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Fluorometric Detection of Adenosine Triphosphate with 3-Hydroxy-4′-(dimethylamino)flavone in Aqueous Solutions

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An effect of appearance of new band in the excitation spectra of 3-hydroxy-4′-(dimethylamino)flavone (FME probe) in presence of adenosine triphosphate (ATP) is described. Considerable shift of new band up to the red and increase of fluorescence intensity points to the formation of FME-ATP associate, in which FME molecule undergoes to a strong electrostatic stabilization by tetra-charged ATP anion. It is shown the FME anion formation is possible under influence of ATP in the studied conditions. The dynamics of the observed effect is studied in mitochondria. The registered phenomenon allows the quantitative evaluation of ATP concentration in the range of 10^{-3}–10^{-5} M. In contrast to ATP, other nucleoside phosphates do not give a new band in the excitation spectra of FME probe. This implies the possibility of the *in vivo* determination of the ATP concentration.

KEY WORDS: ATP; adenosine triphosphate detection; 3-hydroxyflavone; fluorescence spectra.

INTRODUCTION

It is well known that adenosine-5′-triphosphate (ATP) plays a prominent role in the functioning of living cells. As in many of scientific tasks, it is important to know not only a total ATP concentration, but also the local one, or the dynamics of its change. The determination of total ATP concentration can be estimated by the use of several methods, such as liquid chromatography,^{31}P-NMR spectroscopy, or bioluminescence [1,2]. The determination of local ATP concentration or the dynamic of its change is more complex due to the imposed space and/or time limitations. As far as we know, there is no approach of satisfactory resolutions at present. Nevertheless, for the series of other analytes fluorescent microscopy and fluorescent probe applications were shown to be effective in the measurement of analyte local concentration [3].

There have been several attempts to elaborate fluorescent probe for measurement of ATP concentration in liquids. Lehn, [4] and Czarnik's group [5] obtained important results in this direction. Both groups created fluorescent sensors for multi-charged anion concentration measurements, and tetra-charged ATP anion was one effectively determined by them. Albelda and co workers also developed and studied a series of ATP chemosensors [6]. All groups elaborated the aromatic polycathionic probes that in water solutions can bind a number of anionic species, among them phosphate, pyrophosphate, polycarboxylates, etc. Unfortunately, these sensors have two disadvantages. The first, they did not work selectively with ATP anion, and they have not been efficient for the concentration range of ATP existing in the living cells.

To compile an ATP fluorescence probe construction the unique structure and composition of ATP should be counted. In aqueous solutions at physiological pH values it exists as a tetra-charged anion in which two parts formally can be identified. Phosphate and ribose residues represent a charged hydrophilic portion of ATP, while adenine residue is uncharged, plane and more hydrophobic one. The hydrophilic part stabilizes the molecule in water

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and forms electrostatic field close to it. The hydrophobic part creates necessary prerequisites for ATP association with other hydrophobic molecules, if they exist in the surroundings. This association actually is a precondition for biochemical reactions with ATP participation.

Taking into account the features of the structure and composition of ATP, one can consider that an ideal fluorescent probe *P* for ATP detection should be sensitive to the electrostatic field in the surroundings. In order to associate with ATP, the probe should contain a plane hydrophobic, as well as the positively charged parts.

3-Hydroxyflavones (3HFs, flavonols) are known as fluorescent probes with record sensitivity to the electric fields in liquid media. This was shown for local fields, generated by the surrounding molecules [7–20] or for dipole potential of lipid membrane [21], as well as for general ones, caused by charged poles of electro-optical cell [22–24]. The mentioned results point to good aspects of the utilization of 3-hydroxyflavones in the design of probe for tetra-charged ATP anion determination. Going in this direction in the present work we succeeded in the detection of a bright and unique phenomenon of the probe–ATP complex formation using one of common probes, 3-hydroxy-4′-(dimethylamino)flavone (FME) (Scheme 1).

MATERIALS AND METHODS

3-Hydroxy-4′-(dimethylamino)flavone was prepared according to Ref. [25] and purified after additional crystallizations from ethanol. Its purity was checked by thin-layer chromatography on silicagel-60, F-254 (Selecto Scientific, USA), in chloroform–methanol mixtures from 98:2 to 9:1 v/v. Sucrose (Merck), 4(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES), *tris*-(hydroxymethylamino)-methane (TRIS), and nucleoside phosphates were purchased from Sigma-Aldrich.

Fluorescence spectra were recorded using Hitachi MPF-4 apparatus with a home-made system of computer data acquisition. All experiments were performed at 37°C. Starting solution (1.5 mL) in all cases contained sucrose (0.25 M), HEPES or TRIS (0.1 M, pH 7.4) and FME probe (5×10^{-6} M). If used, the concentrations of other components were 10^{-5} M for $CaCl_2$, 3×10^{-3} M for $MgCl_2$, sodium succinate, SDS, and nucleoside phosphates each.

Titration with ATP was made by using 1.5 mL of starting solution and the same solution containing 5.6 mM or 40 mM of ATP. To obtain a needed ATP concentration it was added to starting solution stepwise by aliquots (2, 4, 6, 10, 20, 50, 100, 200, and 600 μL) with intermediate recording of the spectra.

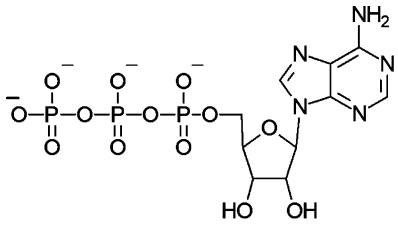
Quantum-chemical simulations of molecular geometry were performed by AM1 semi-empirical method [26] using the MOPAC 6.0 program.

Effective stability constant pK_S value were calculated by the Fletcher–Pawl algorithm using a nonlinear least squares method, as developed in A. Doroshenko's program [27, 28], which minimizes the sum of squared deviations of the experimental and calculated fluorescence data (I) in the approximation of one-step (1:1) complexation process. The program utilizes the common formula [29]:

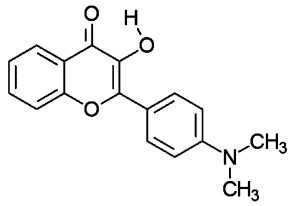
$$I = \frac{I_C[\mathrm{ATP}] + I_{\mathrm{FME}}K_S}{[\mathrm{ATP}] + K_S},$$

where I_C is the fluorescence intensity of the FME–ATP complex, I_{FME} the fluorescence intensity of the free probe, and [ATP] the concentration of ATP.

Uterus mitochondria were isolated from rats, which were estrogenized in 24 h before the experiments. Isolation procedure was performed in ice-cold sucrose buffer (250 mM sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.4) by two sequential centrifugation, first at $1000\times g$, then supernatant was centrifuged at $12{,}000\times g$. Pellets were resuspended at 500 μL of EDTA-sucrose buffer. The isolated mitochondria were stored in ice and used for experiments for up 2 h after the isolation procedure. Experiments with the probe were performed at 37°C. First excitation spectrum of the probe (1 μM) in sucrose buffer with mi-



ATP



FME

Scheme 1.

tochondria (protein concentration −0.2 mg mL^{-1}) was recorded. Then 50 μL aliquot of solution, which contains Ca, Mg, ATP, and succinate, was added and two excitation spectra were recorded after 2 and 5 min, respectively. Final concentrations were 0.01 mM for Ca, 3 mM for Mg, ATP, and succinate each.

RESULTS AND DISCUSSION

In most of organic solvents, as well as in water, in lipid vesicles or in cell membranes the absorption or fluorescence excitation spectrum of FME probe present a band with the maximum around 400–410 nm (see Table I). In highly polar aqueous sucrose solutions the maximum in excitation spectrum moves to 415–420 nm. Similar shift of excitation and absorption maxima is caused by the formation of anion of FME probe in aqueous solutions at high pH values (Table I, Fig. 1). In addition, in basic conditions, the fluorescence maximum of FME probe is localized at longer wavelength, at 555 nm. Analyzing the collected data, one could conclude that anion formation under highly basic conditions causes the most pronounced red shift of the maximum (up to 425 nm) in the absorption and excitation spectra of FME probe.

We found that the presence of ATP in water at pH∼7.4 causes a highly specific effect on FME fluorescence, expressed as an appearance of new maximum in excitation spectrum at 470–480 nm of considerably increased intensity (Fig. 2). This effect is reproduced with small changes in presence of some ions (calcium, magnesium, sodium, and succinate) in their physiological concentrations, or sucrose, as well as at the replacement of HEPES buffer by TRIS or carbonate. The effect is so stable that is reproduced even in the suspension of mitochondria and in the presence of detergent such as sodium

Table I. Band Maxima Positions (nm) in Absorption and Emission Spectra of FME Probe

Conditions	Absorption	Emission
Water, pH 7.2	407 [30]	538
0.25 M sucrose, pH 7.4	421	540
Water, pH 10.5	425 [30]	555 (547 [30])
Methanol	406 [31]	528 [31]
Ethanol	407 [25]	520 [25]
Acetonitril	398 [25]	507, 560 [25]
Benzene	403 [31]	463, 566 [31]
Methylcyclohexane	398 [25]	551 [25]
Lipid vesicles	410 [30]	500–525, 565 [12–14,30]
EYPG vesicles—HEPES	450[a] [30]	508, 565 [30]

[a]In the pH range from 7 to 9 exists as a shoulder.

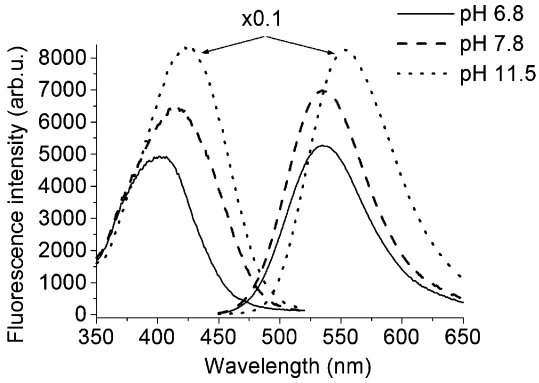


Fig. 1. Fluorescence excitation and emission spectra of FME probe in aqueous solution of HEPES buffer at pH 6.8 (*solid*), 7.8 (*dash*) and 11.5 (*dot*). Emission wavelength: 554 nm for all excitation spectra. Excitation wavelength: 410 nm for all emission spectra.

dodecylsulfate (SDS). Much less expressed, but with the same character is the influence of ATP on the absorption of FME probe (spectra not shown).

Such observations point to the formation of molecular associates between FME and ATP under experimental conditions. The association causes an appearance of new excitation maximum shifted to the red and considerable increase of emission intensity of the FME probe, whereas in the case of lipid vesicles or detergent micelles only the increase of emission intensity was observed [9,12–15]. Both effects, confirming the FME–ATP complex formation point to a special orientation of the FME molecule relative to the charged residue of ATP. In this orientation the FME probe undergoes a strong electrostatic stabilization by tetra-charged anion of ATP that causes considerable red shift of the excitation spectrum. The conception of electrostatic influence finds confirmations in the results of the other research group [30]. In their study, very similar effect was registered with the FME probe in anionic egg yolk phosphatidylglycerol (EYPG) vesicles. Under used conditions, on the increase of pH a new excitation maximum of FME probe appeared at 460 nm, which is close enough to the data observed in the present work. Full-scale study made by steady-state and time-resolved fluorescence spectroscopy allowed authors to assume the anion formation from the FME probe and the stabilization of the anion on the vesicle surface under the electrostatic field of phospholipid-HEPES self-organized structure [30]. The similarity of spectral effects of the FME probe in both the mentioned experiments points to the possibility of anion formation in the case of FME–ATP interaction also. However, this phenomenon needs more detail study.

Having so pronounced effect of FME–ATP complex formation in the fluorescence excitation spectra, no less

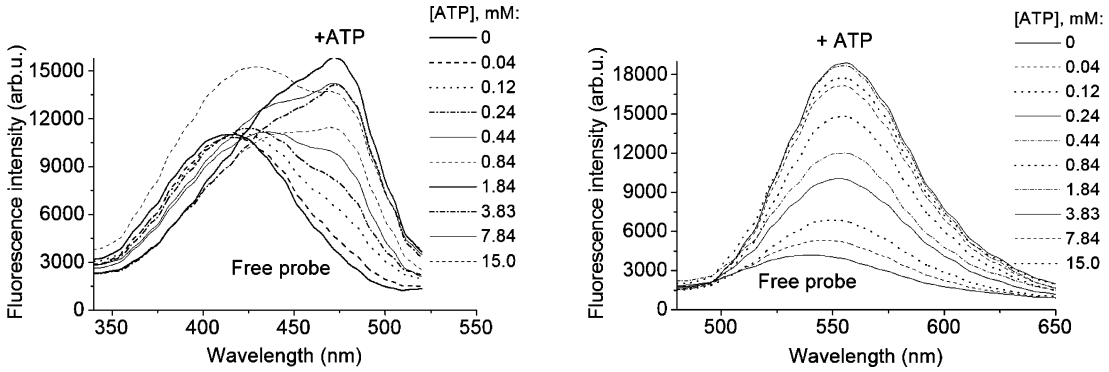


Fig. 2. Fluorescence excitation (*left*) and emission (*right*) spectra of FME probe in aqueous solution of sucrose and HEPES-carbonate buffer at the increase of ATP concentration. Emission wavelength is 554 nm for all excitation spectra. Excitation wavelength: 470 nm for all emission spectra. Concentrations of components are 250 mM (sucrose), 10 mM (HEPES), 40 mM (carbonate), pH 7.4.

effect could be expected in the fluorescence emission. However, interaction with ATP causes only the increase of intensity in the fluorescence spectra of FME probe, while band shape and the position of maximum undergo small changes, coinciding with ones for anion specie (see Table I). Such observations points to the possible reorganization of FME–ATP complex in the excited state. The nature of the forming exciplex is now under investigation.

Important information about FME–ATP complex can be obtained from the analysis of its possible spatial structure. To study the stereochemistry of the complex we made a simulation of its structure in vacuum. As a result, several most stable structures were obtained. One of them is presented in Fig. 3. In all the structures, the aromatic units of FME and ATP lie in the closest proximity, showing that intermolecular stacking interactions can play significant role in the complex formation. Such an orientation allows the positive part of molecular dipole of FME to be localized in close proximity to negatively charged phosphate residues. This gives a strong stabilization of S_1 state of FME molecule, thus leading to the decrease of S_0–S_1 energy gap and to the red shift of absorption and excitation maxima.

Appropriate selectivity of the probe–analyte interaction is one of the most important requirements in the case of analysis of so complex objects as living cells. There are a great number of organic molecules composing the cell, above all proteins, or components of lipid

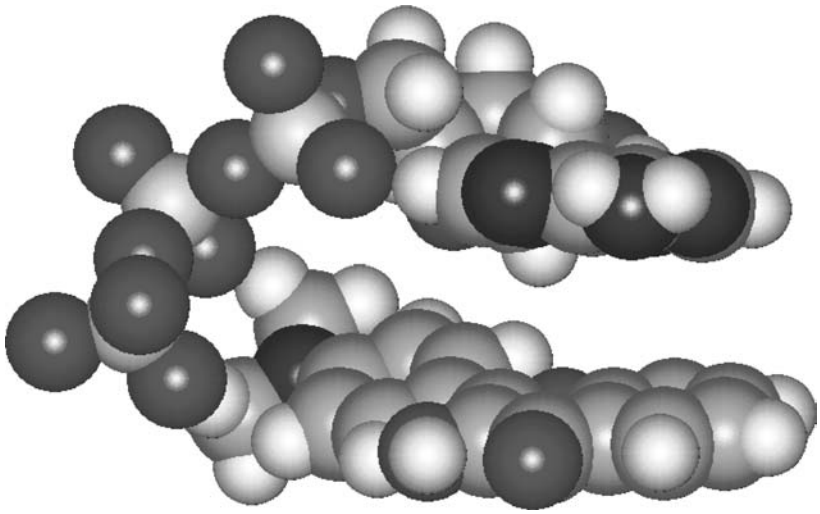


Fig. 3. Possible geometry of the complex between ATP (*top*) and FME (*bottom*) molecules obtained by quantum chemical calculations. Hydrogen atoms are in white, carbon and phosphorus in gray, oxygen and nitrogen atoms are in black.

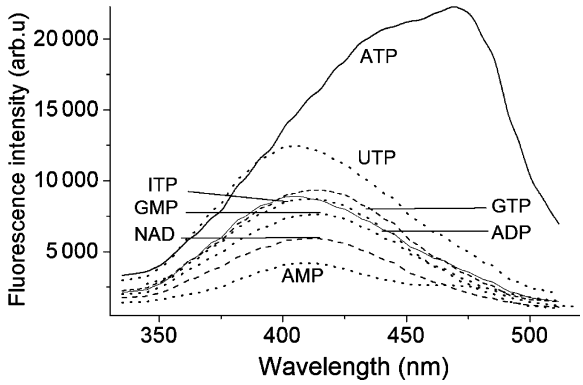


Fig. 4. Fluorescence excitation spectra of FME probe in the presence of nucleoside phosphates. Adenosine-5′-triphosphate (ATP), inosine-5′-triphosphate (ITP), guanosine-5′-triphosphate (GTP), uridine-5′-triphosphate (UTP), adenosine-5′-diphosphate (ADP), adenosine-5′-monophosphate (AMP), β-nicotinamide adenine dinucleotide (NAD), deoxyguanosine-5′-monophosphate (GMP). Emission wavelength: 540 nm, pH = 7.4 in all cases. Concentrations of components: 250 mM (sucrose), 10 mM (HEPES), 40 mM (sodium hydrocarbonate), and 3 mM (all nucleotides).

membranes and nucleoside phosphates, which can also bind the FME probe and influence its fluorescence. To elucidate the applicability of the discovered effect for the ATP determination in living cells, it is important to know a degree of its specificity. To answer this question the emission spectra of FME in the presence of several nucleoside mono-, di-, and triphosphates were obtained (Fig. 4). The results demonstrated that only ATP produces such specific effect in the fluorescence excitation spectra, while other compounds only cause some increase of emission intensity. So high selectivity of FME–ATP interaction is unexpected for us. It points to the key role of the nucleic base structure in the generation of the mentioned effect.

Another important test on selectivity considered the interaction of FME probe with ATP in the presence of lipids, proteins, lipopolysaccharides, and other species composing the mitochondria. Performing this study, we compared fluorescence excitation spectra of FME probe in mitochondria suspended in sucrose-HEPES buffer in absence and in presence of ATP in its optimal concentration.

In aqueous sucrose solution the FME probe has a maximum in the excitation spectrum localized at *ca.* 420 nm (Fig. 2). The presence of mitochondria causes a shift of the maximum up to 405–407 nm testifying to the penetration of the probe molecules into less polar interior of mitochondria membrane (Fig. 5a). However, subsequent addition of ATP to the FME–mitochondria system causes an appearance of new band in the spectrum after 2–5 min of incubation, showing stable effect of complex formation between ATP and FME in the presence of cell organelles. From the last results, we can conclude that in studied conditions FME probe has the highest affinity to ATP anion among all the substances that are present in the solution. We suppose that due to this feature FME molecules abandon the lipid interior of mitochondria and bind ATP.

We have monitored the dynamics of the probe emission changes using the excitation wavelength 480 nm upon the addition of mitochondrial membrane activators, such as Ca^{2+}, Mg^{2+} cations or succinate and ATP anions (Fig. 5b). The obtained kinetic curve shows that Ca^{2+}, Mg^{2+} and succinate in their active concentrations do not influence the emission intensity of FME probe, causing only a small decrease of the fluorescence at the applied excitation wavelength. At that time, the presence of 3 mM of ATP in the bulk solution causes a sharp rise of fluorescence intensity over the next 7 min after addition. Summarizing the results collected from the data in Fig.

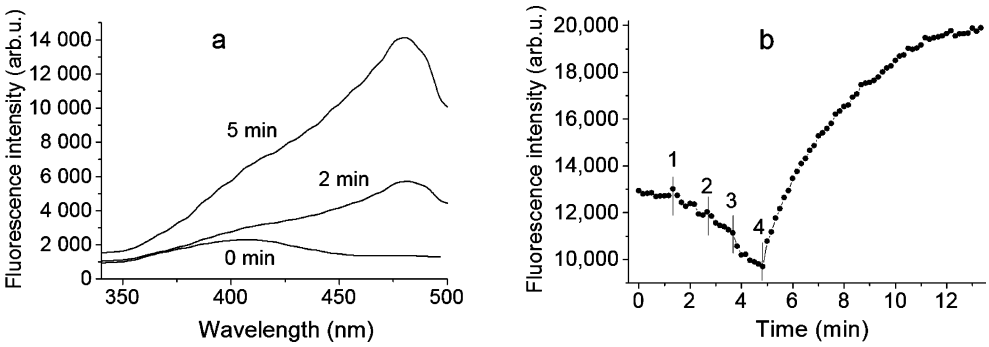


Fig. 5. (a) Changes in fluorescence excitation spectra of FME probe in mitochondria on the incubation with ATP. (b) Dynamics of fluorescence intensity changes at the addition of $MgCl_2$ (1), $CaCl_2$ (2), sodium succinate (3), and ATP (4). Excitation wavelength: 470 nm, emission wavelength: 540 nm. Concentrations of components: 250 mM (sucrose), 100 mM (HEPES), 0.01 mM (Ca^{2+}), 3 mM (Mg^{2+}, succinate and ATP).

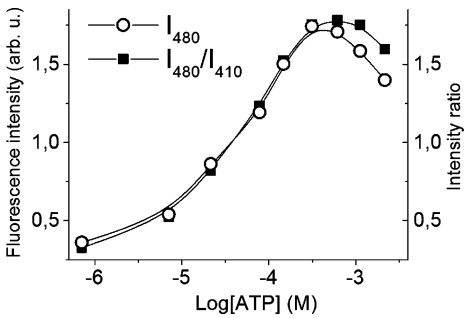


Fig. 6. Fluorescence intensity of FME probe registered at 480 nm excitation wavelength (I_{480}) vs. logarithm (Log) of ATP concentration (*open circles*). Ratio of intensities I_{480}/I_{410} vs. logarithm of ATP concentration (*closed squares*). To superpose the curves, the data of intensity were divided by 12,800. Emission wavelength is 554 nm in all cases.

5a and b we conclude that they confirm the availability of interaction between FME probe and ATP anion in the presence of mitochondria in the solution.

An important feature of the probe is the affinity to analyte, which can be evaluated by the stability constant of the probe–analyte complex. To estimate whether the studied effect can be applied for estimation of ATP concentration in the solution we evaluated an effective stability constant for the FME complex with ATP by the dependence of fluorescence intensity upon logarithm of ATP concentration (Fig. 6). The obtained curve shows that the complex formation takes place in the range of ATP concentration from 10^{-6} to 10^{-3} M. Some fall of emission intensity is observed at higher ATP concentrations, the matter of which now is under investigation. Evaluation of the stability constant from the data in the region of 10^{-6}–10^{-3} M gives a value of K_S equal to 2.5 $\times$ 10^4 L mol^{-1}, and shows the possibility of estimation of ATP concentration in aqueous solutions in the range of 10^{-5}–10^{-3} M. The obtained value points to the possible saturation of FME probe by ATP at its cellular concentrations, usually consisting the range of 10^{-3}–10^{-2} M. In this case, the FME probe allows determining the presence of ATP only. Meantime, it should be taken into attention that high concentrations of organic substances in living cells could diminish the affinity of FME probe to ATP, making possible an evaluation of ATP concentrations in the cellular range. So, the level of FME–ATP affinity in living cells needs further elucidation.

In some cases, the ratio of fluorescence intensities in two separate wavelengths, instead of the absolute intensity, can be more suitable in the analysis. Then the value of the analytical signal will not depend on the cell volume, inhomogeneous redistribution of probe molecules, or on the changes of viscosity of the medium. In the case of the FME probe, it is suitable to compare the fluorescence intensities on excitation at 410 and 480 nm in the excitation spectra. The dependence of intensity ratio I_{480}/I_{410} on ATP concentration is presented in Fig. 6. Using these data, the same value of dissociation constant was obtained as it was done by the fluorescence intensity.

CONCLUSIONS

Thus, our results point to the formation of an associate of specific structure between tetra-charged anion ATP and FME probe in aqueous solutions. The complex formation causes a strong electrostatic stabilization of FME molecule and possibly is accompanied by anion formation. It is reflected in the fluorescence excitation spectrum of FME probe as an appearance of new band shifted to the red by 60 nm with total increase of emission intensity. The observed phenomenon gives an opportunity of the quantitative evaluation of ATP concentration in the range from 10^{-5} to 10^{-3} M. Since the presence of cell organelles, such as mitochondria or of most of nucleoside phosphates does not prevent the evaluation of ATP concentration, the possibilities exist to carry out the measurements *in vivo*. Evidently, at this point, an application of FME probe for ATP determination *in vivo* is an open question, since several theses should be elucidated in this way. And they are under elucidation now.

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REFERENCES

1. P. E. Stanley, and S. G. Williams (1969). Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem.* **29**, 381–391.
2. F. R. Leach (1981). ATP determination with firefly luciferase. *J. Appl. Biochem.* **3**, 473–481.
3. R. P. Haugland (1998). *Handbook of Fluorescent Probes and Research Products*, 6th edn., Molecular Probes, Inc., Eugen.
4. M. R. Hosseini, *et al*. (1988). Multiple Molecular Recognition and Catalysis. Nucleotide binding and ATP hydrolysis by a receptor molecule bearing an anion binding site, an intercalator group and a catalytic site. *J. Chem. Soc., Chem. Commun*. 596–598.
5. M. E. Huston, *et al*. (1989). Chelation enhanced fluorescence detection of non-metal ions. *J. Am. Chem. Soc*. **111**, 8735–8737.
6. M. T. Albelda, *et al*. (1999). Thermodynamics and fluorescence emission studies on potential molecular chemosensors for ATP recognition in aqueous solution. *J. Chem. Soc. Perkin Trans.* **2**, 2545–2549.
7. M. Sarkar, and P. Sengupta (1991). Influence of different micellar environments on the excited-state proton transfer luminescence of 3-hydroxyflavone. *Chem. Phys. Lett*. **179**, 68–72.

8. V. G. Pivovarenko, *et al*. (1997). Flavonols as models for fluorescent membrane probes. 1. The response to the charge of micelles. *Cell. Mol. Biol. Lett.* **2**, 355–364.
9. S. M. Dennison, *et al*. (1999). Intramolecular excited-state proton transfer and charge transfer fluorescence of a 3-hydroxyflavone derivative in micellar media. *Spectrochim. Acta A* **55**, 903–909.
10. A. S. Klymchenko, and A. P. Demchenko (2002). Probing AOT reverse micelles with two-color fluorescence dyes based on 3-hydroxychromone. *Langmuir* **18/15**, 5637–5639.
11. J. Guharay, *et al*. (1997). Excited state proton transfer fluorescence of 3-hydroxyflavone in model membranes. *Spectrochim. Acta A* **53**, 457–462.
12. O. P. Bondar, *et al*. (1998). Flavonols—New fluorescent membrane probes for studying the interdigitation of lipid bilayers. *Biochim. Biophys. Acta* **1369**, 119–130.
13. G. Duportail, *et al*. (2001). Neutral fluorescence probe with strong ratiometric response to surface charge of phospholipid membranes. *FEBS Lett.* **508**, 196–200.
14. G. Duportail, *et al*. (2002). On the coupling between surface charge and hydration in biomembranes. Experiments with 3-hydroxyflavone probes. *J. Fluorescence* **12**, 181–185.
15. A. Klymchenko, *et al*. (2002). Novel two-band ratiometric fluorescence probes with different location and orientation in phospholipid membranes. *Chem. Biol.* **9**, 1199–1208.
16. A. P. Demchenko, *et al*. (2002). In R. Kraayenhof, A. J. W. G. Visser, and H. C. Gerritsen (Eds.), *Fluorescence Spectroscopy, Imaging and Probes—New Tools in Chemical, Physical and Life Sciences*, Springer-Verlag, Heidelberg, 101–110.
17. A. S. Klymchenko and A. P. Demchenko (2002). Electrochromic modulation of excited-state intramolecular proton transfer: The new principle in design of fluorescence sensors. *J. Am. Chem. Soc.* **124**, 12372–12379.
18. A. D. Roshal, *et al*. (1998). Flavonols and crown-flavonols as metal cation chelators. The different nature of Ba^{2+} and Mg^{2+} complexes. *J. Phys. Chem. A* **102**, 5907–5914.
19. A. D. Roshal, *et al*. (1999). Flavonols as metal-ion chelators: complex formation with Mg^{2+} and Ba^{2+} cations in the excited state. *J. Photochem. Photobiol. A Chem.* **127**, 89–100.
20. X. Poteau, *et al*. (2004). The photophysics of some 3-hydroxyflavone derivatives in the presence of protons, alkali metal and alkaline earth cations. *J. Photochem. Photobiol. A Chem.* **162**, 431–439.
21. A. S. Klymchenko, *et al*. (2003). Ultrasensitive two-color fluorescence probes for dipole potential in phospholipid membranes. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11219–11224.
22. N. A. Nemkovich, *et al*. (2001). Site selectivity in excited-state intramolecular proton transfer in flavonols. *J. Photochem. Photobiol. A Chem.* **139**, 53–62.
23. N. A. Nemkovich, *et al*. (2002). Dipole moments of 4′-aminoflavonols determined using electro-optical absorption measurements or molecular Stark-effect spectroscopy. *J. Photochem. Photobiol. A Chem.* **140**, 19–24.
24. N. A. Nemkovich, *et al*. (2003). Determination of the dipole moments of the molecules of 4′-substituted 3-hydroxyflavones using the electrooptic absorption method. *J. Appl. Spectr.* **70**, 230–237.
25. S. M. Ormson, *et al*. (1994). Switching between charge- and proton-transfer emission in the excited state of a substituted 3-hydroxyflavone. *J. Photochem. Photobiol. A Chem.* **81**, 65–72.
26. M. J. S. Dewar, *et al*. (1985). AM1: A new general purpose quantum mechanical molecular model. *J. Am. Chem. Soc.* **107**, 3902–3908.
27. A. O. Doroshenko (1999). *Spectral Data Lab Software*, Kharkiv.
28. D. M. Himmelblau (1975). *Applied Nonlinear Programming*, McGraw-Hill, New York; (1972) Rus. Ed.: Moscow, Mir.
29. I. Ja. Berstein, and Yu. L. Kaminskij (1986). *Spectrophotometric Analysis in Organic Chemistry*, Khimija, Leningrad.
30. V. V. Shynkar, *et al*. (2004). Anion Formation of 4′-(dimethylamino)-3-hydroxyflavone in phosphatidylglycerol vesicles induced by HEPES buffer: A steady-state and time-resolved fluorescence investigation. *J. Phys. Chem. B* **108**, 18750–18755.
31. V. G. Pivovarenko, A. Wróblewska, and J. Błazejowski (2004). The effect of hydrogen bonding interactions between 2-[4-(dimethylamino)phenyl]-3-hydroxy-4*H*-chromene-4-one in the ground and excited states and dimethylsulfoxide or methanol on electronic absorption and emission transitions. *J. Mol. Struc.* **708**, 175–181.