analysis. Interscience Publishers, New York 1954, Vol. I, p. 341.

11) *B. L. Strehler* and *W. D. McElroy* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 871.

12) *B. L. Strehler* in *W. D. McElroy* and *H. B. Glass:* Phosphorus Symposium. The Johns Hopkins Press Baltimore, Md. 1952, Vol. II, p. 491.

13) *R. Grenell* in *H. E. Himwich:* Tranquilizing Drugs. Amer. Assoc. Adv. Sci., Washington, D. C. 1957, Publ. No. 46, p. 61.

14) *D. Billen, B. L. Strehler, G. E. Stapleton* and *E. Brigham*, Arch. Biochem. Biophysics *43*, 1 [1953].

15) *F. D. Carlson* and *A. Siger*, J. gen. Physiol. *43*, 301 [1959].

16) *L. B. Nanninga* and *W. F. H. M. Mommaerts*, Proc. nat. Acad. Sci. USA *46*, 1155 [1960].

Principle

Luciferase in the presence of luciferin, oxygen, magnesium and adenosine triphosphate (ATP) catalyses the formation of adenyl-luciferin from ATP and luciferin. Adenyl-luciferin is oxidized by atmospheric oxygen to adenyl-oxyluciferin and this process is accompanied by emission of light.

(1) $$\text{ATP} + \text{luciferin} \underset{\text{enzyme}}{\overset{Mg^{++}}{\rightleftharpoons}} \text{adenyl-luciferin} + \text{pyrophosphate}$$

(2) $$\text{Adenyl-luciferin} \xrightarrow{O_2} \text{adenyl-oxyluciferin} + H_2O + \text{light}$$

Reaction (1) is reversible and the equilibrium lies far to the right. Reaction (2) is practically irreversible, and its product, adenyl-oxyluciferin, is a potent inhibitor of the luminescent reaction.

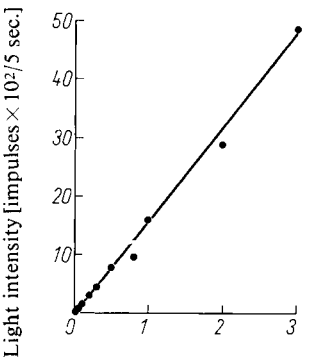

Fig. 1. The relationship between light intensity and ATP concentration. Light production measured with a quantum counter.

Abscissa:
ATP concentration [μg.~P/5 ml.].
1 μg.~P is equivalent to 8.15 μg. ATP.

The *Michaelis* constant for ATP is 5×10^{-5} M at pH 7.4. At ATP concentrations which are a little below the *Michaelis* half saturation value, the rate of the over-all reaction is proportional to the ATP concentration. In the presence of ATP the reaction reaches its maximum rate almost immediately and then decreases essentially as a first order process with time as

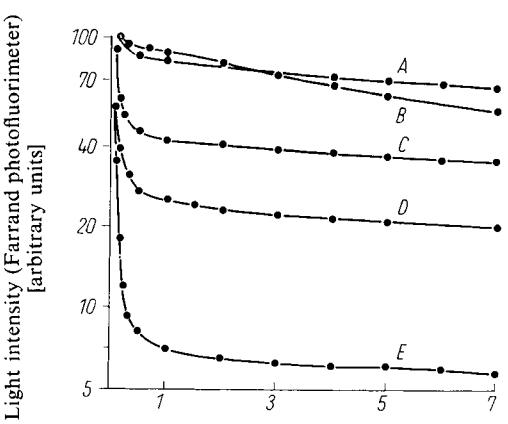

Fig. 2. Time course of the luminescence in the presence of various buffers (pH 7.4).
Curve A: succinate + 0.02 M phosphate
Curve B: succinate + 0.0125 M arsenate
Curve C: succinate + 0.01 M phosphate
Curve D: succinate + 0.005 M arsenate
Curve E: succinate buffer

Abscissa:
Time after mixing luciferase and ATP solutions [min.]

ATP is consumed or as inhibitors accumulate. The determination of ATP therefore involves the measurement of the relative intensity of the light emitted by a luciferase solution within a few seconds after the addition of an ATP solution (in the presence of adequate amounts of Mg^{++}, oxygen and luciferin).

The proportionality between the light intensity and the ATP concentration is shown in Figure 1. The time course of luminescence in the presence of various buffers is shown in Figure 2.

Since the intensity of the emitted light is proportional to the ATP concentration under defined conditions, with the method described here it is also possible to determine any substance which affects the ATP concentration. By addition of the appropriate coupling enzymes or enzyme systems the following substances can be determined: ADP, AMP, creatine phosphate and glucose. In the same way the activity of the following enzymes or enzyme systems can be assayed: hexokinase, myokinase, various ATPases or apyrases and ATP synthesizing systems such as phosphorylating mitochondria[4] or phosphorylating chloroplasts[17].

Apparatus

The sensitivity of the method depends on the sensitivity of the instrument used for the measurement of the light. These light measuring devices consist of the following essential parts: a lightproof housing for the photomultiplier; a lightproof sample chamber; a shutter between the two; a photomultiplier; a stablized high voltage supply and an instrument for measuring the amount of current flowing through the photomultiplier.

For routine assays of ATP concentrations of the order of 1 μg./ml. the Farrand fluorimeter*) or a modified Aminco photometer**) are suitable. The use of a quantum counter, which counts individual photoelectrons, increases the sensitivity by a factor of 100 to 1000. The essential component of this counter is a photomultiplier which is immersed in a Dewar flask containing liquid nitrogen (see Fig. 3).

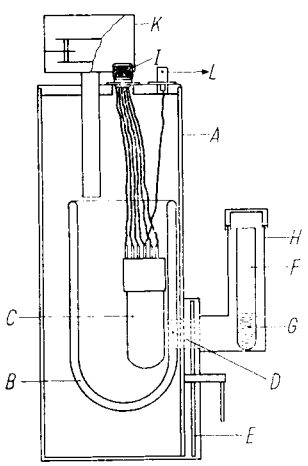

Fig. 3. Quantum counter (for details, see Text).
A: Lightproof housing for the photomultiplier
B: Dewar flask containing liquid nitrogen
C: Photomultiplier
D: Unsilvered window in the Dewar flask
E: Shutter
F: Test tube
G: Sample
H: Lightproof housing for the sample
I: High voltage source
K: Lightproof filling device for liquid nitrogen
L: To the counter

The light reaches the photosensitive surface of the photomultiplier through an unsilvered window in the Dewar flask. Operation at the temperature of liquid nitrogen has the advantage of a much lower rate of emission of thermal electrons from the photocathode and dynodes than occurs at room temperature. Thus the background counting rate is reduced. The single photo-electron pulses, after amplification, consist of about a million electrons which arrive practically simultaneously at the anode. These pulses last for less than a micro-second. Each impulse is then amplified by a linear

*) Manufacturer: Farrand Optical Co., Inc., New York 70, N. Y., USA.
**) Manufacturer: American Instrument Co., Silver Spring, USA. Md., USA.

17) *B. L. Strehler* and *D. D. Hendley* in *W. D. McElroy* and *H. B. Glass:* Symposium on Light and Life. The Johns Hopkins Press, Baltimore, Md. 1961, p. 601.

amplifier, for example, an AlA preamplifier-Al amplifier combination. Such amplifiers, which were primarily developed for scintillation counting, are available commercially from the Radiation Counter Laboratories, Chicago, USA. They can be used for the counting of individual light quanta without major changes.

After amplification the impulse passes through a discriminator which discards all pulses which have too low an amplitude and therefore did not originate from the photocathode of the photomultiplier. The pulses passed by the discriminator are given a uniform size and are directed to the output terminal of the Al amplifier. They are then counted with a scaler which gives counts per unit time, or an integrator may be used which gives a DC voltage proportional to the counting rate. This voltage may then be employed to drive a recorder.

A recorder is particularly useful when the time course of very low levels of luminescence have to be measured (*e.g.* ATP formation as a result of the action of physical or chemical factors on biological material, *e.g.* illumination of luciferase-chloroplast mixtures). A quantum counting photometer with a simple recorder costs about $ 2500. Of particular importance in the design of such an instrument

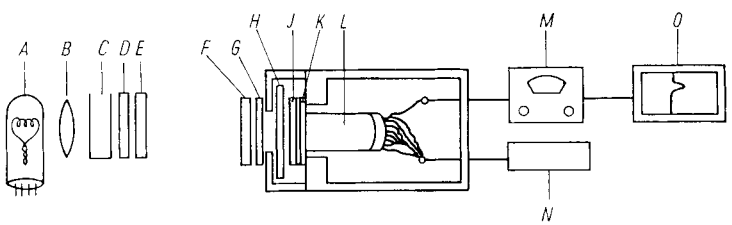

Fig. 4. Apparatus for the continual measurement of the ATP concentration in illuminated chloroplasts

A: Tungsten filament lamp
B: Condensing lens
C: Water (heat filter)
D: Wratten filter No. 25
E: Wratten filter No. 26
F: Wratten filter No. 29
G: Corning filter No. 2408
H: Sample cuvette (light path: 2 mm.)
J: Corning filter No. 9782
K: Wratten filter No. 65
L: Photomultiplier
M: Aminco photometer
N: High voltage source (Baird Atomic Model 312)
O: Recorder

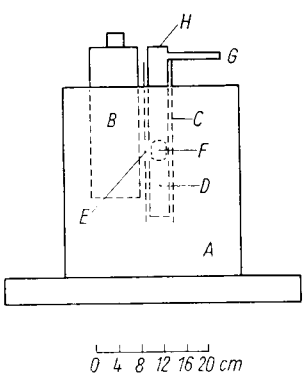

Fig. 5. A simple housing for photomultiplier and sample

A: Lightproof wooden housing
B: Photomultiplier (inserted into a hole in the top of the housing in such a way that light is excluded)
C: Tube mounted rigidly in the top of the housing
D: Rotatable, but lightproof tube inserted in tube C (container for the test tube with sample)
E: Opening in tube C
F: Opening in tube D
G: Handle for rotatable tube D
H: Lightproof cap for tube D

The tubes C and D with their openings E and F form the equivalent of a shutter, which is opened and closed by rotation of tube D.

is the complete protection of the photomultiplier from external light, either during the measurements or during the introduction of the sample. If light falls on the photomultiplier during the changing of the sample it may yield some low level phosphorescence which can give false results or may even damage the photomultiplier. With a properly constructed housing the background counting rate with a cooled 1P21 or 1P22 photomultiplier is three or four impulses/min. The use of the more expensive

1P21 photomultiplier has no appreciable advantage over the much less expensive 1P22, since both tubes have practically no dark current at the temperature of liquid nitrogen. For further details of the construction and applications of a quantum counter, see[18,19].

A relatively simple apparatus[17] for the continuous measurement of the ATP concentration in chloroplasts is illustrated in Figure 4. An easily constructed adaptation of the Aminco photometer suitable for the routine assay of microgram quantities of ATP is shown in Figure 5.

A) Determination of Adenosine-5′-triphosphate

Reagents

1. Adenosine triphosphate, ATP
   crystalline sodium salt, ATP-$Na_2H_2 \cdot 3\ H_2O$; commercial preparation, see p. 1006.
2. Luciferase
   In most cases it is not necessary to use a highly purified preparation. For methods of preparing the enzyme, see "Appendix", p. 572.

For other reagents, see the sections "Experimental material" (p. 564), "Enzymatic reaction" (p. 564, 565) and "Appendix" (p. 570).

Preparation of Solutions

Use distilled water free from heavy metal ions.

I. ATP standard solution

a) Stock solution (1.6×10^{-4} M):
Dissolve 25 mg. ATP-$Na_2H_2 \cdot 3\ H_2O$ in 250 ml. distilled water. Store the solution frozen at $-20°$C.

b) Dilute solution (1.6×10^{-6} M):
Dilute 5 ml. solution a) to 500 ml. with distilled water. Store the solution between 0 and 4° C and prepare freshly each day. For the determination of purity, see "Appendix", p. 570.

II. Luciferase

Solutions, see "Appendix", p. 572.

For other solutions, see the sections "Enzymatic reaction" (p. 565) and "Appendix" (p. 572).

Stability of the solutions

The stability of dilute ATP solutions is variable, even at $-20°$C. Therefore prepare the standard ATP solution freshly each day. The luciferase preparations prepared according to p. 572 keep for a year at $-20°$C.

Procedure

Experimental material

Because of its inhibitory and denaturing action on enzymes trichloroacetic acid is not suitable for the extraction of ATP. In many cases, especially with highly dispersed suspensions (tissue cultures, blood, bacteria, algae, mitochondria, *etc.*) it is sufficient to heat the samples

[18] *B. L. Strehler*, Arch. Biochem. Biophysics *34*, 239 [1951].
[19] *W. E. Arthur* and *B. L. Strehler*, Arch. Biochem. Biophysics *70*, 507 [1957].

at 100° C for between 5 and 15 min. (boiling water bath). However, it is important that the samples are brought as quickly as possible to 100° C so that enzymatic degradation of substrates in the sample during the heat inactivation of the proteins is avoided. It is preferable to inject the sample into two or three volumes of boiling water. Immediately after heating, transfer the sample to an ice bath or deep-freeze and store there until the assay.

Before using this method for the extraction of ATP it should be compared with other procedures to see if similar results are obtained. In studies on the effect of light on photosynthetic phosphorylation in green algae, heating the sample at 100° C for 10–15 min. was found to be sufficient to extract all the ATP. For larger, and especially for solid samples this simple extraction by heating is obviously not sufficient.

Tissues which contain high concentrations of enzymes capable of reacting with ATP require special treatment. *Carlson*[20] has developed a procedure for the analysis of ATP in muscle: freeze the tissue sample in dry ice-petroleum ether, grind in a cold mortar with frozen 8% perchloric acid (1 ml. perchloric acid/100 mg. muscle). Thaw the mixture and then allow to stand for 10 to 15 min. at 0° C. Filter, neutralize the filtrate with 1 N NaOH or KOH and dilute to 25 ml. with distilled water. The solution can be stored at −20° C.

In certain cases, for example, in the assay of ATP in chloroplasts, extraction is not necessary. Usually the samples need not be deproteinized. The luciferase preparation is contaminated with appreciable quantities of myokinase, pyrophosphatase and apyrase[8,9]. If the sample contains light-scattering or absorbing particles (chloroplasts, mitochondria, suspended lipids), a known amount of ATP should be added to the reaction mixture after the determination of the ATP contained in the sample. The experimental results are then related to this internal standard.

Enzymatic reaction

Preliminary remarks: Four methods for the determination of ATP are described below. They are based on two principles: 1. The sample containing ATP is rapidly injected into the luciferase solution. The maximum light intensity observed is a measure of the ATP concentration. However, the maximum intensity depends on the rate at which the sample and enzyme are mixed and also on the presence of ADP. 2. The rapid decay of luminescence is retarded by use of a high concentration of arsenate (or phosphate) and magnesium in the assay mixture[4]. The ATP concentration is obtained by extrapolation of the light intensity to zero time. Also with this method, the sample and enzyme must be mixed rapidly and in a reproducible manner.

Method:

a) If a Farrand fluorimeter is available, proceed as follows:

dilute

0.2 ml. luciferase solution (preparation a, p. 572)

to 0.6 ml. with distilled water. Immediately before the measurements add

0.2 ml. of sample,

mix rapidly and measure the light intensity at 5, 10, 15, 20, 25 and 30 sec. after mixing in the sample.

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[20] *F. Carlson*, personal communication.

b) If a quantum counter is available, proceed as follows:

dilute the

luciferase solution (preparation b, p. 572)

five-fold with arsenate-magnesium buffer*).

Add

$^1/_5$ volume of sample

and proceed as described under a).

c) According to *McElroy*[21]

mix

0.1 ml. luciferase solution (preparation c, p. 572)

with

2.1 ml. glycylglycine-magnesium buffer**).

Place the solution in the instrument in front of the photomultiplier. With a 0.25 ml. syringe rapidly inject

0.2 ml. sample

and proceed as described under a).

d) For continuous measurement of the ATP concentration in chloroplast preparations[17] proceed as follows:

Prepare the luciferase solution according to procedure a) (p. 572), but use phosphate buffer without adding $MgSO_4$.

Mix the following solutions:

0.2 ml. luciferase solution
1.4 ml. suspension medium+)
0.1 ml. PMS solution++)
0.1 ml. AMP solution©).

Add to this mixture in subdued light

0.2 ml. chloroplast suspension (about 700 μg. chloroplasts/ml. suspension).

Pour the mixture into a cuvette (2 mm. light path) and place in the apparatus shown in Fig. 4. The result of a typical experiment is illustrated in Fig. 6.

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*) Arsenate-magnesium buffer (0.1 M arsenate; 0.05 M Mg^{2+}; pH 7.4): Dissolve 42.5 g. $Na_3AsO_4 \cdot 12\,H_2O$ and 10 g. $MgCl_2 \cdot 6\,H_2O$ in 500 ml. distilled water, adjust to pH 7.4 with 1 N HCl and dilute to 1000 ml. with distilled water.

**) Glycylglycine-magnesium buffer (0.025 M glycylglycine; 0.1 M Mg^{2+}; pH 7.5):
Dissolve 1.65 g. glycylglycine and 12.3 g. $MgSO_4 \cdot 7\,H_2O$ in 400 ml. distilled water, adjust to pH 7.5 with 1 N NaOH and dilute to 500 ml. with distilled water.

+) Suspension medium:
Dissolve 0.139 g. K_2HPO_4, 0.495 g. NaCl, and 0.341 g. $MgCl_2 \cdot 6\,H_2O$ in distilled water, add 95 ml. 0.1 M tris buffer (pH 8.0) and make up to 500 ml. with distilled water.
Tris buffer (0.1 M; pH 8.0):
Dissolve 6.05 g. tris-hydroxymethyl-aminomethane in 400 ml. distilled water, adjust to pH 8.0 with 30 ml. 1.0 N HCl and dilute to 500 ml. with distilled water.

++) Phenazine methosulphate, PMS (5×10^{-4} M):
Dissolve 0.145 g. PMS in 1000 ml. distilled water.

©) Adenosine monophosphate, AMP (2×10^{-2} M):
Dissolve 1.03 g. AMP-Na_2 in 125 ml. distilled water.

[21] *W. D. McElroy*, unpublished.

Calculations

Since the luminescence obtained with luciferase is directly proportional to the ATP concentration (in the physiological concentration range), the light intensity produced by the sample is compared to that produced by a standard. It is advisable to construct a standard curve to check whether the relationship between the luminescence and the ATP concentration is linear.

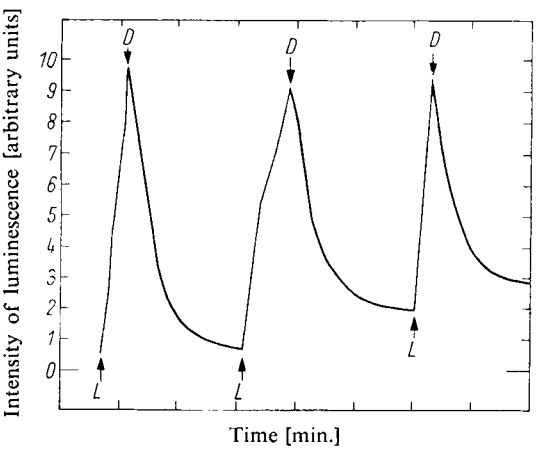

Fig. 6. Time course of the ATP concentration in chloroplasts illuminated at intervals (measured with the apparatus shown in Fig. 4). The figure shows the fall in ATP concentration in the dark due to the action of myokinase contained in both the chloroplasts and the luciferase solution. It also illustrates the gradual rise in the base line luminescence due to the accumulation of ATP and ADP.
L = Start of illumination
D = End of illumination

Example

ATP synthesis in *Chlorella pyrenoidosa*[22]. The reaction mixture prepared according to method a) contained 2 mg. algae (wet weight). The following values were measured:

Duration of illumination [sec.]	Luminescence [counts/15 sec.]
0	1052
10	1362 (Δ_{20} = 1410 counts/15 sec.)
20	2462
30	2311
60	2091

$$\Delta_1 = \frac{\Delta_{20}}{\text{duration of illumination}} = \frac{1410}{20} = 70 \text{ counts/15 sec./sec. illumination}$$

0.152 μg. ~ P*) gave Δ_1 = 5000 counts/15 sec. Therefore: $\frac{70 \times 0.152}{5000} = 0.002$ μg. ~ P*)/sec. illumination was synthesized in the reaction mixture. This contained 2 mg. (= 2000 μg.) algae (wet weight). Therefore the algae synthesized 10^{-6} times**) their weight of ATP.

Other Determinations

Adenosine diphosphate (ADP): First determine the ATP content of the sample as described above. Then convert the ATP to ADP with ATPase prepared from crayfish muscle according to *Lohmann*[23]. Boil the reaction mixture for 5 to 10 min., and determine the total ADP (ATP + ADP) as described for ATP. The luciferase preparation contains myokinase which converts ADP to ATP. Obtain the ADP content by comparison with a standard curve. The time course of the luminescence with ADP is illustrated in Fig. 7.

*) 1 μg. ~ P is equivalent to 8.15 μg. ATP

**) $\frac{0.002}{2000} = \frac{2 \times 10^{-3}}{2 \times 10^{3}} = 10^{-6}$

22) *B. L. Strehler*, Arch. Biochem. Biophysics *43*, 67 [1953].
23) *K. Lohmann*, Biochem. Z. *282*, 109 [1935].

Specificity

The reaction is specific for ATP, although substances which can alter the concentration of ATP available to the luciferase affect the luminescence. ADP in the absence of myokinase, creatine phosphate in the absence of creatine kinase, adenosine tetraphosphate, inosine triphosphate, cytidine triphosphate, uridine triphosphate, acetyl phosphate and other phosphorylated intermediates do not react.

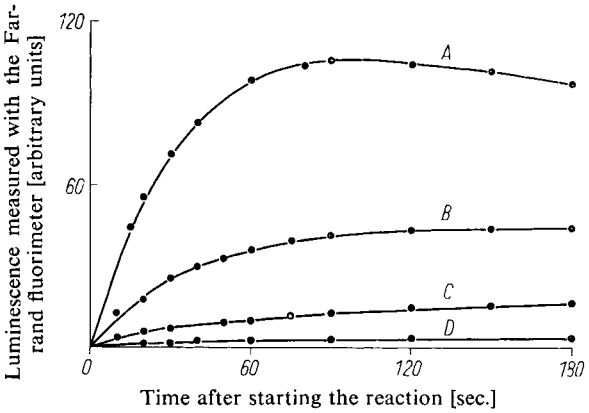

Fig. 7. The time course of the luminescence in the ADP assay. The reaction mixture (0.8 ml.) contained: 0.2 ml. luciferase solution (preparation b), p. 572; 0.2 ml. arsenate buffer (0.1 M; pH 7.4; 0.05 M $MgSO_4$) and ADP: curve A: 0.650 μmoles; curve B: 0.260 μmoles; curve C: 0.130 μmoles; curve D: 0.065 μmoles.

Sources of Error

Possible sources of error are:

1. Precipitation of buffer, enzyme or activator.
2. Depletion of oxygen due to respiratory activity of the sample or the luciferase.
3. Quenching of luminescence by monovalent anions, turbidity, absorption or natural inhibitors (see Fig. 8).
4. Utilization of ATP by side reactions or binding of ATP at inactive sites[9] (high concentrations of arsenate buffer decrease this error).
5. Luminescent or phosphorescent impurities[24].
6. Presence of ammonium ions in photosynthetic phosphorylation test systems.
7. Hydrolytic enzymes in the sample. This source of error can be obviated if the samples are deproteinized with perchloric acid.
8. Injection of the sample into the reaction mixture without proper protection from light; damage to the photomultiplier by over-exposure to light; faulty performance of the instrument, leading to non-linear response.

Nearly all such sources of error can be eliminated or corrected by use of internal standards (see p. 35) or standard curves.

Other Methods of Determination

Other methods for the estimation of ATP can be found on pages 539, 543 and 551. The ion exchange method of *Cohn* and *Carter*[25] which is described in the "Appendix" (p. 570) is also useful. However,

[24] *B. L. Strehler* and *W. A. Arnold*, J. gen. Physiol. *34*, 809 [1951].
[25] *W. E. Cohn* and *C. E. Carter*, J. Amer. chem. Soc. *72*, 4273 [1950].

the most sensitive appears to be the determination of ATP with luciferase, especially if a quantum counter is available. It has the advantages of being rapid and simple and it permits the continual measurement of ATP concentration in certain systems.

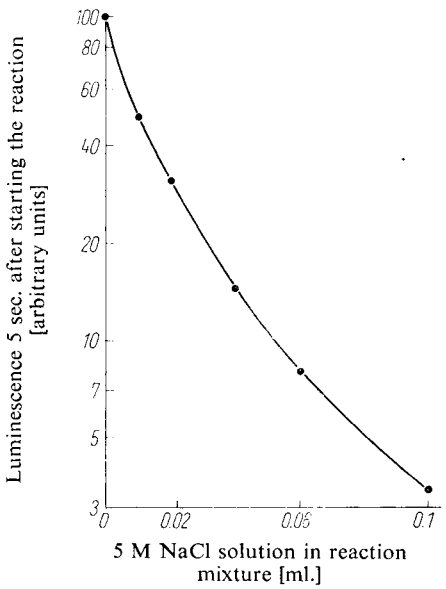

Fig. 8. Effect of chloride ions on the intensity of the luminescence. The reaction mixture (0.8 ml.) contained 0.2 ml. luciferase (preparation b, p. 572); 0.2 ml. ATP solution containing 250 μg. ~ P/ml. *); 0.2 ml. 0.1 M arsenate buffer (pH 7.4); 2 mg. $MgSO_4$ and the amounts of 5 M NaCl solution stated on the abscissa.

B) Determination of Creatine Phosphate

Reagents

See under A (p. 563). In addition:

3. Creatine phosphate
   sodium salt; commercial preparation, see p. 1008.
4. Adenosine monophosphate, AMP
   sodium salt, AMP-Na_2; commercial preparation, see p. 1005.
5. Sodium arsenate, $Na_3AsO_4 \cdot 12\ H_2O$
6. Creatine kinase
   lyophilized powder from muscle[26]; commercial preparation, see p. 973.

Preparation of Solutions

See under A (p. 563). In addition:

III. Creatine phosphate standard solution (10^{-4} M):
   Dissolve 1 mg. creatine phosphate (Na salt) in 5 ml. distilled water. Prepare freshly each day. For the determination of the content of the solution, see "Appendix", p. 571 and 610.

IV. Adenosine monophosphate (2×10^{-2} M AMP):
   Dissolve 40 mg. AMP-Na_2 in 5 ml. distilled water.

*) 1 μg. ~ P corresponds to 16 mμmoles ATP.

26) *K. Lohmann*, Biochem. Z. *271*, 264 [1934].

V. Sodium arsenate (0.1 M; pH 7.4):
Dissolve 42.5 g. $Na_3AsO_4 \cdot 12\,H_2O$ in 500 ml. distilled water, adjust to pH 7.4 with 1 N HCl and dilute to 1000 ml. with distilled water.

VI. Creatine kinase, CPK:
Dissolve the CPK preparation in sufficient distilled water so that the intensity of the luminescence is readily measurable during the 10–30 sec. reaction time. Prepare the solution freshly each day.

Procedure

Enzymatic reaction

The following should be prepared: experimental tube, control tube without creatine kinase and AMP (especially if the sample contains much ATP and ADP*), and a standard tube containing creatine phosphate standard solution (III) instead of the sample. A standard curve should also be prepared (see Fig. 10).

Pipette into a test tube:

0.20 ml. luciferase solution (preparation a, p. 572)
0.20 ml. sodium arsenate solution (V)
0.12 ml. AMP solution (IV)
0.01 ml. creatine kinase (VI)
0.02 to 0.2 ml. sample
distilled water to 1.0 ml.

Mix thoroughly and read the luminescence every 10 sec. The time course of the luminescence is shown in Fig. 9.

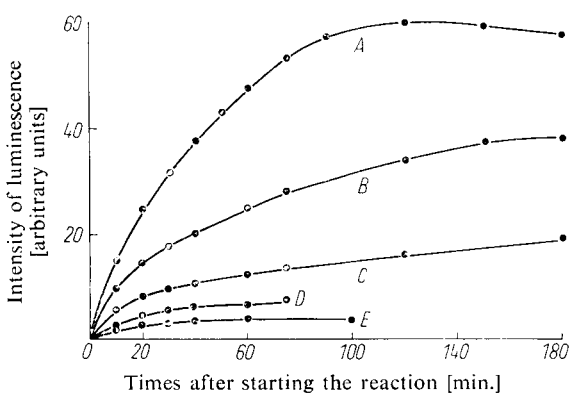

Fig. 9. Time course of the luminescence in the creatine phosphate assay. The reaction mixture (1 ml.) contained: 0.01 ml. of a crude creatine kinase solution; 0.2 ml. 0.1 M Na arsenate solution (pH 7.4); 10 mg. $MgSO_4$; 1 mg. AMP; 0.2 ml. luciferase solution and the following amounts of creatine phosphate:
Curve A: 0.664 μmoles;
Curve B: 0.332 μmoles;
Curve C: 0.199 μmoles;
Curve D: 0.132 μmoles;
Curve E: 0.0664 μmoles.

Calculations

The creatine phosphate content of the sample is read off from a standard curve (see Fig. 10).

Sources of Error

See p. 567.

*) Alternatively, the ATP and ADP contained in the sample can be converted to AMP with ATPase (from crayfish [23]) and myokinase, and then the ATPase is inactivated by boiling.

Other Methods of Determination

Creatine phosphate can also be determined by the method described in the "Appendix" (p. 571.) Another method can be found on p. 610.

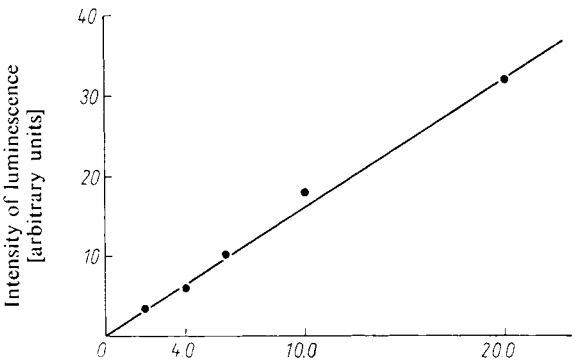

Fig. 10. Relationship between luminescence (30 sec. after mixing) and creatine phosphate content. For constituents of the assay mixture see Legend to Fig. 9.

Appendix

Assay of the Purity of the ATP Standard Solutions [25]

Reagents and Solutions

1. Dowex 1 (NH_4^+ form), 200–400 mesh
2. Hydrochloric acid, 3×10^{-3} N; 10^{-2} N; 1 N
3. Ammonium chloride (0.01 M):
   Dissolve 0.053 g. NH_4Cl in 100 ml. distilled water.
4. Sodium chloride-HCl (0.02 M NaCl; 10^{-2} N HCl):
   Dissolve 0.1169 g. NaCl in 100 ml. 10^{-2} N HCl.
5. Sodium chloride-HCl (0.2 M NaCl; 10^{-2} N HCl):
   Dissolve 1.169 g. NaCl in 100 ml. 10^{-2} N HCl.

Procedure

Suspend Dowex 1 in distilled water, prepare a 1.5 cm. high column (*ca.* 1 cm. diameter) and convert the resin to the chloride form with *ca.* 50 ml. 1 N HCl. Wash the column with water until the washings are neutral. Adjust the pH of the ATP solution to 8 to 9 and pour the solution on the column *). Wash the column with *ca.* 50 ml. distilled water and then elute successively with 100 ml. of each of the following solutions: 0.01 M NH_4Cl (elutes adenosine and adenine); 3×10^{-3} N HCl (elutes AMP and inorganic phosphate); 0.02 M NaCl in 10^{-2} N HCl (elutes ADP); 0.2 M NaCl in 10^{-2} N HCl (elutes ATP) **).

Determine the adenine nucleotide content of the eluate by measurement of the optical density at 260 mμ (pH 2). Under these conditions the molar extinction coefficient of adenosine and its phosphorylated derivatives is about 14.2×10^6 cm^2./mole.

*) To ensure that all the nucleotides are adsorbed on the resin the anion concentration of the solution should not exceed 0.01 N.

**) According to *Cohn* (personal communication) the adenosine phosphates can also be separated by elution with 0.1 M Na_2SO_4 in 10^{-2} N H_2SO_4, with 1.5 M ammonium formate or with 0.2 M ammonium formate in 4 M formic acid. These reagents are approximately as effective as 0.1 M NaCl in 10^{-2} N HCl in eluting ATP from the column.

Assay of the Purity of the Creatine Phosphate Solutions [27]

Reagents and Solutions

1. Ammonium molybdate:
   Dissolve 25 g. $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ in 500 ml. 10 N H_2SO_4 and dilute to 1000 ml. with distilled water.
2. Ferrous sulphate:
   Dissolve 5 g. $FeSO_4 \cdot 7\ H_2O$ in 50 ml. distilled water containing 1 ml. 1 N H_2SO_4. Prepare the solution freshly each day.
3. Hydrochloric acid, 0.4 N.
4. Sodium hydroxide, 0.4 N
5. Sodium chloride, 0.4 M
6. α-Naphthol, recrystallized, 1% (w/v).
7. Diacetyl (butane-2,3-dione).

Procedure

a) Fiske Subbarow phosphate determination:

Pipette into 10 ml. test tubes:

Tube A	*Tube B*
2.0 ml. creatine phosphate standard solution (III)	2.0 ml. distilled water
1.0 ml. 0.4 N HCl	2.0 ml. 0.4 M NaCl solution

Heat tube A in a water bath at 65° C for 10 min., cool and add
1.0 ml. 0.4 N NaOH.

Pipette into both tubes
1.0 ml. molybdate solution (1)
0.1 ml. $FeSO_4$ solution (2).

Mix, allow to stand for 5 min. and read the optical density at 600 mμ or a suitable adjacent wavelength (A against B). Obtain the phosphate content of tube A from a standard curve.

b) Determination of creatine:

Pipette into 10 ml. test tubes (graduated to 10 ml.):

	Tube C	*Tube D*	*Tube E*
creatine standard solution (III)	2.0 ml.	2.0 ml.	—
distilled water	1.0 ml.	1.0 ml.	3.0 ml.
0.4 M NaCl solution	—	2.0 ml.	2.0 ml.

Warm tube C to 65° C in a water bath, add
1.0 ml. 0.4 N HCl

and keep at 65° C for 9 min. Then add
1.0 ml. 0.4 N NaOH

and quickly cool to room temperature in an ice bath.

To all three tubes in the dark, add
2.0 ml. 1% α-naphthol solution[+)]
1.0 ml. diacetyl (butane-2,3-dione)
distilled water to 10 ml.,

[+)] If an unknown sample is analysed instead of the creatine phosphate standard solution and it contains proteins or other SH groups, add 1 ml. 0.5 M Na-p-chloromercuribenzoate solution.

[27] *A. H. Ennor* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 850.

and keep in the dark for 20 min. at 20° C. Measure the optical density at 520 mμ (C and D against E). With the difference $E_C^{520} - E_D^{520}$ obtain the creatine content from a standard curve. If the standard curve has been prepared with a pure solution of creatine, then the amount of creatine corresponding to $E_C^{520} - E_D^{520}$ must be multiplied by 1.122, because the creatine phosphate forms some creatinine on hydrolysis. Naturally, this correction is not necessary if the standard curve is prepared with creatine phosphate.

Preparation of Luciferase

Method a

This simple method was described by *Strehler* and *Totter*[4], who used preparations obtained in this way to determine ATP with the Farrand fluorimeter.

Grind 50 mg. of vacuum-dried firefly lanterns*) (10 or 12 lanterns) with 5 ml. ice-cold 0.1 M Na arsenate buffer**) or Na phosphate buffer+) (pH 7.4) for 5 to 10 min. Filter the mixture into a test tube standing in an ice bath. Dissolve 50 mg. $MgSO_4 \cdot 7\ H_2O$ in the filtrate. The preparation keeps for several days at 4° C.

Method b

A purer preparation can be obtained according to[6,10]. This method involves fractional precipitation with ammonium sulphate, and the final preparation contains relatively little apyrase. However, it is not suitable for the continuous measurement of the ATP concentration in chloroplasts, since NH_4^+ ions inhibit the photosynthetic phosphorylation[28].

Grind thoroughly 4 g. vacuum-dried firefly lanterns with sand and extract with two 50 ml. portions of distilled water. Adjust the extract to pH 6 with 1 N HCl, centrifuge and discard the precipitate. To the supernatant add 10 g. $(NH_4)_2SO_4$, allow to stand for 15 min. at 0° C, centrifuge and discard the precipitate. Adjust the pH of the supernatant to 7.5 with 1 N NaOH, again add 10 g. $(NH_4)_2SO_4$, allow to stand for 15 min. at 0° C, centrifuge and discard the precipitate. Adjust the pH of the supernatant to 4.5 with 1 N HCl, add 30 g. $(NH_4)_2SO_4$, allow to stand for 10 min. at 0° C, filter and discard the filtrate. Dissolve the precipitate in 50 ml. distilled water, store the clear amber-yellow coloured solution in small portions at −20° C (keeps for several years) or lyophilize and store the residue in a deep-freeze.

Method c

McElroy[21] recommends the following modification.

Grind thoroughly 5 g. dried firefly lanterns in a mortar and extract with 25 ml. cold distilled water. Adjust the pH of the suspension to *ca.* 7.7 with 1 N NaOH, centrifuge for 10 min. in the cold at 3000 g and save the supernatant. Extract the precipitate once again with 25 ml. cold distilled water and centrifuge as already described. Combine the supernatants and use this crude enzyme preparation for the determinations. Store frozen! On thawing, centrifuge off any precipitate which appears and adjust the pH of the supernatant to *ca.* 7.5.

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*) Obtainable from Sigma Chemical Co. St. Louis, Mo.; Schwarz Bio-Research Inc., Mt. Vernon, New York; Worthington Biochemical Corp., Freehold, New Jersey, USA.

**) Na arsenate buffer (0.1 M; pH 7.4):
Dissolve 42.5 g. $Na_3AsO_4 \cdot 12\ H_2O$ and 10 g. $MgCl_2 \cdot 6\ H_2O$ in 1000 ml. distilled water.

+) Na phosphate buffer (0.1 M; pH 7.4):
Dissolve 14.42 g. $Na_2HPO_4 \cdot 2\ H_2O$ and 2.62 g. $NaH_2PO_4 \cdot H_2O$ in 1000 ml. distilled water.

28) *A. T. Jagendorf* in *C. Fuller* et al.: The Photochemical Apparatus, Its Structure and Function. Brookhaven National Laboratory, Upton, N. Y. 1958, p. 236.