1. INTRODUCTION

"In the beginning when God created the heavens and the earth, the earth was a formless void and darkness covered the face of the deep, while a wind from God swept over the face of the waters. Then God said, "Let there be light"; and there was light. And God saw that the light was good; and God separated the light from the darkness. God called the light Day, and the darkness he called Night. And there was evening and there was morning, the first day."[*]

1.1. LIGHT

1.1.1. **Definition**

Light is the part of the electromagnetic spectrum of energy that is able to excite the receptors in the eye and produce a signal in the brain that will lead to the process of vision. Of course, this is the definition of visible light or the visible part of the electromagnetic spectrum, but sometimes the words are used indistinctly. Electromagnetic energy was defined by J. C. Maxwell as a wave in movement that has an electrical component that creates a magnetic field and a magnetic component that creates an electrical field. The energy behaves as an undulation that travels in space and time. Because of this, light is defined by wave parameters, such as wavelength (λ), which is the distance between successive peaks of a wave, and frequency (ν), which is the number of oscillations of the wave in a unit of time. Both parameters are related by:

$$v = \lambda \nu$$

where (v) is the velocity of the wave, and ν is related to angular frequency (ω) by:

[*] Moses. *Genesis*. 1, 1-6.

$$\nu = \omega / 2\pi$$

Radiations with greater frequency are more energetic than radiations with lower frequency. For wavelengths, it is just the opposite: a shorter wavelength displaces more energy than a longer wavelength.

The whole electromagnetic spectrum according to wavelength can be classified as follows:

Far infrared:	10 to 1000 μm;
Middle infrared:	1 to 10 μm;
Near infrared:	0.7 to 1 μm or 700 to 1000 nm;
Visible:	400 to 700 nm;
Ultraviolet:	200 to 400 nm;
Vacuum ultraviolet:	100 to 200 nm;
Extreme ultraviolet:	10 to 100 nm;
Soft X-rays:	1 to approx. 20-30 nm (some overlap with EUV).

1.1.2. **Historical overview**

Light is indispensable for life, at least for life on Earth, and mankind has realized its importance since the very beginning. Light and color have played a role in healing for centuries. At the temple of Heliopolis in ancient Egypt, patients were treated in rooms specifically designed to break up the sun's rays into the colors of the spectrum. Phototherapy was also practiced in ancient Greece, China, Rome

and India. In ancient Rome the *solarium* was an important part of a house or gymnasium.

The history of Science has used many theories to explain the origin, meaning and nature of light from ancient Greece to the XXI century. While our knowledge of light is great, it has still not been fully explained or fully understood.

Early philosophers thought that light originated in the eyes and was used as a beam to explore objects and obtain information about the outside world. Empedocles (5th century BC) was the first to think that day and night were originated by the planet earth getting in the way of the sun. The light was composed of small 'atoms' that originated in the sun and interacted with matter. This was the beginning of the corpuscular theory of light.

The first person to provide a new hypothesis was *Abu Ali al-Hasan ibn al-Haytham*, who lived between 965 and 1038. This scientist wrote *The Treasury of Optics* and developed the image of a 'dark room' where images were formed.

For a more detailed study of the phenomenon we have to wait until Johannes Kepler (1571-1630), who suggested that an image of the objects was formed in the retina. By measuring the movements of the moons of Jupiter, Galileo Galilei (1564-1642) provided the basis for the first measurement of the speed of light, which was taken by the Danish astronomer Ole Rømer. Rømer's value was very close to 3×10^8 m/s, which is the actual value of the speed of light.

The first modern scientific theory of light, however, was created by Sir Isaac Newton (1642-1727). Newton wrote his first paper on the nature of light, gave his first lecture in the Lucasian Chair of Mathematics in Cambridge on Optics, and

wrote a fundamental book on the subject entitled *Optiks*. He made important observations in the theory of colors by decomposing a white beam into the whole spectrum when it traversed a prism, thus showing that white light was a summation of fundamental colors. He supported the idea that light was made up of a stream of corpuscles, perhaps because his laws of Mechanics explained its behavior better. However, another theory from another scientist, Rene Descartes (1596-1650), discussed a wave theory based on the notion of'ether' filling the Universe and light 'pushing' it in its displacement. Robert Hooke, a British scientist and probably the first person to look at a cell through a microscope, supported and developed a wave theory of light that totally opposed Newton's.

Christaan Huygens (1629-1695), a Dutch researcher working on many subjects of Natural Sciences, designed successful telescopes and made important astronomical observations. Building from Descartes idea, Huygens was the first to develop a complete wave theory of light that succeeded in explaining reflection and refraction (the change in direction when light changes medium). The problem with this new theory was that it contradicted Newton's, and Newton was the greater scientist who ever lived.

Thomas Young (1773-1829), a British medical doctor, made important contributions to many scientific fields. For example, he estimated the size of molecules for first time, invented the theory that colors were made in the retina from a combination of yellow, green and red, and deciphered the code contained in the Rosetta stone, an achievement that was fundamental to the understanding of ancient Egyptian documents. He carried out the first experiments on interference

(the double slit experiment that confirmed the wave nature of light) and realized that each color of light corresponds to a defined wavelength, and that the amount by which light bends when it is diffracted or refracted depends on its wavelength. He calculated the wavelength of red light to be 650 nm and the wavelength of violet light to be 450 nm, These calculations are in good agreement with the values accepted today. He also explained that diffraction is comparable to the wavelength involved, and successfully explained Newton's experiments with his wave theory of light. During his lifetime his theory was controversial because it again contradicted Newton's. Now we know, however, that his descriptions were exactly right.

Michael Faraday (1791-1867) was a British experimental scientist who conducted important research into electricity and magnetism and defined the theory that a magnetic field creates an electric current and an electric current creates a magnetic field, which he called 'electromagnetic induction'. He explained the concept of lines of force and argued that light can be explained in terms of vibration of these electric lines of force.

All Young and Faraday's work was condensed and fully explained, both mathematically and physically, by the Scottish physicist John Clerk Maxwell (1831-1879), who described the vibrations of electric and magnetic fields in a set of four equations published in 1864, four years before Faraday died. Maxwell is known as the greatest theoretical physicist between the times of Newton and Einstein. He also developed several techniques and worked in various research subjects such as the design of an interferometer to measure the relative speed

between the earth and ether, which was believed to fill all space, using the speed of light. His fundamental contribution to Science, however, was undoubtedly the theory of Electromagnetism, which Einstein used to develop his Special Theory of Relativity. Maxwell made the analogy between the wave behavior of sound and electricity on the one hand and waves in a fluid on the other and related all of them with mathematic formulae. He theoretically obtained the speed of an electromagnetic wave in a vacuum from the permittivity (electric constant) and permeability (magnetic constant) of the medium. He found that the wave was traveling at 2.99792457 x 10^8 m/s, which is exactly the speed of light measured in the vacuum. Therefore, he associated light with electromagnetism and described light as electromagnetic radiation that behaves according to the rules of all electromagnetic and wave phenomena. Maxwell's himself explain his thoughts in this way: "*This velocity is so nearly that of light that it seems we have strong reason to conclude that light itself (including radiant heat and other radiations, if any) is an electromagnetic disturbance in the form of waves propagated through the electromagnetic field according to electromagnetic laws"*[†].

The age of modern physics began in 1877 when the German-American scientist Albert Michelson (1852-1931), in collaboration with Edward Morley (1838-1923), carried forward Maxwell's method to measure the speed of earth and ether using light. In his lifetime Michelson made several measurements of the speed of light. The last one, when he was 73 years old, gave an experimental value of 2.99796 ± 0.00004 x 10^8 m /s, which fits the modern accepted value of 2.997925 x 10^8 m/s. However, the measurement with Maxwell's interferometer produced

[†] J. C. Maxwell. *A treatise on electricity and magnetism.*

surprising results. The method needed to measure the speed of light forwards in the direction of the rotation of the earth, and backwards, in the anti-rotation direction. The difference would be used to calculate the relative motion of earth in ether. However, as the measured velocities were the same in both directions, the speed of the earth's rotation did not affect the speed of the light. This result, together with Maxwell equations and the equations known as Lorentz's Transformations, which describe mathematically the way electromagnetic fields are seen by different observers at different positions and moving at different velocities, led Albert Einstein (1879-1955) to propose his Special Theory of Relativity in 1905 when he was just 26 years old. This Theory considers (c*),* the speed of light in a vacuum, as a universal physical constant. Einstein developed Maxwell equations and reported that the absolute nature of time and length does not exist. He suggested, therefore, that all motion is relative but that (c) is a constant that does not depend on the speed with which the source of waves is moving but progresses always at the speed determined by Maxwell equations.

At that time the wave theory of light was so well established that again it was difficult for a particle theory to get considered. However, Max Planck (1858-1947), a German physicist of the old school, was not able, in his research in 1895, to explain the radiation from a hot (black body) object using the wave theory of light. When in 1900 he found the solution to the dilemma, the conclusion was surprising: bodies cannot emit any amount of electromagnetic energy that they like; they can only emit energy (or absorb, depending on the way the equations

are run) in packets of a finite size that he called *quanta*. The energy (E) of each quantum is expressed by [1 chapter 3]:

$$E = h \nu$$

where (ν) is the frequency and (h) is the Planck constant (6.6256×10^{-34} Joules. second).

The quanta were called photons, derived from the Greek word for light, *photos*.

Using photons, in 1905 Einstein succeeded in explaining the photoelectric effect i.e. the way that electrons are displaced from a metallic surface when illuminated. Photons of a certain amount of energy, which depends on its frequency ($E = h \nu$), knock out of a metal electrons that carry the same energy.

Newton's theory was back, and the light was made of corpuscles.

This explanation and the definition of absorption, emission and stimulated emission (which provided the basis for laser emission) were the beginning of Quantum Electrodynamics, the new theory of matter and energy. This was fully developed by the American physicist Richard Feynman (1918-1988), perhaps the most important theoretical physicist after Einstein. Feynman defined matter and its interaction with energy in terms of photons and electrons. When an electron absorbs a photon, it increases its energy by reaching an upper energy level in the atom; when an electron falls from an upper energy level to a lower energy level, it loses its energy by emitting a photon (Fig. 1). So easy and yet so complicated at the same time.

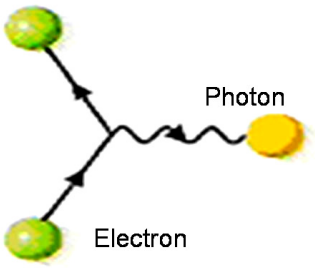

Figure 1. Feynman diagram of the photon absorption/ emission process by an electron.

This again changed the way scientists looked at light phenomenon. Today we still ask: What is light, a wave or a particle, or both? This dilemma is artificially solved by saying that light has a duality—the "wave-particle duality". Sometimes it behaves as a wave, and experiments designed to measure wave cannot "see" corpuscles, and sometimes it behaves as a particle, and these experiments cannot measure waves. Of course, this duality reflects ignorance about its true nature.

1.2.LASER

1.2.1. **Definition and historical overview**

Laser is an acronym of Light Amplification of Stimulated Emission of Radiation but it is now accepted as a regular word is most languages. The stimulated emission of radiation, defined by Einstein in 1905, involves the absorption of photons of a defined wavelength in a material. In this atom or molecule, one electron per photon absorbed reaches an upper energy level. If electrons are

maintained at that excited level for certain time, the material then moves to a state called “inversion of population”, which means that more electrons will be found in the excited state that in the ground state. If photons continue to reach the material, this will make all the electrons in the excited level fall to the ground state at the same time, and all of these electrons will emit a photon of the same wavelength the excitation photon.

After an inversion of population, the other thing a laser needs is an optical feedback. The material in which a stimulated emission is produced is inside a cavity, which has a mirror at both ends. One of the mirrors is fully reflecting and the other is partially reflecting. Stimulated photons will travel inside the cavity reflected by the mirrors. The intensity will be amplified by more stimulated photons coming from the material. When the intensity is high, the partially reflecting mirror will allow these photons to leave the cavity and the laser beam will appear. The material needed to produce a laser emission has to be transparent to the wavelength of stimulated photons, but to produce absorption, and consequently emission, it needs to be doped will impurities such as metals, which are responsible for continuing the exchange of energy.

Charles Townes used the stimulated emission process to construct a microwave amplifier called MASER. Microwave is a less energetic electromagnetic radiation with a lower frequency than light. Townes’ MASER device produced a coherent beam of microwaves for use in communications. In 1958 Townes and Schalow published a paper extending their ideas to optical frequencies and in 1960

Theodore Maiman of Hughes Research Laboratories produced the first laser using a ruby crystal as the amplifier medium and a flash lamp as the source of photons. A beautiful, intense red light emerged from within the cavity.

The first gas laser was developed in 1961 by Javan, Bennet and Harriott of Bell Laboratories using a mixture of helium and neon gases. This was a Helium-Neon laser that emitted in the red part of the spectrum as well as the green and yellow parts when some parameters were modified.

The first semiconductor laser was developed by R. Hall at the General Electric Research Laboratories in 1962. In 1963 C. K. N. Patel of Bell Laboratories developed the CO_2 laser. In 1964, Sorokin and Lankard of the IBM Research Laboratories constructed the first liquid laser using an organic dye, thus leading to the category of broadly tunable lasers, which can emit at several different wavelengths.

The first vacuum ultraviolet laser was reported to occur in molecular hydrogen by R. Hodgson of IBM and independently by R. Waynant at the United States Naval Research Laboratories in 1970.

The list was growing exponentially. Nowadays lasers are obtained from various media and materials offering a broad spectrum of wavelengths.

Lasers do not necessarily emit very powerful beams but they have powerful properties. Consider the light from a candle illuminating objects in any direction. If we could concentrate the light from a candle onto a single beam of light 3 mm in diameter, the light would be 1,000,000 times more intense than what we normally see from the candle.

A laser is monochromatic (i.e. the light is emitted in one pure color), directional (i.e. the beam has a single direction) and the emitted light is coherent (i.e. all its photons or waves are in the same phase). These characteristics produce a summation of effects or constructive interference in the laser beam.

1.2.2. **Classification of lasers**

Lasers can be obtained from several kinds of materials. They can be classified into laser systems involving low-density gain media and laser systems involving high-density gain media.

Low-density gain media lasers represent approximately half of the existing commercial lasers and are obtained from a gaseous (low-density) medium.

The most important lasers using gases are Helium-Neon, Argon ion, Helium-Cadmium, Copper vapor, carbon dioxide (CO_2) and "excimer" (excited dimer) lasers.

High-density gain media lasers are obtained from high-density media, liquids (organic dyes) and solids (mostly crystals). The most important lasers obtained from crystals are Ruby, Neodymium YAG, Alexandrite and Erbium YAG.

One particular kind of solid lasers are diode lasers, which are small and very operative. They are not pumped optically (with photons) but with an electrical current that mobilizes the electrons in the media and leads to photon emission. They are all made by semiconductor crystals (e.g. AlAs, AlGaAs and GaAs). Because of their origin they are also called semiconductor lasers [1 chapter 14,2].

1.2.3 **Lasers applications**

1.2.3.1. Non-medical applications

Because of their characteristics of monochromaticity, unidirectionality and coherency, lasers are used in communication systems, radar and military targeting applications, surveying instruments and everyday devices such as CD and DVD players and recorders, supermarket check-out scanners and optical pointers. They are also used in art restoration and in powerful industrial machines to cut iron and steel blocks. In Analytical Chemistry they are used in laser spectroscopy to excite molecules and analyze their chemical behavior and in Physics to test optical properties and the energy state of matter.

1.2.3.2. Medical applications of lasers

The medical applications of lasers are many and, thanks to the greater availability of sources and wavelengths, they are growing every day.

A laser is a very specialized light source that should be used only when its unique properties are required. When used correctly it can provide important benefits for patients with a wide range of pathologies and diagnoses.

To understand the mechanisms, we can divide lasers in medicine into two groups:

- thermal lasers, and
- non-thermal lasers

1.2.3.2.1. *Thermal lasers*

Thermal effects begin when the power of the light source is greater than or equal to 500 mW. The optical mechanism is the absorption of the light in the body that produces the heat, which is the actual mechanism of action. In this category we include all surgical lasers used to cut and ablate tissue (light blade) or remove undesired elements from the skin or tissues, such as blood vessels, pigmented lesions or hair. They are also used to coagulate vessels in the retina due to diabetic proliferation and in transmyocardial laser revascularization.

1.2.3.2.2. *Non-thermal lasers*

The mechanism involved in non-thermal lasers is photon absorption by an atom or by a molecule in a), b) and c) below. Even where it is not clear that absorption produces the experimental effects (see *d)* below) the effects may be explained by absorption and the electronic excitation of an atom or molecule.

Non-thermal applications of lasers can be divided into:

a) Ultraviolet laser ablation, in which lasers convert tissue into plasma by absorption at very superficial levels in the tissue in very short interacting times. A typical example is refractive surgery of the eye using excimer lasers.

b) Optical tissue diagnostics, such as spectroscopic diagnostics of malignant tumors or atherosclerotic plaques, and light scattering studies for obtaining the characteristics of tissues.

c) Photodynamic therapy, a therapeutic method that uses a photosensitizer introduced into a cell. When illuminated, the molecule absorbs light energy and the cell starts to produce singlet oxygen, which kills the cell via a direct cytotoxic effect. This technique is basically used to treat cancer and macular degeneration in the retina [3].

d) Light-stimulation or photobiomodulation in tissues, wound healing and some kinds of pain treatment.

Lasers and other light sources have proved effective for treating wounds [4] and several kinds of pain, nerve regeneration and inflammation in the nervous system [5], collagen synthesis in fibroblasts [6], stimulating the formation of DNA and RNA in the nucleus [7], and local effects in the immune system [8], among others.

Clearly the mechanism is not produced by a heating effect because the power of the light sources used in these studies is below 30 mW. The increase in temperature in the tissue of about 0.1 ^{0}C is more likely produced by the response to light, which stimulates cell metabolism, than by absorption itself. [8].

Because the effect observed was a modulation of the response, which adapted to the actual necessities of the tissue, it was called *photobiomodulation* or *Low Level Laser Therapy (LLLT)* to differentiate it from the thermal effect of lasers.

We mentioned earlier that light therapy was practiced in ancient times, with examples in ancient Egypt, Greece and Rome.

In the last 130 years, colored light and full spectrum light therapy have been used in Medicine. In 1876 Augustus Pleasanton stimulated glands, organs and the nervous system with blue light. In 1877 Seth Pancoast used red and blue light to try to balance the autonomic nervous system. In 1878 Dr. Edwin Babbitt published *The Principles of Light and Color* in which he elucidated a system for applying colored light to the body using solar elixirs, which were colored bottles containing water "charged" by the sun. He treated many stubborn medical conditions that were unresponsive to the conventional treatments of the time.

In modern times, after the first lasers of the sixties, light therapy took off with the Hungarian Medical Doctor Endre Mester. Mester was a surgeon who began treating surgical wounds with lasers and observed that the healing time was short and the quality of the tissue improved. In 1966 he published the first scientific paper on the stimulatory effects of non-thermal ruby laser light (694 nm) on wounds. Mester demonstrated that cells in culture and tissue can be stimulated by a dose of laser light. After this pioneering work, several pathologies were treated with lasers. Some treatments were more successful than others and some produced no results at all.

Complete ignorance of the actual mechanism behind the light effects led some less rigorous practitioners to propose things like the *magic ray*, which was supposed to be able to treat almost everything, including growing hair in alopecia

cases, removing it in hypertrychosis (of course, I am referring to non-thermal lasers: hair removal due to a thermal laser effect has excellent results), treating hypo- and hypertonic problems, high and low blood pressure, anxiety and depression and so on. The treatment of some pathologies produced no results and the concept of *Low Level Laser Therapy* was greatly damaged.

On the other hand, medical courses in Universities do not teach students how to treat pathologies such as pain due to inflammatory processes (arthritis, for example) using physical therapies (e.g. lasers, ultrasound, magnetic and electric fields) but using pharmacotherapy. This is extremely lucrative for pharmaceutical companies but it reduces the possibilities and benefits available to patients and prevents doctors from giving advice about a therapy they do not know about.

Some pathologies has been well studied, however. Rigorous work has been done and the positive effect of lasers has been demonstrated. Despite all the theories for explaining the effects of light, the basic mechanism has still not been demonstrated.

We will now describe the proposed mechanisms for non-thermal laser effects in cells and tissues and discuss the processes that cannot be explained by any of these mechanisms.

1.2.3.2.2.1. The photochemical effect

With this effect visible light is absorbed by metals or molecules with a metallic atom and this enhances chemical reactions in which these atoms or molecules are involved.

The photochemical effect obeys three basic laws, which are the *Laws of Photochemistry*:

The first law of photochemistry (Grotthus-Draper Law, but proposed originally by Einstein) states that light must be absorbed by a molecule or atom before photochemistry can occur.

The second law of photochemistry (Stark-Einstein Law) states that light absorbed need not produce an effect but if it does, only one photon is required for each absorbing molecule.

The third law of photochemistry is the *reciprocity rule* (Bunsen-Roscoe Law), which states that the photochemical effect is independent of the intensity of light and the irradiation time when dose is kept constant [7].

1.2.3.2.2.2. Karu's mechanism due to absorption in mitochondrial cytochrome chain

Karu's theory is based on the photochemical effect but it is so elaborate that it needs to be referred to separately. The Estonian photochemist Tiina Karu developed a theory that the absorption of visible and near-infrared light (632.8 and 810 nm) by the cytochromes in the mitochondrial membrane plays a fundamental role in the light response in cells and tissues. Cytochromes are molecules that have some metallic atoms in their structure and are able to absorb

visible and near-infrared light. When irradiating HeLa cells (a continuous line of tumor cells), an increase in ATP synthesis is observed 20 minutes after irradiation. After measuring the spectrum of cytochrome c oxidase, it is observed that the absorption of light converts the molecule from its oxidized form to its reduced form. In Karu's theory, this process makes the cell start ATP synthesis and the cell improves its metabolism [9].

1.2.3.2.2.3. The photophysical effect

With this effect, light with a wavelength greater that 1000 nm (near-middle infrared) produces vibration in certain parts of molecules or cell structures such as membranes. The absorption of middle-infrared photons leads to vibration of parts of a molecule, and a rotation and displacement of the molecule when wavelengths are increased (microwaves). However, these wavelengths are rarely used in studies of biomodulation, and the effect cannot occur at shorter wavelengths.

1.2.3.2.2.4. The bioelectric effect

With this effect, light is considered as an electromagnetic phenomenon that interacts with electrically charged ions and voltage-dependant ionic channels, which are responsible for the observed light effects. This hypothesis is not developed or sustained in experimental work. We must also consider that some of these channels are ATP-dependant (Na^+/K^+ ATPase pump) [10].

1.2.3.2.2.5. The hormonal explanation

Under this hypothesis, light stimulates some myelinic fibers in the nervous system. This stimulates the hypothalamus to produce ACTH, MSH, FSH and endorphins. [8]

1.2.3.2.2.6. Light biomodulation and non-explained processes

Light has demonstrated important effects in treating damaged or diseased tissues whose processes of healing and repair require large amounts of energy [8,11], modulating inflammatory response [5], normalizing tissue sufferance from poor oxygen or foodstuffs, and treating metabolic processes such as diabetes [12]. As we have said, these effects are not well explained and cannot be related to any of the above mechanisms.

Responses to light when irradiating enzymes [13] are also unexplained. Some authors have described biochemical and biological responses to irradiation with laser light that are independent of mitochondrial activity and that do not identify any known chromophore [14,15].

However, for all these processes to occur, ATP is required.

1.3. LIGHT - MATTER INTERACTION

The energy carried in an electromagnetic wave (or in photons if we use the particle nomenclature) must somehow interact with matter to produce an exchange of energy between light and medium. Otherwise, no effect due to irradiation can be expected

When light reaches a tissue or a specific kind of matter, several processes can take place.

1.3.1. **Reflection**

This defines the light that is sent back when it interacts with matter. The incident and reflecting angles are the same. The properties of reflection are used when a beam is conducted inside an optical fiber. Light is introduced into the fiber at an angle that produces several reflections along the path and confines the light inside the material.

1.3.2. **Refraction**

This refers to the light that alters its direction when it changes from one medium to another with a different refractive index. The refractive index (n) of a medium is the factor by which light bends when traveling inside that medium due to interaction with atoms or molecules. It is defined by

$$n = c/v = (\varepsilon/\varepsilon_0)^{1/2}$$

where (c) is the speed of light in vacuum, (v) is the speed of the light in the medium, and (ε) and (ε_0) are the permittivity or electric constants of the medium and the vacuum. The refractive index is used in gem identification, lens design and microscope optics, where an immersion oil with a different refractive index to that of air needs to be placed between the sample and the objective in order to conduct the light.

1.3.3. **Transmission**

This is the percentage of light that penetrates the matter or tissue and is not absorbed in it. It is the inverse concept to that of absorption. A medium that transmits 100% of the light (i.e. is transparent) has 0% absorption, and a medium that absorbs 100% of the light (i.e. is opaque) has 0% transmission.

1.3.4. **Scattering**

This is the light that, once inside a medium, changes its direction due an interaction with the structures it encounters in its path. Scattered light is light that can be dispersed forwards or backwards. The process is not as uniform as refraction or reflection since it depends strongly on the uniformity of the medium and on the size and collocation of the particles that constitute this medium [16, 17]. Light can be scattered by large structures and fundamentally by electrons, which interact with the electric field that light produces.

1.3.4.1. Scattering processes due to interaction of an electromagnetic field with a chemical bond: interaction with bound electrons

An important point of the interaction of light with transparent materials is the dispersion of light or the wavelength dependence of the refractive index (RI). So far as problems involving light are concerned, the correct picture of an atom is that the electrons behave as thought they were held by springs. When the frequency of the incident light is far from resonance (the natural oscillating frequency of the electrons) the electric field that light produces in the medium is

not absorbed but changes the oscillation of the bound electrons, producing oscillating dipole moments. The acceleration of these oscillating charges radiates new light (dipole radiation). The new radiated light produces a new electric field, which is equivalent to a phase shift of the original wave emitted by the source. Since this change in phase is proportional to the thickness of the medium, the effect is the same to having a different velocity inside the medium. The acceleration of the electrons is proportional to the incoming electric field, and thus, to the frequency of the light. The amount of new radiation will be, then, wavelength dependent. In dense media (i.e. solids and liquids) the oscillation of electrons will not only be modified by the radiating field, but also by the new radiation produced by other electrons in the vicinity, which will produce a complex response of the medium to the electromagnetic energy. This molecular origin of the RI explained by classical physics still maintains validity in quantum mechanics [16, 17]

1.3.4.2. Scattering processes due to the interaction of an electromagnetic field with a chemical bond: interaction with free electrons

Free electrons in a medium produce an electrical current and absorption of light due to the conversion of energy into heat. They can also scatter the incident light by an electrical interaction but, quantitatively, the effect is small. However, for X-rays, which interact strongly with any media, the effect of scattering by free electrons is large [18, chap. XV].

Four types of scattering due to bound electrons have been described:

1.3.4.3. Rayleigh scattering:

This phenomenon was first described by J.W. Strutt, Lord Rayleigh, in 1871. When the Bohr condition in the atom, which states that the energy of the incoming photon (hν) has to match the differences in energetic levels in the atom, is not even approximately obeyed (the medium is transparent to the incident wavelength), a photon absorption-emission process in an atom can be produced in two stages:

1. The electromagnetic wave loses energy (hν) and the atom changes from state *n* to state *m* (photon absorption where $energy_n < energy_m$), but v_{nm} is not even approximately equal to *v*.

2. The atom changes from state *m* to state *n* (photon emission) and the medium gains energy hν.

Energy is not even approximately conserved in either of the stages, but it is conserved in the process as a whole. This is all we require, since the intermediate stage is not observable. Light is scattered forward when the medium is perfectly homogenous.

At the end of the process, the outgoing number of photons is the same, but the velocity of the light in the medium is slowed down by the absorption-emission process. This difference in kinetic energy and momentum is the interactive mechanism between electromagnetic energy and the medium.

Rayleigh scattering is selective because certain particles are more effective at scattering a particular wavelength of light. Air molecules, such as oxygen and nitrogen, are small so are more effective at scattering shorter wavelengths of light (blue and violet). Because of the Rayleigh scattering by air molecules, the sky is blue on a clear, sunny day [18, chap. XV].

1.3.4.4. Mie Scattering

This is the simplest type of scattering and is produced by spherical particles. It is based on Maxwell equations after analyzing the changes in the electric and magnetic fields due to interaction with the medium. Mie Scattering is responsible for the white appearance of the clouds. Cloud droplets are large enough to scatter all visible wavelengths more or less equally. This means that almost all of the light that enters the clouds will be scattered. Because all the wavelengths are scattered, the clouds appear white. Mie scattering is also responsible for the dispersion of the incident light in the skin when it interacts fundamentally with structures whose sizes are almost equal to that of the wavelength [18, chap. XV].

1.3.4.5. The Compton Effect

This is the increase in the wavelengths of X rays and gamma rays when they collide with and are scattered from loosely bound electrons in matter. This effect is only explained when photons rather than waves are considered. According to the quantum theory, in a collision a photon can transfer part of its energy and linear momentum to a loosely bound electron. Since the energy and magnitude of

the linear momentum of a photon are proportional to its frequency, after the collision the photon has a lower frequency and, therefore, a longer wavelength. The increase in the wavelength does not depend on the wavelength of the incident rays or on the target material. It depends only on the angle formed between the incident and scattered rays. A large scattering angle will produce a large increase in wavelength. The Compton Effect is used to study electrons in matter and to produce variable energy gamma-ray beams [18, chap. XV].

1.3.4.6. The Raman Effect

This is the appearance of additional lines in the spectrum (with different colors) of monochromatic light scattered by a transparent material medium. The energy, and therefore the frequency and wavelength, of the scattered light is changed (wavelength is increased) as the light imparts rotational or vibrational energy to the scattering molecules or takes energy away. The line spectrum of the scattered light has one prominent line corresponding to the original wavelength of the incident radiation, plus additional lines to each side of it corresponding to the shorter or longer wavelengths of the altered portion of the light. This Raman spectrum is characteristic of the transmitting substance. Raman spectrometry is a useful technique in physical and chemical research, particularly for characterizing materials [18, chap. XV].

1.3.5. **Absorption and interaction**

We must first define the mechanisms of absorption and polarization. We will do this using both the wave and particle behavior approaches.

Absorption refers to the amount of light that interacts with a medium and, as this is not transmitted, reflected or scattered, it is assimilated by the medium. Absorption occurs when the electromagnetic wave in a medium is extinguished [19].

The interaction and absorption of a wave with a medium are basically related to the electric and magnetic characteristics [expressed by the *permittivity* or electric constant (ε) and by the *permeability* or magnetic constant (μ)] of the medium in which the light penetrates.

The speed of the light in the medium is defined by

$$v = (\mu\ \varepsilon)^{-1/2}$$

We can then define, according to the electromagnetic theory of light, two different kinds of media (conductors and non-conductors), depending on whether the light induces an electrical current when it traverses them. We must therefore consider two different kinds of interaction. A special case of light-matter interaction is also described when the wave interacts with very long chains of alternated double-single bonds in the molecules called polyenes.

1.3.5.1. Absorption of electromagnetic radiation: electrically conductors, opaque or magnetic (metallic) media

"*If the medium, instead of being a perfect insulator, is a conductor whose conductivity per unit of volume is C, the disturbance will not consist only of*

electric displacements but of currents of conduction, in which electric energy is transformed into heat, so that the undulation is absorbed by the medium."[‡]

These media are perfectly described by Maxwell, who states that a magnetic media is opaque to light because some free electrons travel from one atom to another, thus producing an electrical current and extinguishing the wave [19]. Maxwell electromagnetic theory is phenomenological, it is not related to the intimae constitution of matter. Quantum mechanics theory explains the atomic structure of matter and relates it to the absorption and emission of electromagnetic energy. However, some of the processes explained by classical physics are still valid in quantum mechanics, and the polarization of a dielectric medium by the oscillating electric field that light produces is one of them [16, 17]

Molecules made up of metals, such as metallic salts in a solution, have this behavior.

When considering the absorption of photons, and therefore the particle behavior of light, we must describe two mechanisms: electronic excitation and the photoelectric effect.

1.3.5.1.1. Electronic excitation (atomic energy levels)

Bohr's levels of energy in atoms are those in which the difference in energy between level *m* and level *n*, $E_m - E_n$, is almost exactly matched by a photon of energy $E_{m-n} = h\nu$. No other energy produces this effect, and the frequency that

[‡] J. C. Maxwell. A treatise on electricity and magnetism. [798] Relation between electric conductivity and opacity, pp. 445-446.

produces this excitation is called resonance angular frequency o $\omega_{0,}$ which has a very narrow band.

This characteristic of each atom or molecule is defined by its absorption spectrum obtained by spectroscopy, which shows peaks of absorption and transparent zones where light is not absorbed. For a photon to be absorbed in a molecule, its energy must be greater that the energy contained in the chemical bonds that constitute the molecule. For example, the energy of a P-O bond is 500.74 kJ/mol. The energy of a UV photon of 200 nm is 598 kJ/mol and the energy of a blue photon of 400 nm is 299 kJ/mol. The UV photon can then be absorbed but the blue photon definitely cannot.

After absorbing a photon, the atom or molecule is excited to an upper energy level and the decay of the energy can produce another photon, be relaxed as heath or vibration in the molecule or be converted into chemical energy by allowing the excited molecule to react faster with other molecules. In Physical Chemistry, this kind of conversion of energy is known as Photochemistry. If there is no absorption, the laws of Photochemistry state that a photochemical effect is not expected [1, 18, chap. XVIII, 20].

Enzymes whose structures contain a metallic atom, such as cytochrome c oxidase, which has a copper atom or hemoglobin, which is a porfirin with iron atoms, absorb visible light due to the electronic excitation of the metallic atom.

Chemical substances that can absorb photons are known as chromophores. The strict definition of a chromophore (from the Greek words *khrôma*, meaning color and *phoros*, meaning to bring) is a chemical group that gives color to a molecule.

The color of a molecule or substance is produced by the light that is reflected or transmitted, which is the light that is not absorbed by a particular chemical group. Therefore, transparent media cannot be chromophores and the only colored molecules are those that absorb light at different wavelengths. Transparent media either reflect all the light or the light passes through them, and we do not see a color coming from them. When we speak about color, we are speaking about the absorption of visible light. A molecule that absorbs in the UV or the IR is not strictly a chromophore because we are looking at a transparent medium for visible wavelenghts. If we could look at the substances through a UV or IR detector, we would be able to see a "color".

In Biology, chromophores are classified according to whether they are exogenous or endogenous to the body, and whether they are specific (their main function is to absorb light) or non-specific (they have other functions but also absorb light). The following are examples of chromophores:

- Endogenous chromophores*:*

  a) Specific chromophores: melanin, chlorophyll and other photosynthetic pigments, rhodopsin [21 chap. XIII].

  b) Non-specific chromophores: hemoglobin, porphyrine, tyrosine, catalytic enzymes, flavoproteins, asparagines, ceruloplasmine, superoxid dismutase. Any protein that has a metal as the prosthetic group absorbs light, like cytochromes in the inner membrane of the mitochondria.

- Exogenous chromophores:

Organic dyes used in photodynamic therapy, photosensitizers, and some drugs that produce a response to light for example, after solar exposition.

The skin has an *optical window*, which is defined as the range of wavelengths that are able to penetrate the skin and reach deeper structures because they are not absorbed at the superficial levels. This range is approximately between 450 and 1400 nm. Fig. 2 shows this optical window and the lasers with emission wavelengths in this range [10].

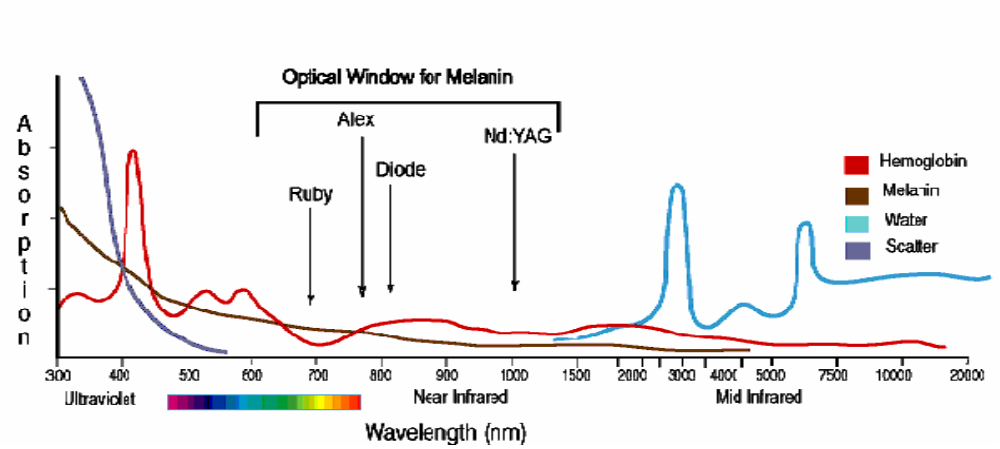

Figure 2. Optical window of light transmission in the skin. The range is from 300 to 1400 nm. Some commercial lasers are able to emit at these wavelengths.

1.3.5.1.2. The Photoelectric effect

This phenomenon involves the emission of electrons when light is focused onto a solid *metallic* surface. The number of electrons emitted is proportional to the intensity of the light, but their kinetic energy depends only on the wavelength of the light. This light behavior, which is related to photons, cannot be explained by

a continuous electromagnetic wave. This effect is the basis for television tubes [18, chap. XVII].

1.3.5.1.3. Absorption of light in polyenes

A long chain of conjugated (alternated double-single) bonds interacts with the electromagnetic field and as a result light is absorbed. In this case, the medium is non-metallic and no electrical currents are observed.
One efficient light trapping process that has this kind of interaction is photosynthesis. Chlorophylls are highly effective photoreceptors because they contain extremely long networks of alternating single and double bonds. Such compounds are called polyenes. These have very strong absorption bands in the visible region of the spectrum where the solar output reaching Earth is also maximal. The peak molar absorption coefficient of light, i.e. the efficiency in absorbing *chlorophyll a*, is over 10^5 M^{-1} cm^{-1}, which is among the highest observed for organic compounds. The wavelengths that are absorbed in these molecules depend on the length of the chain of single-double bonds.

1.3.5.2. Interaction of electromagnetic irradiation with mater: transparent, dielectric or non-magnetic (non-metallic) media

This defines the effects of light in transparent media in which no absorption is found. A medium is defined as transparent for a specific wavelength when it has no atomic or molecular light absorption. The wave is therefore not extinguished but its velocity, direction and polarization are affected. There is no absorption

when the resonance angular frequency (ω_0) of the atom or molecule that constitutes the medium is far from the wavelengths that irradiate the medium (see 1.3.5.1.1).

The velocity of the light in these kinds of media is defined by

$$v = \varepsilon^{-1/2}$$

The velocity of the wave in a transparent medium only depends inversely on the square root of its permittivity or electrical constant ε. In this medium, no electrical currents are produced due to the electromagnetic disturbance, since dielectric molecules are not conductors but insulators. Molecules in this type of media can be dipolar, but any chemical bond in the molecule is polarized by light (electrons in the bond act as small dipoles). Depending on their size, molecular dipoles will or will not be affected in their displacement or orientation by the electric field. Molecules cannot follow the oscillations of light because of the high frequency of visible wavelengths (in the range of terahertz, 10^{14} Hz), and organic molecules usually stop following the movement of an electrical current when it arrives at the gigahertz range (10^9 Hz). The frequency at which a molecule ceases its movement produced by an electrical current is called the relaxation frequency, which is specific for the molecule. However, bound electrons in chemical bonds will oscillate even at high frequencies of visible light [16, 17, 22].

The speed of light in transparent media depends on the wavelength, and shorter wavelengths interact greater with the medium than longer ones.

The interacting mechanism of light is an electromagnetic disturbance that creates a displacement of charges in the medium that depends on the number of electrons

that can be displaced, the intensity of the force that attaches the electrons to the atom, and the intensity of the electromagnetic field, which is given by the following equation:

$$P = (Ne^2/k)\,E$$

where (P) is the polarization of the material; (*N*) is the number of electrons that can be displaced; (*e*) is the electric charge of the electron; (*k*) is the restoring force that, like a spring, pulls the electron back to the atom; and (E) is the intensity of the electric field produced by the light.

The molecular origin for non-absorptive electromagnetic interaction with a medium is related to the characteristics of the chemical bonds that constitute the molecule. Any molecule will interact with light because of its bond electrons but if the molecule has a single-double bond structure, interaction will be very significant. A single bond between two molecules A and B always has the σ bond structure, in which the electrons are found in a certain region of the space in a straight line between the two atoms. On the other hand, a double bond consists of a σ bond and a π bond. A π bond is the overlap of two *p* orbitals—one from each atom that forms the bond.

The optical response of σ bonds is very different from that of π bonds because σ electrons (electrons contained in the σ bond) tend to be localized in space. In contrast, π electrons tend to be delocalized. Because they are delocalized, they tend to be less tightly bound and therefore tend to produce larger linear and nonlinear optical responses. π electrons are delocalized because they can be found anywhere in the alternated single-double bond structure, and the single bond can

be exchanged with the double bond. The actual form of a conjugated bond is the superposition of different resonance configurations. The double A=B bond is therefore a σ bond in the center, and two superposed π bonds around the σ bond (A≡B).

Ideally, the bond is considered to be a point charge of charge q located between the two atoms. r_A and r_B are the covalent radii of atoms A and B and $d = r_A + r_B$ is the bond length. The bond charge is given by:

$$q = en_v\,(1/\varepsilon + \tfrac{1}{3} f_c)$$

where n_v is the number of electrons per bond, ε is the dielectric constant of the medium and f_c is the fractional degree of covalence in the bond. In the presence of an electric field (E) parallel to the axis, the charge is seen to move by an amount $\delta r = \alpha_{\parallel} E/q$, where $\alpha_{\parallel}$ is the polarizability measured along the bond axis. The ion-to-bond-charge distances r_A and r_B therefore change by amounts

$$-\Delta r_A = \Delta r_B = \delta r = \alpha_{\parallel} E/q. \qquad [16, 17, 23]$$

Dipolar molecules in liquid state, such as water, acetone, alcohols or acetic acid, have this behavior, as do non-metallic solutions (but not electrolytes). Important biologically interesting examples are all dipolar molecules in a solution, such as nucleotides, aminoacids, DNA, enzymes and other proteins. Nucleotide phosphates such as ATP, ADP and AMP are also good examples [22].

1.3.5.3. <u>Difference between light interaction (i.e. polarization) and light absorption due to chemical conjugated bonds</u>

The fundamental difference here is that a double-single bond interacts with light but will not capture light because after this bond the wave can travel freely. The alternated double-single bond can find another molecule with the same characteristics and there will be another interaction, and so on. The sum of these interactions leads to a decrease in the speed of light in the medium and a displacement of charges in the material. This polarizes the medium but does not extinguish the whole wave.

In a polyene, double-single bonds lay one beside another in a very long chain. Light that interacts with the first bond immediately interacts with the second one, then with the third one, and so on until the velocity slows to zero and there is no energy left. The wave will extinguish, so the light will be absorbed and its whole energy transferred to the medium.

1.4. ATP: A KEY MOLECULE

1.4.1. **Energy currency**

Living things require a continuous input of free energy for three major purposes: the performance of mechanical work in muscle contraction and other cellular movements, the active transport of molecules and ions, and the synthesis of macromolecules and other biomolecules from simple precursors. The free energy used in these processes, which maintain an organism in a state that is far from equilibrium, is derived from the environment. *Chemotrophs* such as mammalian cells obtain this energy via the oxidation of foodstuffs, whereas *phototrophs* such as plants obtain it by trapping light energy. Part of the energy derived from the

oxidation of foodstuffs and from light is transformed into a highly accessible form before it is used in motion, active transport and biosynthesis. The free-energy donor in most energy-requiring processes is adenosine triphosphate (ATP). The central role of ATP in energy exchanges in biological systems was perceived in 1941 by Fritz Lipmann and Hermann Kalckar [24].

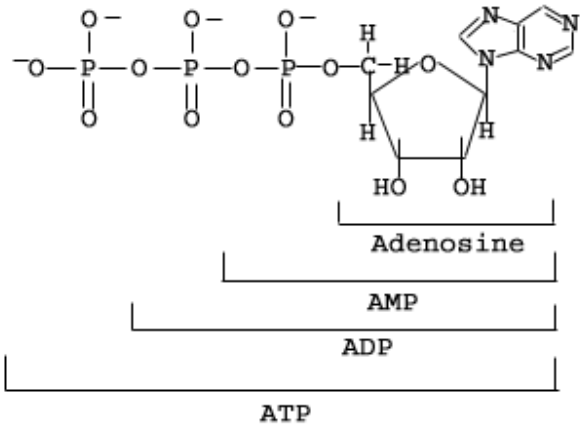

Figure 3. ATP has an adenine attached to a ribose that is called adenosine. The number of phosphates names the molecule and determines its biological function.

ATP is a nucleotide consisting of an adenine, a ribose and a triphosphate unit (Fig. 3). The active form of ATP is usually a complex of Mg^{2+} or Mn^{2+} that binds the phosphates. To consider the role of ATP as an energy carrier, we can focus on its triphosphate unit. ATP is an energy-rich molecule because its unit contains two phosphor-oxygen bonds (the phosphate linked to the adenosine to form AMP has different characteristics because it binds a CH_2O^- group and not another PO^- group). A large amount of energy is liberated when ATP is broken down into

ADP plus orthophosphate (P_i) or into AMP plus pyrophosphate (PP_i): 7.3 kcal/ mol for each bond [24].

ATP is then the energy currency in all metabolic cell reactions, and the key molecule for most cell processes. [25, 26].

It can be also considered as a molecule that translates different kinds of energy into a common language. In this sense it must be considered a key molecule.

1.4.2. **ATP synthesis**

ATP is mostly synthesized in the mitochondria, after a series of redox reactions in the electronic transport chain located in the inner membrane of the organelle [27]. In aerobic conditions, 36-38 molecules of ATP are synthesized from one molecule of glucose [28]. In anaerobic conditions, when the oxygen levels are insufficient for cell metabolism and ATP synthesis by mitochondrial respiration, a molecule of glucose is fully oxidized in the cytoplasm to produce two molecules of ATP and pyruvate in a process called glycolysis [21].

1.4.3. **ATP and electromagnetic energy**

The chemical bonds contained in an ATP molecule and their intrinsic energies are shown in Table 1. The energies of photons of certain wavelengths are shown in Table 2.

Bond	kJ/mol
P-O	500.74
P=O	501.99
C-H	338.71
O-H	428.48
C-N	770.37
C-O	1077.1
N-H	314.01
C-C	602.9

Table 1. Intrinsic energies of the chemical bonds that form ATP, expressed in kJoules per mol.

Wavelength (nm)	kJ/mol
200	598
400	299
635	188.32
655	182.54
700	171
830	144.07
1000	120

Table 2. Intrinsic energies of light at certain wavelengths, expressed in kJoules per mol.

For a photon to be absorbed in a molecule, the energy of the photon must be greater than or equal to the intrinsic energy of one of the chemical bonds that constitute that molecule [29]. For ATP, 635 (188.32 kJ/mol), 655 (182.54 kJ/mol) and 830 nm (144.07 kJ/mol) light have less energy than the intrinsic energy for the N-H bond (314.01 kJ/mol), which is the least energetic one in ATP. Light at these wavelengths is therefore not absorbed by the ATP molecule.

The lowest intrinsic energy bonds for ATP are C-H (338.91 kJ/mol) and N-H (314.01 kJ/mol) bonds. Therefore, for absorption to occur by the ATP molecule, the irradiation light must be in the UV part of the spectrum. ATP has a strong absorption in the UV, with two important peaks at 210 and 260 nm. If we consider visible light and near-infrared light, ATP is not colored and has no absorption in that range of the spectrum. *ATP is therefore not a chromophore* for these wavelengths. ATP absorbs in the middle-infrared part of the electromagnetic spectrum, at 8000 to 11000 nm, which corresponds to microwave [30].

1.4.4. **ATP as a dipole**

ATP is a dipolar molecule with a measured dipole moment of 30 Debyes and a relaxation frequency of 50 MHz. The entire molecule will therefore not oscillate due to visible or near-infrared light interaction because it is too big to follow optical frequencies. It will stop oscillating at radio or low microwave frequencies. The dipolar moment corresponds to a certain permittivity or dielectric constant of the medium. Electrons in chemical bonds may interact with electromagnetic

energy and ATP molecules can be then polarized by the light. Since dipoles are insulators, no significant electrical current may be light induced [22, 31, 32]. ATP also has a response to electrical currents. It has been shown that the mechanism that transports the molecule from inside to outside the mitochondria after ATP is synthesized through induced micro-electrical currents [33].

2. OBJECTIVES

In this thesis I have sought to demonstrate that there is and interaction between visible and near-infrared light and ATP, a non-chromophore for these wavelengths. This interaction will be demonstrated through:

1. **The measure of the Refractive index of an ATP solution and the behaviour of ATP in the luciferine-luciferase reaction.**

2. **Spectroscopy and fluorescent measurements to show the kind of interaction.**

3. **The biological significance of light interaction with ATP will be demonstrated by analyzing the kinetic parameters of a fundamental cell reaction, the phosphorilation of glucose mediated by hexokinase.**

In this thesis I have sought to demonstrate that**:**

4. **A molecule that is not a chromophore for a studied range of wavelengths can interact with these non-absorptive wavelengths and be polarized through the electric field that light produces in the medium. This interaction can be demonstrated by measuring the refractive index of a dielectric medium that ATP in solution is, and whether this refractive index changes after exposing the solution to the studied range of wavelengths.**

5. **The interaction of non-absorptive wavelengths modifies important physical properties of a molecule that are not produced by absorption of resonant frequencies. ATP show intrinsic fluorescence when excited with ultraviolet light. It will be studied whether visible and near-infrared wavelengths are able to modify the intrinsic fluorescence of ATP.**

6. **This interaction between ATP and the electric field induced by light in the medium can produce measurable and significant differences in the kinetic parameters of biochemical reactions in which ATP is involved. This may be demonstrated by adding light-irradiated ATP to the reaction and measuring its kinetic parameters. These measurements will be performed in two different reactions: the luciferine-luciferase reaction and the phosphorilation of glucose mediated by hexokinase.**

3. MATERIAL AND METHODS

3.1. TEMPERATURE MEASUREMENT: CONTROL

The bath temperature of distilled water and ATP samples at all ATP concentrations was monitored before, during and after laser irradiation with visible and infrared wavelengths (635, 655 and 830 nm) in order to determine whether laser irradiation produced a change in the temperature of the preparation. During the experiment, the thermistor probe (YSI Reusable Temperature probe - YSI Incorporated 402; resolution ± 0.2°C) was placed in contact with the solution and attached to a centralized data logger (Letica - TMP 812, Rovira i Virgili University, Reus, Spain).

3.2. ATP IMPURITIES MEASUREMENT: CONTROL

ATP powder obtained from Sigma had several impurities. These were metallic and non-metallic atoms. Sigma informed that the contaminating atoms were chloride, aluminum, calcium, copper, iron, lead, magnesium, zinc, potassium (0.1275 %) and inorganic phosphorus (0.1 %). The absorbance at 635, 655 and 830 nm of a cocktail of the contaminating atoms at the actual concentration of the experiments was measured using a Shimadzu ultraviolet-visible near-infrared scanner spectrophotometer UV-310PC (Food and Drug Administration, FDA, Division of Physics, Rockville USA).

3.3. THE LUCIFERINE-LUCIFERASE REACTION

3.3.1. Reagents

A Sigma FL-AA ATP bioluminescent assay kit was used:

a) The *assay mix* was a lyophilized powder containing luciferase, luciferine, $MgSO_4$, DTT, EDTS, bovine serum albumin and tricine buffer salts. This is stable indefinitely if it is stored desiccated below 0ºC and protected from light.

b) The *ATP standard* was a pre-weighed lyophilized powder. Each bottle contained approximately 1 mg ($2.0x10^{-6}$ mols) of ATP. This is stable if desiccated below 0ºC.

3.3.2. The reaction

The firefly reaction is a standard model for quantifying ATP concentration based on the reaction:

$$\text{Luciferine} + \text{ATP} + O_2 \xrightarrow{\text{luciferase}} \text{Oxiluciferine} + \text{AMP} + \text{PPi} + CO_2 + \text{luminescence}$$

3.3.3. Luminescence detection

A LKB Wallac 1250 luminometer was used to measure light at 562 nm. A LKB Bromma 2210 two-channel recorder was used to obtain the graph of the luminescence signal (Histology and Neurobiology Unit of the Faculty of Medicine and Health Sciences of the Rovira i Virgili University).

3.3.4. Light sources

Two AsGaAl diode lasers were used—one at 635 ±10 nm (16 mW output power) and one at 830 ±10 nm (32 mW). The spot area in both lasers was 0.25 cm^2. Irradiation was made from the top of the cuvette with the light source directly in contact with the solution. The radiant exposure was 1 J/cm^2, 4 J/ cm^2 and 6 J/ cm^2. The power was measured with a Melles Griot 13 PEM 001 power/energy meter.

3.3.5. Technical procedure and experimental groups

The *assay solution* was the assay mix diluted in 5 ml of sterile aqua distillate at pH 7.8 and stored at 0ºC.

Three different *ATP solutions* were used: 2, 0.2 and 0.02 nM. These were stored in ice for 1 hour before use.

Five groups were formed. Two of these were system controls and three were control-experimental groups. The three control-experimental groups were used as controls when the ATP was not irradiated and as experimental groups when ATP was irradiated.

The five groups were as follows:

1. The assay solution alone (system control group);

2. The three ATP solutions alone (system control group);

3. 2 nM ATP solution plus assay solution (control group (non-irradiated ATP) and experimental group (irradiated ATP);

4. 0.2 nM ATP solution plus assay solution (control group (non-irradiated ATP) and experimental group (irradiated ATP);

5. 0.02 nM ATP solution plus assay solution (control group (non-irradiated ATP) and experimental group (irradiated ATP);

ATP samples were made by mixing 100 µl of non-irradiated or irradiated ATP solution (at different radiant exposures) with 100 µl of non-irradiated assay solution.

The total number of recordings for the experiment was 314. These were taken as follows:

First group: 1 measurement

Second group: 1 measurement

Third group: 2 nM ATP/ assay solution. 16 measurements for non-irradiated ATP; 48 measurements of 635 nm and 48 of 830 nm at the 3 different radiant exposures.

Fourth group: 0.2 nM ATP/ assay solution. 16 measurements for non-irradiated ATP; 48 measurements of 635 nm and 48 of 830 nm at the 3 different radiant exposures.

Fifth group: 0.02 nM ATP/ assay solution. 16 measurements for non-irradiated ATP; 48 measurements of 635 nm and 48 of 830 nm at the 3 different radiant exposures.

ATP samples were heated to 37 ºC then irradiated at 1 J/cm^2, 4 J/ cm^2 and 6 J/ cm^2 radiant exposure. Room temperature was kept constant and the study was performed in the dark. We used polystyrene cuvettes, which are good for measuring in the visible part of the spectrum (emission was at 562 nm).

The light emission of the reaction measured by the luminometer began immediately after all the reagents were inserted.

A peak in intensity, expressed as peak voltage V_0 of the luminometer, occurred about one second later. As the reaction progressed and the ATP content in the sample decreased, the luminometer voltage V decayed from the V_0 peak voltage to zero. For the analysis we assumed an exponential decay of the luminescence signal

$$V(t) = V_0 \exp(-kt)$$

where k is the rate constant of the light decay (in min^{-1}) and t is the time (in minutes) after the initial peak V_0. It was measured V(t) at t = 1 minute and derived k from

$$k = \ln [V_0 / V_{1\ min}]$$

The area under the luminescence curve was determined by the integral of the measured V(t) curve. This was equal to V_0 divided by k (the unit is Volt minutes).

3.3.6. Statistical study and data analysis

Statistical analyses were conducted with GraphPad Prisma software, using the ANOVA test complemented with the Bonferroni test. The level of significance was 95% ($p<0.05$).

3.4. ABSORPTION OF ATP

We used a Jasco FP-750 spectrofluorometer (Laser Centrum, University of Amsterdam, The Netherlands) to measure absorption, and varied the wavelengths from 200 nm to 900 nm in 1 nm steps. From 200 to 300 nm we studied a concentration of 100 μM to avoid saturation. From 300 to 900 nm, we studied a

concentration of 2 mM to obtain a better signal. We also measured absorption with the same parameters as a ATP-Mg^{2+} 100 μM/ 2 mM solution and a 2 mM $MgCl_2$ solution.

Room temperature was kept constant and the study was performed in the dark. Quartz cuvettes were used because polystyrene cuvettes have absorption in the UV.

3.5. THE HEXOKINASE REACTION EXPERIMENT

3.5.1. Reagents, concentrations and handling

The following chemical compounds were used: hexokinase IV (glucokinase), ATP disodium salt, β-NADP, glucose-6-phosphate dehydrogenase (G6PDH), glucose, Tris-HCl and $MgCl_2$ (Sigma). Solutions were made with distilled water.

The concentrations were: for the buffer, 0.06 M Tris-HCl, 6 mM $MgCl_2$ at pH 8.0; glucose at 200mM, NADH at 80mM, glucokinase 1000 U/ ml, G6PDH 1000 U/ ml, and ATP at 0.5, 1, 5, 10, 50 and 100 mM. The substrates and enzymes were diluted before each measurement when the samples were prepared with Tris-HCl $MgCl_2$ (20-fold). ATP concentrations were 0.025, 0.05, 0.25, 0.5, 2.5 and 5 mM. ATP and the enzymes were prepared fresh daily and stored in ice.

3.5.2. Light sources, power, dose parameters and irradiation procedure

For the hexokinase experiment, two AsGaAl CW diode lasers were used (Sorisa, Spain). One of these diodes emitted at 655 ±10 nm (200 mW output power) and the other emitted at 830 ±10 nm (100 mW). The spot size, 0.25 cm^2, was the same

for both lasers. The power density was 70 mW/cm^2 at the surface of the ATP solution for both lasers. For the red laser, irradiation was through an optical fiber introduced into the cuvette that touched the solution. For the infra-red laser, irradiation was carried out directly from the diode. For the k_m and v_{max} experiments, the radiant exposure was 12 J/cm^2. For the dose-response experiment, the radiant exposures were 1, 3, 6, 9, 12, 15 and 18 J/cm^2.

3.5.3. Experimental groups

For the k_m and v_{max} measurement, control groups were created by adding non-irradiated ATP (at 6 different concentrations) to the reagents and enzymes. The experimental groups were created by adding irradiated ATP (at 6 different concentrations) to the solutions. Ten measurements were taken for each non-irradiated ATP group at six concentrations (60 measurements), ten measurements were taken for each irradiated ATP group at six concentrations with 655 nm laser light (60 measurements), and ten measurements were taken for each irradiated ATP group at six concentrations with 830 nm laser light (60 measurements) (Table 3). Irradiation with both wavelengths was carried out at one fixed radiant exposure. The values of k_m and v_{max} were calculated from the initial velocities of the linear part of the reaction. Calculations were done independently for each control and experimental group.

Group ATP concentration (mM)	*Control* Non-irradiated ATP (*n*)	*Experimental* Irradiated ATP at 655 nm (*n*)	*Experimental* Irradiated ATP at 830 nm (*n*)
0.025	10	10	10
0.05	10	10	10
0.25	10	10	10
0.5	10	10	10
2.5	10	10	10
5	10	10	10

Table 3. Distribution of measurements (*n*) in control and experimental groups at different ATP concentrations in the k_m and v_{max} measurement experiment.

For the dose response measurement the concentration of ATP was 5 mM.

The control group was non-irradiated ATP (10 measurements), and the experimental groups irradiated ATP at 655 and 830 nm and seven different radiant exposures (140 measurements in total for all irradiated groups).

3.5.4. The hexokinase reaction

The main reaction was

$$\text{Glucose + ATP} \xrightarrow{\text{hexokinase}} \text{Glucose-6-phosphate + ADP}$$

The enzymatic cofactor is Mg^{2+}, which binds with ATP to form the ATP- Mg^{2+} complex, which is the true substrate of the reaction. The reaction follows

Michaelis-Menten kinetics against ATP concentrations. The reaction also follows Michaelis-Menten kinetics for glucose concentrations above 10 mM, but at lower glucose concentrations the curve inflects strongly, which indicates a sigmoid or co-operative dependence of the velocity [12]. This is a fundamental difference between this and other hexokinases, which follow Michaelis-Menten kinetics for both ATP-Mg and glucose.

3.5.5. The indicator reaction

An indicator reaction of the kinetics of hexokinase reaction was:

$$\text{Glucose-6-phosphate} + NADP^{+} \xrightarrow{\text{G6PDH}} \text{phosphogluconate} + NADPH$$

In this reaction, the cofactor is also Mg^{2+}. The glucose-6-phosphate product of the main reaction is the substrate of the indicator reaction, so both reactions have parallel kinetics. $NADP^{+}$ absorbs light at 340 nm, and the reduced form (NADPH) does not.

The Cecil 2030 CE spectrophotometer was set to measure the absorption at 340 nm as a function of time. When the reaction occurs, $NADP^{+}$ is transformed into NADPH and the absorption at 340 nm decreases over time. We used this velocity (absorbance *(OD340)*/min) as the parameter for the kinetic calculations.

3.5.6. Experimental procedure

The assay solution included glucose, enzymes (hexokinase and G6PDH), NADP, ATP and buffer. ATP solutions (0.05 ml) at different concentrations (0.025 to 5 mM) were added to 0.85 ml of buffer. ATP solution plus buffer (0.9 ml) was irradiated for the experimental group. In the spectrophotometer, the ATP plus buffer solution (0.9 ml) was placed in a cuvette; NADP (0.05 ml) was then added and the spectrophotometer was started to measure the signal. The glucose plus enzyme solution (0.05 ml with 0.1 U of each enzyme) was then added and the reaction effectively started. The sample in the spectrophotometer had a volume of 1 ml. The first measurement was taken at 20 seconds. The velocity was measured every 20 seconds for 3 minutes. All experiments were performed at room temperature (22^{o} C).

3.5.7. Spectrophotometer for kinetic measurements

The measurements were taken with a Cecil 2030 CE spectrophotometer and the built-in kinetic program was used.

3.5.8. Dose-response measurement

The dose-response measurements were calculated with the initial velocities (OD340/min) in the linear part of the reaction.

3.5.9. Statistical analysis

For statistical analysis, we used the GraphPad Prism software. The measurements of the velocity were tested with one-way ANOVA complemented with Tukey's Multiple Comparison Test. The level of significance was set at $p<0.05$.

3.5.10. Graphic representation and non-linear regression analysis for kinetic measurements (k_m and v_{max}) and fluorescent measurements

The kinetic measurements at the different ATP concentrations were analyzed with GraphPad Prism software and fitted with a non-linear regression curve. This software determines the plateau (v_{max}), and the concentration of ATP at which the velocity is $v_{max}/ 2$ (k_m). The v_{max} parameter is the maximum velocity of a specific reaction at the experimental conditions. It refers to the turnover number of the enzyme or the number of units of substrate transformed into product in a unit of time. The Michaelis constant k_m indicates the dissociation rate of the enzyme-substrate complex (ES) and its strength.

R^2 and F tests were performed to quantify the goodness of fit. R^2 is the fraction of the total squared error that is explained by the model. Values approaching one are desirable. The F Test compares several possible fitting options in non-linear regression. The experimental curve was compared to several models. The results were plotted using Graph Pad Prism and Origin software.

3.6. FLUORESCENCE OF ADENOSINE N-PHOSPHATE MOLECULES

3.6.1. Reagents, concentrations and handling

ATP disodium salt, adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenine, adenosine and $MgCl_2$ (Sigma) were used. The studied concentration for ATP, ADP, AMP, adenine and adenosine was 2 mM. To investigate the binding properties of ATP with Mg^{2+}, the ATP concentration was 20 μM. $MgCl_2$ was used at several concentrations from 20 to 360 μM. Solutions were made with distilled water, prepared fresh daily and stored in ice.

3.6.2. Spectrofluorometer

We used a spectrofluorometer Photon Technology International PTI (Food and Drug Administration, Division of Physics, Rockville, United States) with an 814 Photomultiplier Detection System module and the Felix 32 Analysis Mode software. The slit that used for the measurements was 0.125 mm. The integration time was set at 0.2 s and the measurements were taken every 1 nm.

3.6.3. Light sources and irradiation procedure

Additional irradiation with visible and near-IR light of ATP solution inside the spectrofluorometer was carried out through the excitation lamp that alternated 260 nm light with 655 nm or 830 nm light. The frequency of the alternation was 650 Hz.

3.6.4. Experimental groups

The fluorescence of ATP, ADP, AMP, adenine and adenosine was investigated after light excitation at 260 nm in the spectrofluorometer. In another experiment, ATP (20 μM) light emission was measured against several Mg^{2+} ($MgCl_2$) concentrations (0- 360 μM). The integral of the fluorescence curve from 325 to 475 nm was used in the calculations. The third experiment in fluorescence was performed with ATP (20 μM) as the first solution, and ATP (20 μM) plus Mg^{2+} (40 μM) as the second solution. Both were excited with 260 nm light for the control group, and with 260 nm plus 655 nm and 260 nm plus 830 nm for the two experimental groups. The control groups were excited in both parts of the cycle at 260 nm light, and fluorescence was then measured from 300 to 500 nm. The experimental groups were excited alternately with 655 or 830 nm light in the first part of the cycle and with 260 nm light in the second part. Fluorescence was measured in both parts of the cycle at 300, 325, 350, 375, 400, 425, 450 and 475 nm.

3.6.5. Statistical analysis

For statistical analysis, we used the GraphPad Prism software. The measurements of ATP and ATP-Mg^{2+} after irradiation with 655 and 830 nm light were tested with one-way ANOVA complemented with Tukey's Multiple Comparison Test. The level of significance was set at $p < 0.05$.

3.6.6. Graphic representation and non-linear regression analysis for kinetic measurements (k_m and v_{max}) and fluorescent measurements

ATP light emission with several Mg^{2+} concentrations were analyzed with GraphPad Prism software and fitted with a non-linear regression curve.

R^2 and F tests were performed to quantify the goodness of the fit. The results were plotted using Graph Pad Prism and Origin software.

3.7. THE MICHELSON INTERFEROMETER EXPERIMENT

3.7.1. Solutions

Distilled water and a 1.4 M ATP solution made with distilled water were used.

3.7.2. Light source

A non-polarized Melles Griot Helium Neon gas laser (632.8 nm, 5 mW) was used as the light source (Food and Drug Administration, Division of Physics, Rockville, United States).

3.7.3. Experimental setup

The Michelson interferometer (Fig. 4) is an established setup for observing the phenomenon of the interference that light produces with itself. The beam from the He-Ne laser was split into two beams, and each of these followed a different path. These beams were reflected by mirrors (M_1 and M_2) and made to reconverge. The resulting beam was projected onto a screen. Since each beam follows a different path, when they reconverge light arrives in a different phase. This difference

produces an interference that is observed in the screen by alternated bright and dark lines. A quartz cuvette was placed in one of the two channels and the interference pattern was recorded by a digital camera when the cuvette was filled with air, water and 1.4 M ATP. Light passed through the medium twice, once when going to the mirror M_1 and once when returning from it.

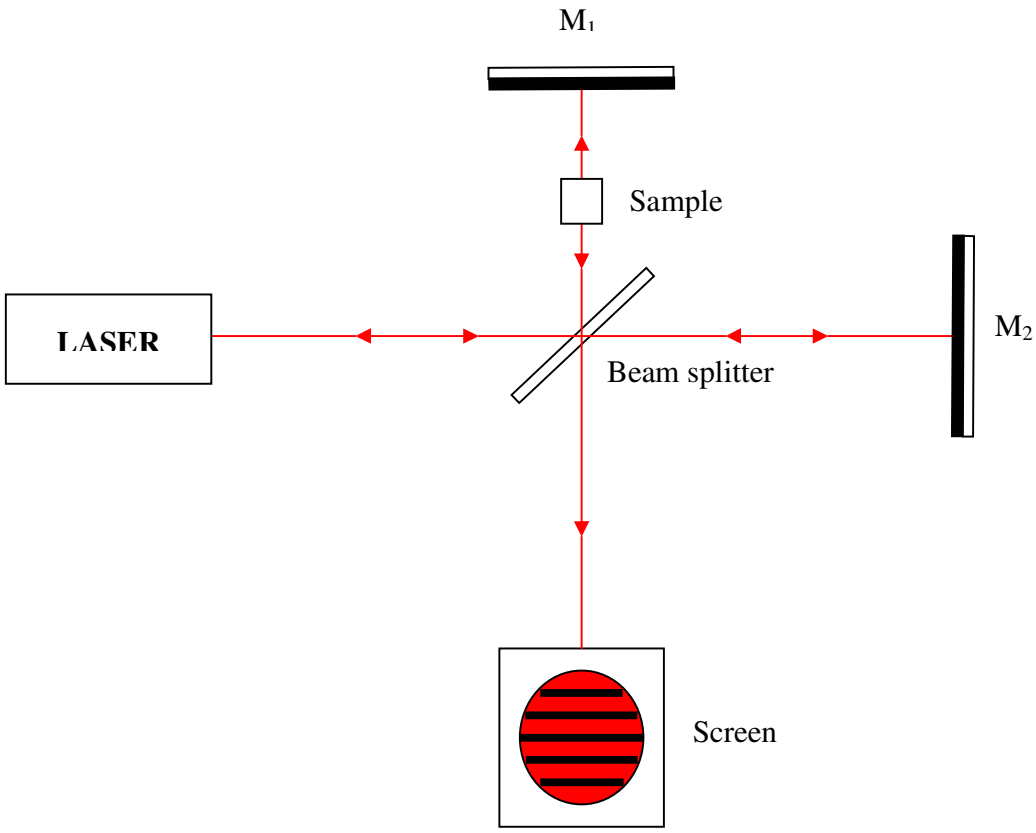

Figure 4. The Michelson interferometer setup, with the light source (laser), the splitter, the two different paths that light follows, the reflecting mirrors (M_1 and M_2), the place for the cuvette (sample) and the screen that showed the interference pattern.

3.7.4. Image analysis

A digital picture of the screen showing the interference pattern was taken, and the images were analyzed using Scion Image software. Three parameters were considered: the thickness of the bright lines, the distance between them (the thickness of the dark lines) and the distance for five consecutive bright lines (the density of the lines).

3.8. THE MEASURE OF THE REFRACTIVE INDEX (RI)

3.8.1. Solutions

Distilled water, NaCl 1.4 M solution and 1.4 M ATP solution (both with distilled water) were prepared.

3.8.2. Light sources and irradiation procedure

A non-polarized Melles Griot Helium Neon gas laser (632.8 nm, 5 mW), a diode laser (405 nm, 2 mW), a Melles Griot Helium Neon gas laser (514 nm, 1 mW), a diode laser (969 nm, 180 mW) and a Thorr DD-II diode laser (810 nm, 160 mW) were used as light sources. All were set to a1 mW power through optical filters. Irradiation was carried out directly in the liquids placed in the device. Irradiation was stopped before every measurement to avoid interference with the measurement process. A control group was made by exposing an ATP solution to ambient light during 10 minutes. RI was measured every 1 minute.

To investigate the effects of the concentration on the refractive index changes, it was used four ATP solutions (1400, 700, 350 and 175 mM) and excited with 514 nm laser light.

3.8.3. Refractometer

A Palm Abbe Digital Refractometer (MISCO Cleveland, Ohio US; Food and Drug Administration, Division of Physics, Rockville, United States) was used to measure the refractive index at 589 nm. The accuracy of the device was ± 0.0001.

3.8.4. Experimental groups

Water and NaCl solution were used as controls and ATP was used as the experimental group. The refractive index of the three liquids was measured before irradiation and after every minute of irradiation for up to 10 minutes. The samples were also heated by applying warm air to determine whether increasing the temperature had any effect on the RI. The temperature during the experiment was also recorded since the interferometer measures sample temperature as well as the refractive index.

4. RESULTS

4.1. TEMPERATURE MEASUREMENT IN THE SOLUTION

Temperatures were recorded for 5-minute periods. There were no significant differences between the sample temperatures of the control preparation and those of the laser-irradiated preparations with the different parameters between 1 and 5 minutes after irradiation. The room temperature during these experiments was 25.9 ± 0.1°C.

4.2. ATP IMPURITIES

Absorbance at 655 and 830 nm of a cocktail of the contaminating atoms (at concentrations equal to those in the ATP compound) was measured. At both wavelengths the absorption was negligible (data not shown).

4.3. LUCIFERINE-LUCIFERASE REACTION

4.3.1. Exponential behaviour of V(t)

Six graphs were plotted and in each graph an exponential decay was observed. It was therefore assumed that all the others were also exponential (see Fig. 5).

In the first and second control groups, no luminescence emission was observed. The control of third group gave the luminescence signals that were used as the base line. V_0 and k were measured and the results were normalized to 100. The standard deviation was 14 for V_0 and this was normalized to 2. The standard deviation was 0.06 for k.

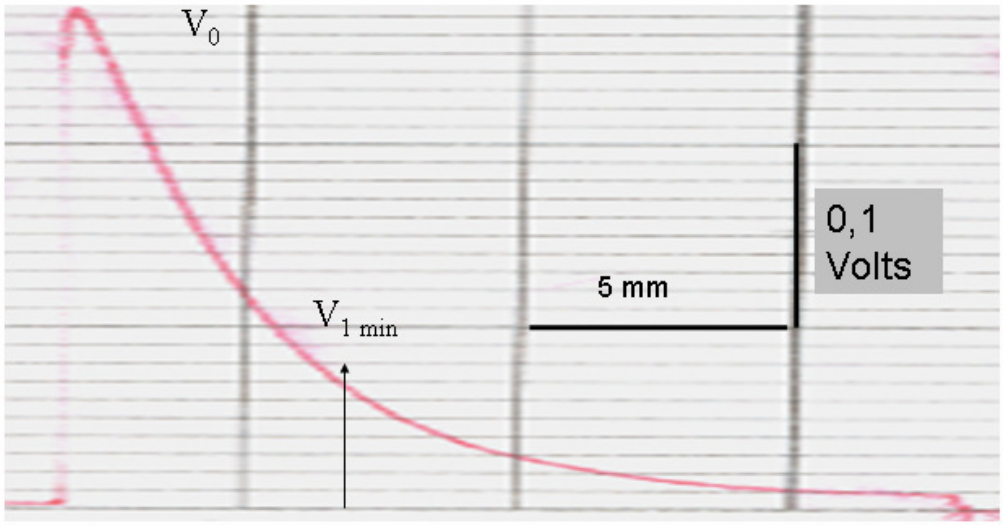

Figure 5. Example of a graphical recording of the outcoming signal of the luminometer in a control measurement. V_0 (initial luminescence peak) and V_{1min} (luminescence after 1 minute) are shown. The scale is 5 mm: minute (x) and 10 mm: 0.1 V (y). This curve shows an exponential decay in luminescence.

4.3.2. Luminescence measurements

The luminescence of the luciferine-luciferase reaction was measured with non-irradiated ATP and irradiated ATP at the three ATP concentrations. Significant differences were observed only at 2 nM (Fig. 6).

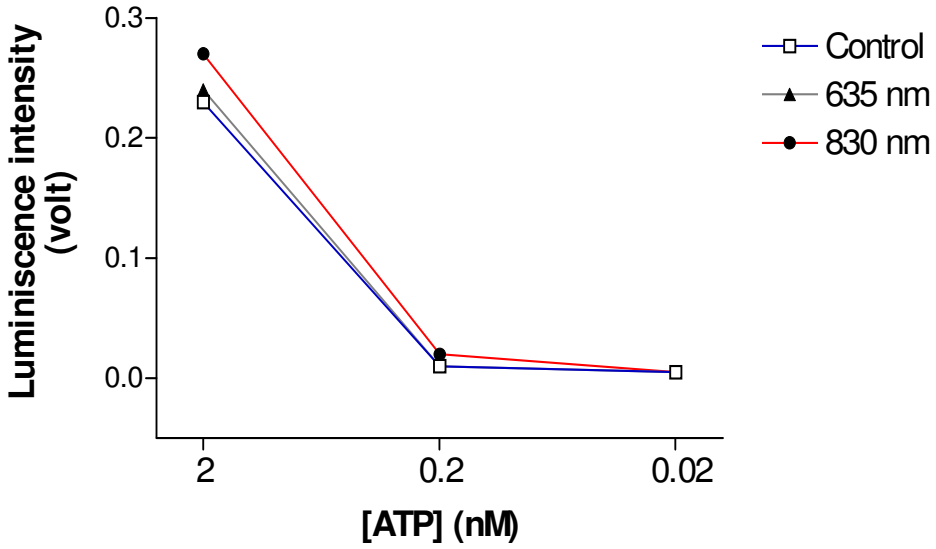

Figure 6. Luminescence measurement of ATP at 2, 0.2 and 0.02 nM in the control groups and with 635 and 830 nm irradiation at 4 J/cm^2. Changes are observed only at the first ATP concentration. This concentration was therefore used in all subsequent experiments.

4.3.3. Luminescence peak (V_0)

At 2 nM concentration, the results showed significant differences in the luminescence peak V_0 between the control and the irradiated groups at 4 and 6 J/cm^2 with the 635 nm laser and at 1, 4 and 6 J/cm^2 with the 830 nm laser. For the control group, values were normalized to 100 and the SEM (Standard Error Mean) was 11.9. Results for the irradiated groups are shown in Table 4 (average and SEM of the experimental values) and in Fig. 7.

	1 J/cm^2	4 J/cm^2	6 J/cm^2
635 nm	120.996 ± 7.94	131.83 ± 7.69	138 ± 9.42
830 nm	130.74 ± 5.24	134.98 ± 5.82	153.2 ± 5.82

Table 4. Normalized to the control (100) values of the V_0 luminescence peak for the three studied radiant exposures and the two wavelengths. Averages and SEM are shown.

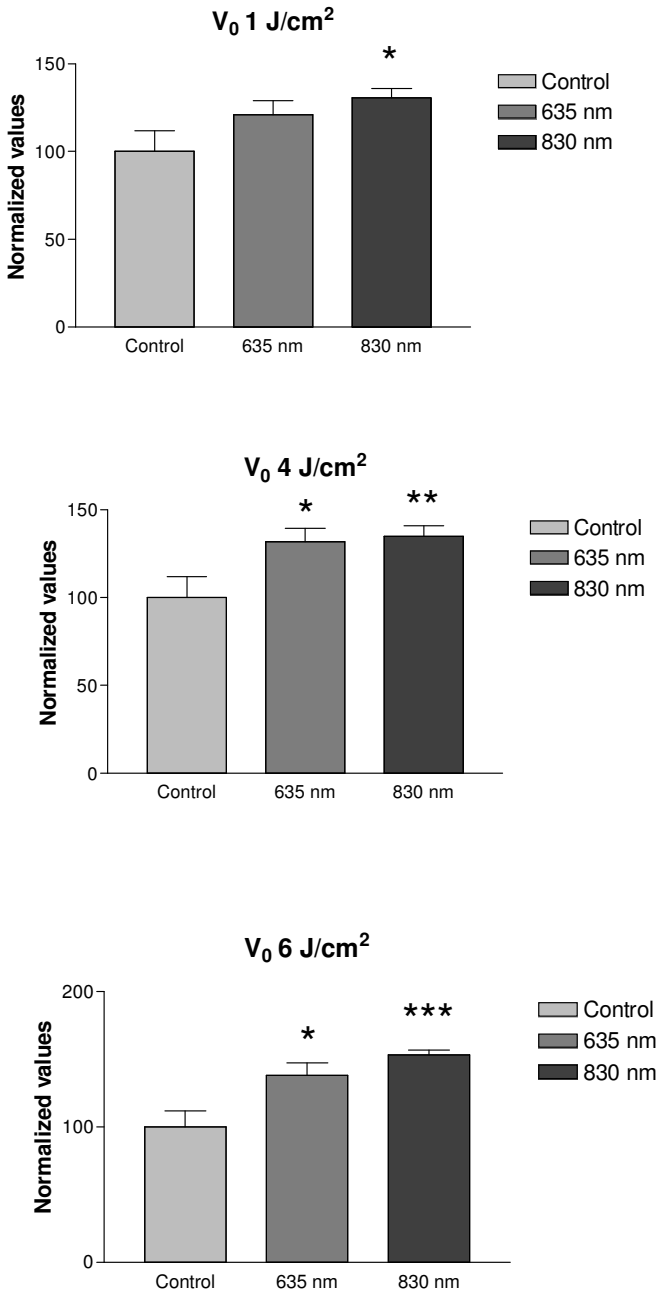

Figure 7. Normalized luminescence peak V_0 with 635 nm and 830 nm laser irradiation. Results were statistically significant at 6 J/cm^2 for the red laser and at 1, 4 and 6 J/cm^2 for the infrared wavelength (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

4.3.4. Reaction rate (k)

The reaction rate constant k was measured at 2 nM ATP. The k values were normalized to 2 min^{-1} ± 0.02, which were the values for the control group. Results were statistically significant with the 635 nm laser at 4 and 6 J/cm^2 and with the 830 nm laser at 1, 4 and 6 J/cm^2. The values for experimental groups are presented in Table 5 and graphically represented in Fig. 8.

	1 J/cm^2	4 J/cm^2	6 J/cm^2
635 nm	2.04 ± 0.018	2.08 ± 0.022	2.108 ± 0.015
830 nm	2.08 ± 0.014	2.091 ± 0.018	2.111 ± 0.0091

Table 5. Normalized values to control (2 min^{-1}) of the kinetic value *k* in the luciferine-luciferase reaction. The results for experimental groups show the average of the measurements and the SEM.

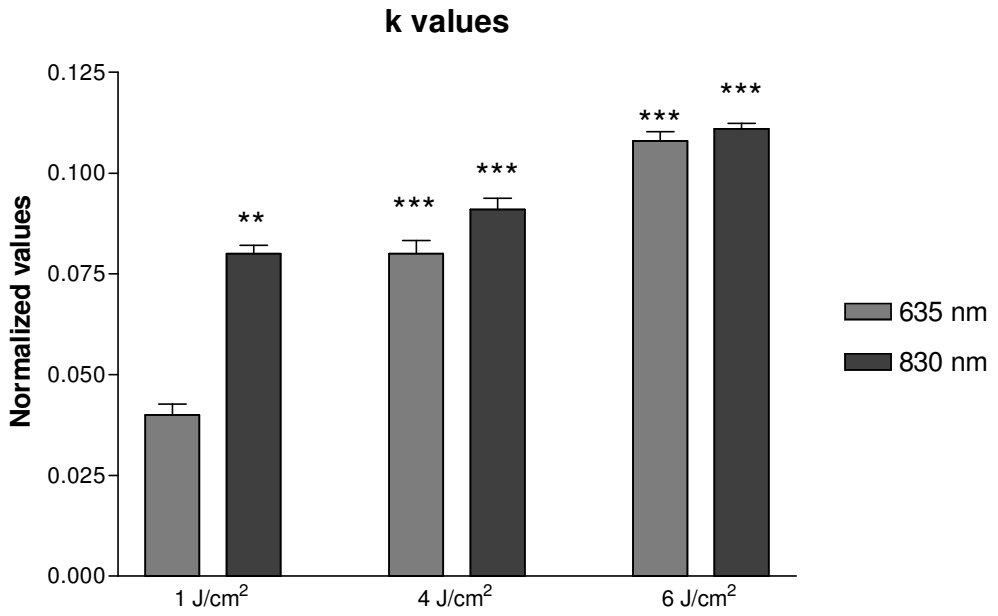

Figure 8. Normalized k values to the control (2 min^{-1}) of the reaction with 635 and 830 nm laser irradiation at 1, 4 and 6 J/cm^2. Both lasers at this dose increased the k of this ATP-dependent reaction at 4 and 6 J/cm^2. The 830 nm laser also did so at 1 J/cm^2 (** $p<0.01$; *** $p<0.001$).

The behavior of V_0 and k at 0.2 and 0.02 nM ATP concentration was not significantly different from that of the control group.

4.3.4. Area under the luminescence curve

In the control groups, the area under the luminescence curve was normalized at 100 % SEM 3.62. Experimental values are shown in Table 6 and represented graphically in Fig. 9. Significant differences were observed in all wavelengths and radiant exposures.

	1 J/cm^2	4 J/cm^2	6 J/cm^2
635 nm	118.622 ± 4.4	126.758 ± 3.49	130.92 ± 6.28
830 nm	125.7 ± 3.74	129.104 ± 3.23	145.14 ± 6.39

Table 6. Area under the V(t) curve values normalized to control (100%). Average and SEM are presented.

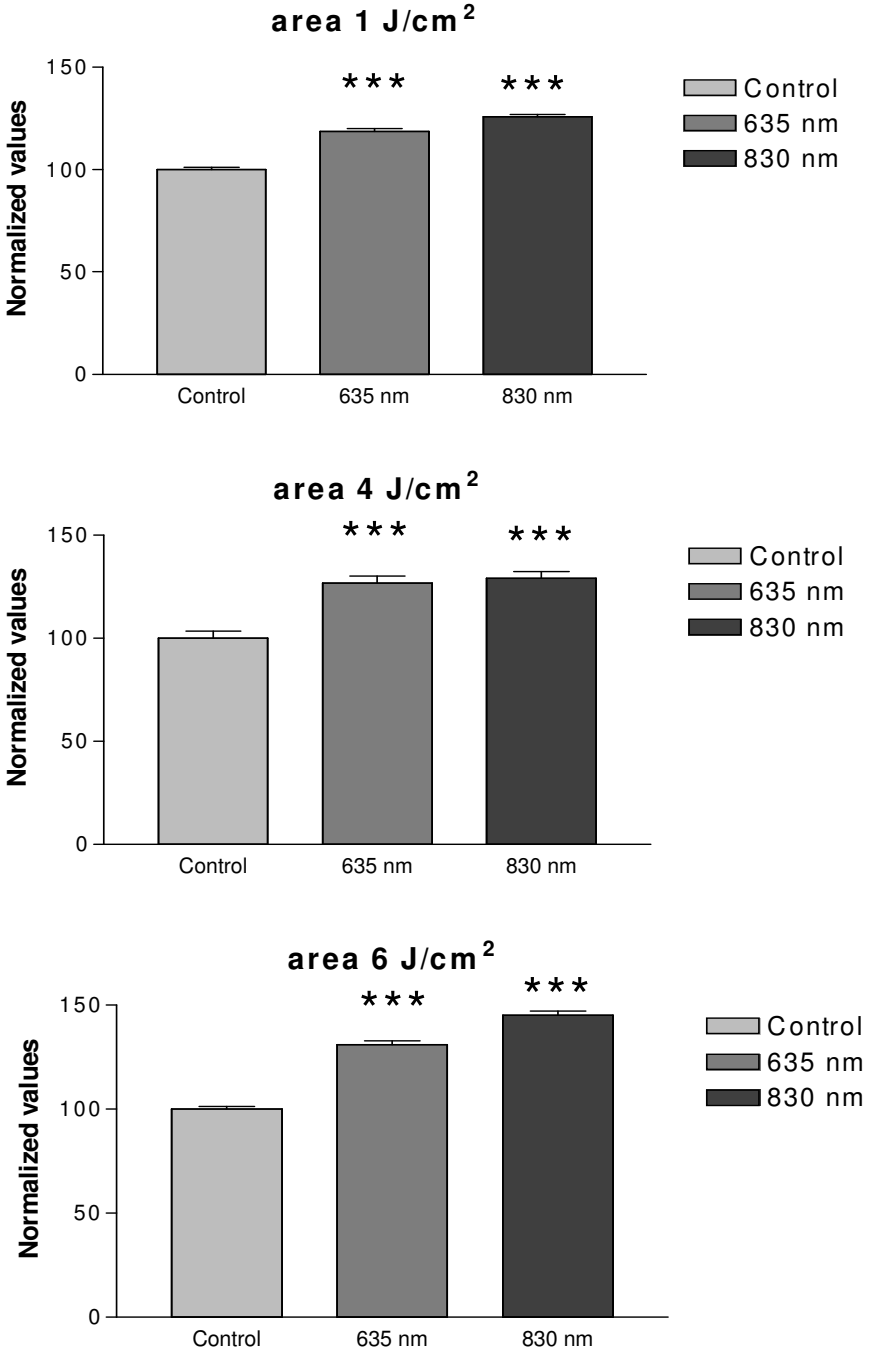

Figure 9. Normalized areas under the V(t) curve, proportional to the number of ATP molecules that reacted. Statistical significance was achieved with both wavelengths at 1, 4 and 6 J/cm^2 (*** $p<0.001$).

4.4. ABSORPTION OF ATP

The absorption values for the ATP molecule at 100 μM concentration are represented in two separate graphs. The first one, in Fig. 10, shows the absorbance values at the UV part of the spectrum from 200 to 300 nm. Fig. 11 shows the absorbance values of ATP from 600 to 850 nm, where the absorbance values compared to the UV are negligible.

The values of ATP-Mg^{2+} were the same as those of ATP.

$MgCl_2$ solution 2 mM showed negligible absorbance values in the range of wavelengths studied (200-300 nm, 600-850 nm).

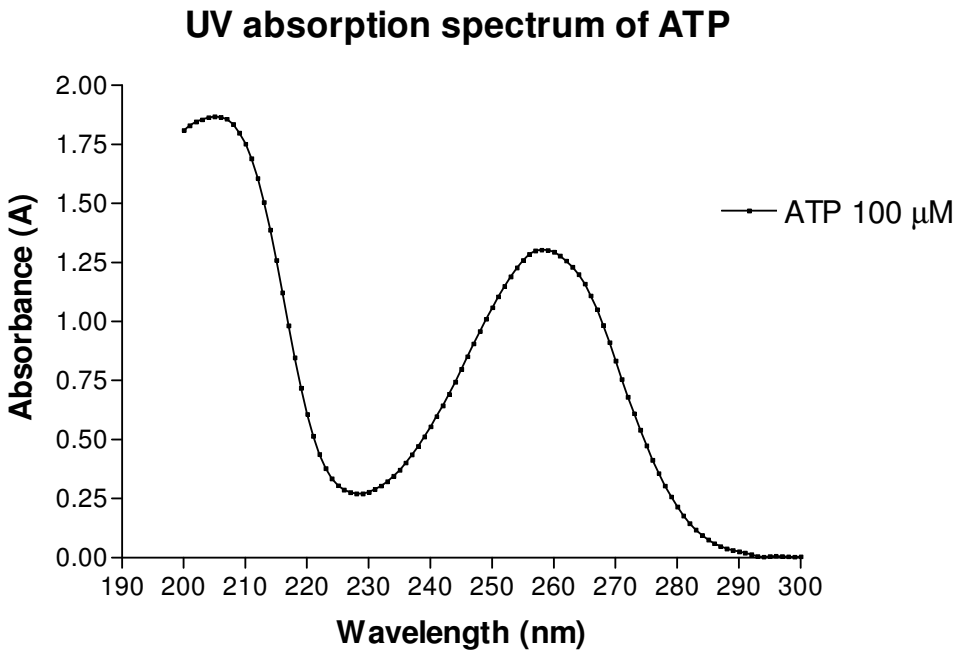

Figure 10. Absorption spectrum of 100 μM ATP in the UV part of the spectrum, from 200 to 300 nm. We can see one absorption peak at 210 nm and one at 260 nm, both with high absorbance values. For ATP-Mg^{2+} at the same concentration, the values are the same.

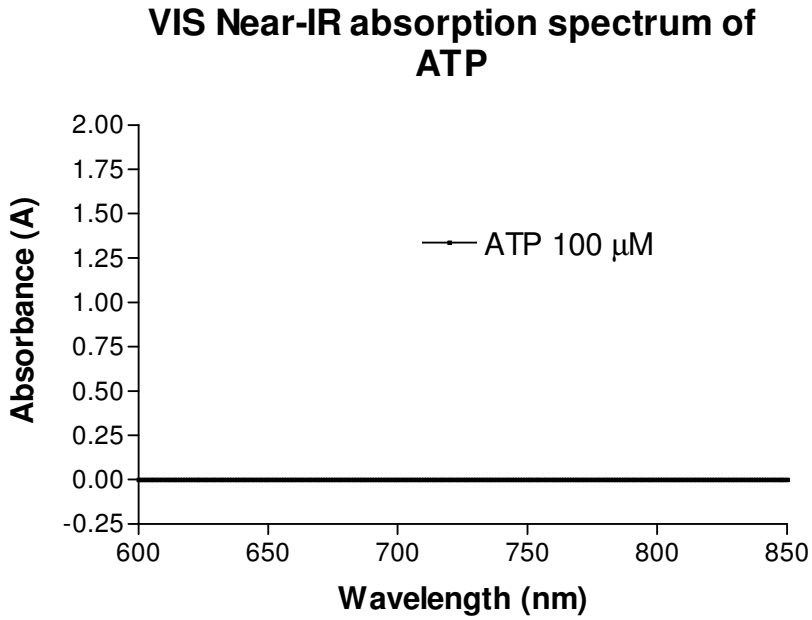

Figure 11. Absorption spectrum of ATP and ATP-Mg^{2+} at 100 µM concentration. The results show that the absolute units of absorbance are negligible compared to UV absorption.

4.5. THE HEXOKINASE REACTION

4.5.1. Kinetics measurements

The velocity of the hexokinase reaction was significantly different (p value from 0.0002 to 0.0362) in control and irradiated groups for all ATP concentrations and from 20 to 160 seconds. From 40 to 140 seconds the velocity was linear. For the initial rates of the linear part of the reaction the p value was 0.0002. At 180 seconds the reaction finished. The average of the initial velocities in the linear part of the reaction and the SEM (Standard Error Mean) for the six ATP concentrations (0.025 to 5 mM) are shown in Table 7.

[ATP] mM	Control Velocity (OD340/min)	SEM (OD340/min)	655 nm Velocity (OD340/min)	SEM (OD340/min)	830 nm Velocity (OD340/min)	SEM (OD340/min)
0.025	0.012	0.001	0.022	0.001	0.018	0.001
0.05	0.027	0.002	0.042	0.004	0.041	0.002
0.25	0.034	0.001	0.045	0.001	0.044	0.002
0.5	0.043	0.003	0.052	0.002	0.050	0.001
2.5	0.071	0.001	0.088	0.002	0.086	0.002
5	0.078	0.002	0.105	0.004	0.093	0.004

Table 7. Average velocity and SEM in the linear part of the hexokinase reaction for the six ATP concentrations.

Fitting to the hyperbolic curve was tested by R^2 values. R^2 was 0.9865 for the control group, 0.9907 for 655 nm and 0.9316 for 830 nm. We performed the F Test to compare fitting options. The chosen curve showed the best significance ($p<0.0001$).

Calculations were done by treating data from the control, 655 nm and 830 nm groups as independent data since there were different and independent reactions. After the data was successfully fitted with non-linear regression we observed that the values of k_m and v_{max} of the reaction were different for each group (Table 8 and Figs. 12 A and B, 13).

	Control	655 nm	830 nm
k_m (mM)	0.385 ±0.011	0.732 ±0.03	0.482 ±0.02
v_{max} (OD340/min)	0.07862 ±0.001	0.1166 ±0.003	0.1044 ±0.003

Table 8. Kinetic values of the reaction for non-irradiated ATP (control) and irradiated ATP groups. The kinetic parameters are significantly different between the control and the irradiated groups. The kinetic parameters of the various experimental groups are also significantly different

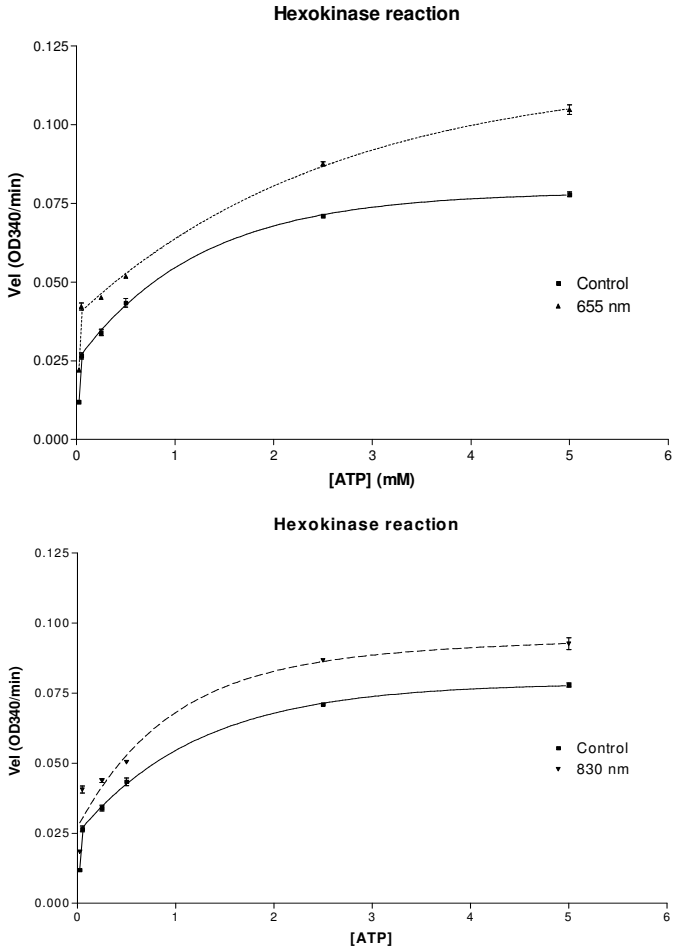

Figure 12 A and B. Michaelis-Menten graphs of the glucokinase reaction comparing the control group with the 655 nm and 830 nm irradiated groups. In the first graph the control group is at the plateau (v_{max}) when the irradiated group has not yet reached it. The second graph shows that 830 nm irradiation has a greater velocity than the control group and that the plateau is reached at a higher velocity.

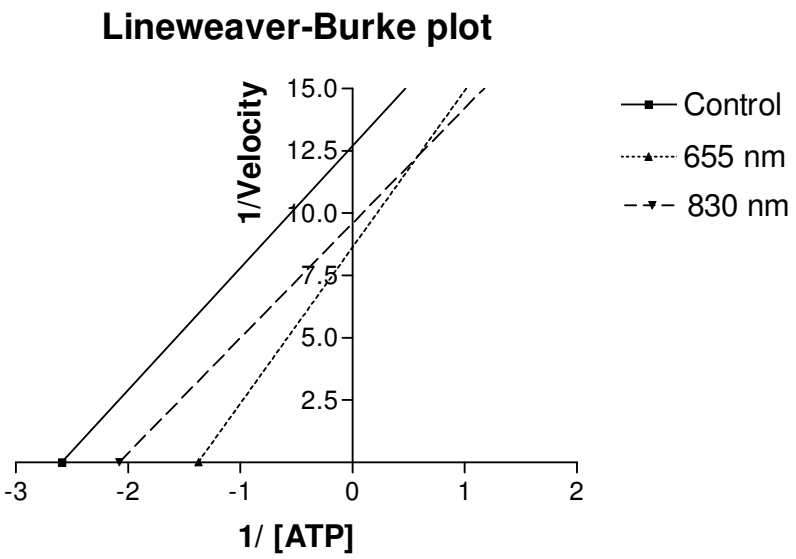

Figure 13. Lineweaver-Burke plot of the kinetic parameters of the hexokinase reaction. This figure shows the k_m and v_{max} values graphically, but it is not used to calculate them, since non-linear regression give more accurate information about the experimental results. The X axis-intercept equals $-1/k_m$ and the Y-intercept equals $1/v_{max}$. The slope equals k_m/v_{max}.

4.5.2. Dose-response measurements

The activation threshold was 6 J/cm^2 for both wavelengths. At less radiant exposures no changes were observed. Both wavelengths showed a plateau that began at 12 J/cm^2 and there was no inhibition threshold (Fig. 14).

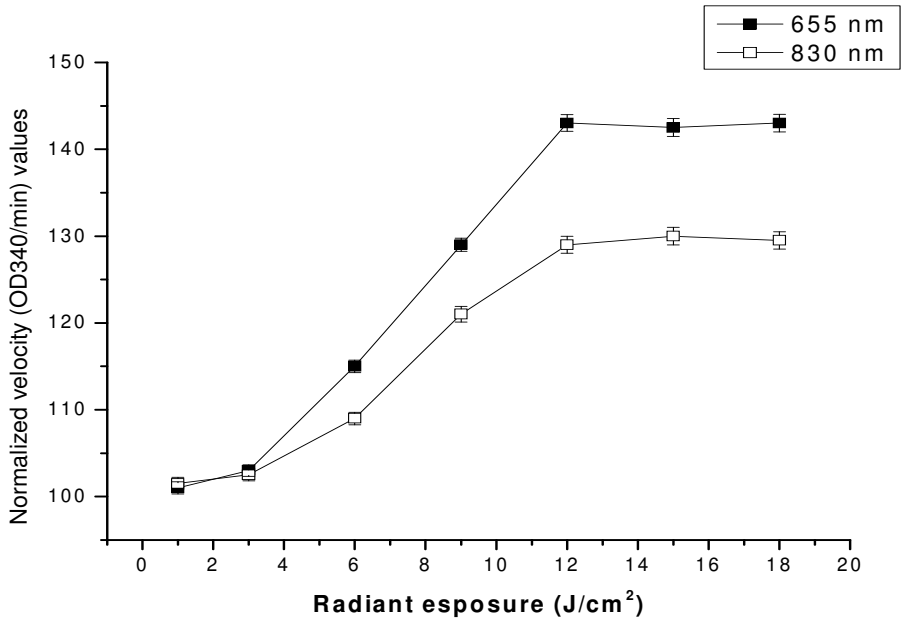

Figure 14. Dose-response curves of the hexokinase reaction were 5 mM. ATP was irradiated with 655 nm or 830 nm light. The data were normalized to control (non-irradiated ATP) velocities (100). The error bars correspond to the SEM.

4.6. FLUORESCENCE OF ADENOSINE N-PHOSPHATE MOLECULES

ATP emitted light in the 310-500 nm range when excited with 260 nm light. ADP showed less fluorescence intensity and AMP had a very weak when excited at 260 nm light. Adenine and adenosine did not have a significant fluorescence (Fig. 15)

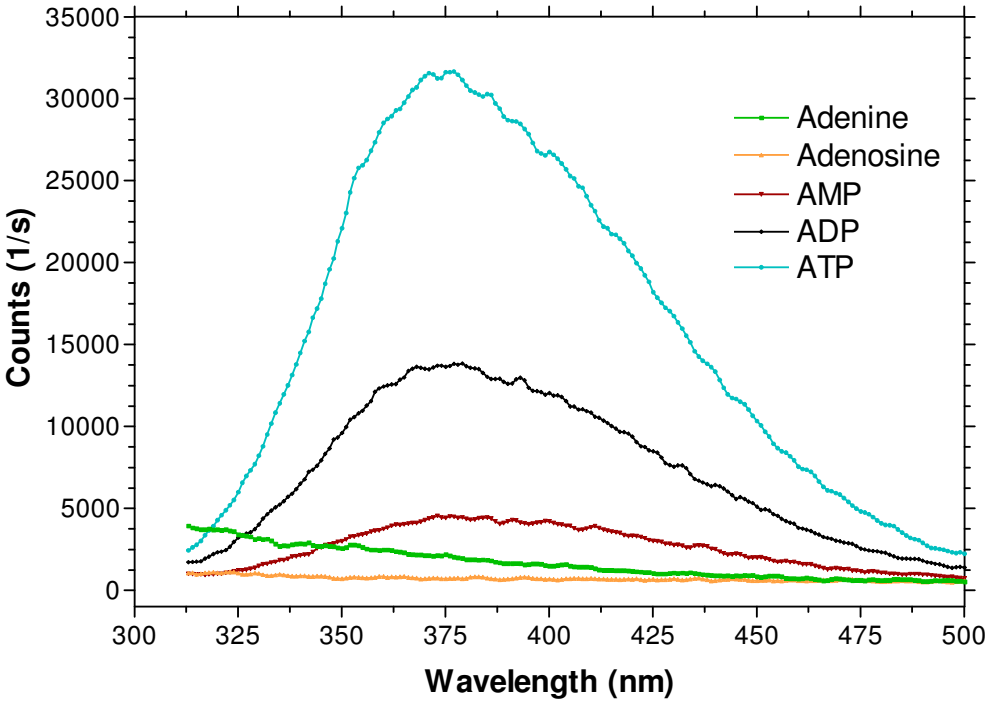

Figure 15. Fluorescence measurements for the three studied adenosine phosphate molecules, ATP, ADP and AMP, adenine and adenosine. The graph shows the range from 300 to 500 nm. ATP has greater fluorescence than ADP and AMP. Adenine and adenosine does not show a significant fluorescence.

When Mg^{2+} was added to the ATP solution, light emission decreased. The shape of the model that best fit the experimental results was a two-way exponential decay (Fig. 16). The R^2 value for the curve was 0.9919, against 0.9806 for one-way exponential decay. The *plateau* was reached at 350215 area values, and the fast decay finished at a Mg^{2+} concentration of 45.32 mM. This was followed by the slow decay of the curve.

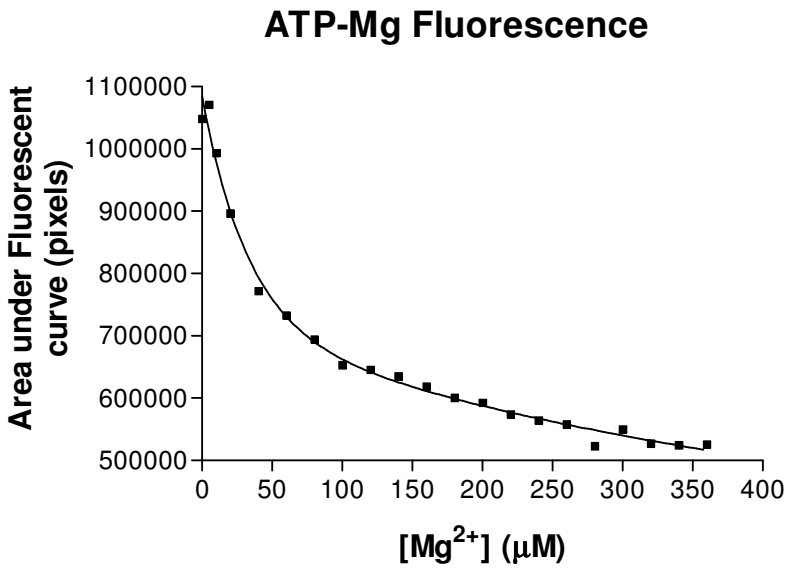

Figure 16. Fluorescence of ATP at different concentrations of Mg^{2+}. The signal decreases exponentially. Decay is fast at low Mg^{2+} concentrations and slow at higher concentrations.

In the next experiment, fluorescence measurements were taken with ATP (20 μM) and ATP (20 μM) plus Mg^{2+} (40 μM) after 655 nm and 830 nm light interaction. ATP only fluoresced after excitation with 260 nm light. After 655 or 830 nm light there was no light emission but this irradiation had an effect on the measured fluorescence after 260 nm excitation (if the solution was Mg^{2+} ATP). There was no significant difference in the light emission of ATP (20 μM) excited with 260 nm or ATP (20 μM) excited with 260 nm plus 655 or 830 nm.

ATP (20 μM) plus Mg^{2+} (40 μM) showed significant differences ($p<0.001$ value) between the control and the irradiated groups in the fluorescence intensity from 350 to 475 nm. There were also differences (p values from 0.05 to 0.001) between

irradiation at 655 and irradiation at 830 nm in the fluorescence intensity from 350 to 475 nm. The fluorescence of the two experimental groups was greater than that of the control group, and 655 nm had a greater effect than 830 nm (Fig. 17).

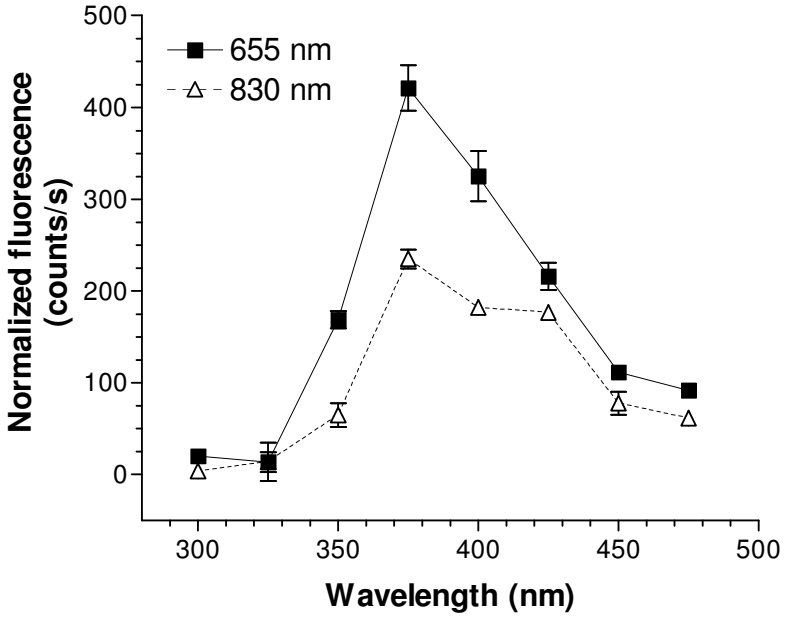

Figure 17. Normalized (to the control, 0) fluorescence intensity of the ATP-Mg^{2+} solution when the sample was irradiated with 655 and 830 nm light. Both wavelengths increased the signal, and the effects of 655 nm were greater than the effects of 830 nm light. Differences between control and irradiated groups were significantly different in the range 350 - 475 nm ($p<0.001$). Experimental groups were significantly different one from each other in the same range of wavelengths (p values from 0.05 to 0.001).

4.7. THE MICHELSON INTERFEROMETER EXPERIMENT

The interference pattern for a cuvette with air, water and 1.4 M ATP is shown in Fig. 18. The measurements performed on those pictures are shown in Table 9.

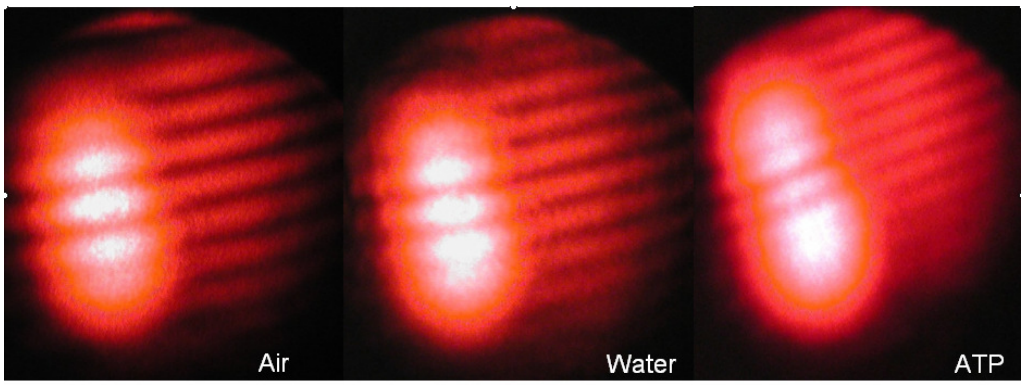

Figure 18. Interference pattern when a cuvette with air (first), water (second) and 1.4 M ATP (third) was placed in the path of the light in the Michelson interferometer. The density of the lines and the thickness of the bright and dark lines were different for each substance.

Media	Thickness of bright lines	Thickness of dark lines	Density (5 lines)
Air	4	1.5	2.4
Water	3.8	1.3	2.1
ATP (1.4 M)	3	1	1.7

Table 9. Measurement of three parameters (in mm) of the interference pattern created in the Michelson interferometer. The thickness of the bright and dark lines and the density of the bright lines were different for the three studied substances.

4.8. MEASUREMENT OF THE REFRACTIVE INDEX (RI)

The RI for water was 1.3327 at 589 nm. For a 1.4 M solution of NaCl, it was 1.3423, and for a 1.4 M solution of ATP, it was 1.4115. The temperature ranged between 21.2 and 21.4 °C when pure water was measured, between 21.3 and 21.5 °C when ATP was measured and between 22.5 and 22.7 °C when NaCl was measured. When the liquids were irradiated, the RI of water and NaCl did not change significantly. For ATP, the control group had a very slight increase of the RI. The RI increased after the solution was irradiated for the studied wavelengths from 405 to 810 nm. 969 nm light did not change significantly the RI. 810 and 969 nm irradiation increased the temperature of the water, NaCl and ATP samples by 0.8 °C (from 22.2 to 23 °C) due to the heat produced by the operation of the diode lasers. The water, NaCl and ATP samples were therefore heated to raise temperature by 1.4 °C. The changes in RI were less than 0.0001 for all the solutions when the temperature increased, which is within the error of the device (Fig. 19). The results of irradiating four ATP concentrations with 514 nm laser light are shown in Fig. 20. The changes in the RI are proportional to the concentration and, thus, to the number of ATP molecules in the medium.

After irradiation, the samples were stored in a fresh and dark place, and the RI of the solutions was measured every 10 minutes. The changes were totally reversed to the original values after 30 minutes.

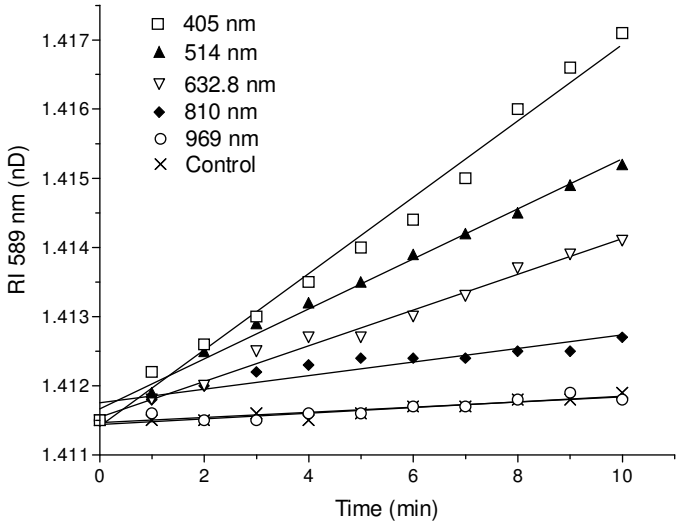

Figure 19. RI of 1.4 M ATP before (0 s) and after irradiation with light at several wavelengths. The effects on the RI are wavelength dependent (proportional to the intrinsic energy of the light).

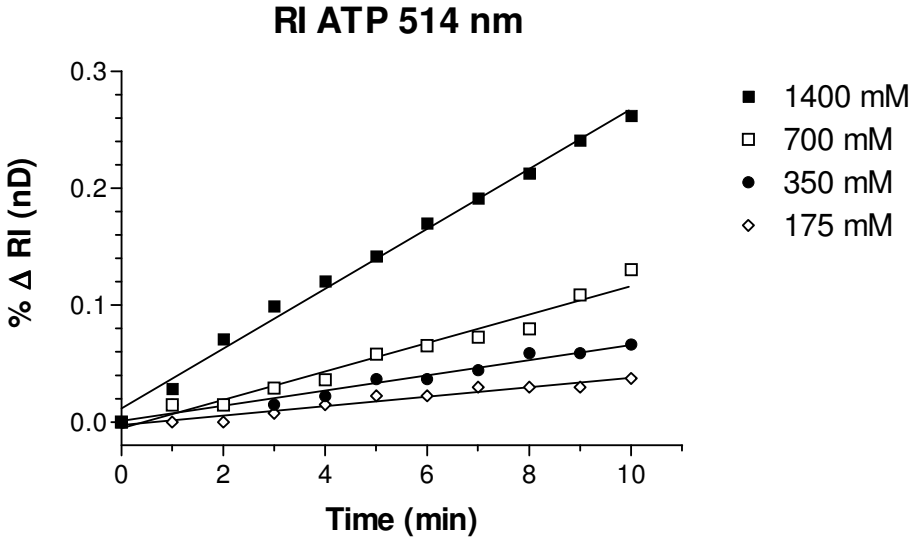

Figure 20. RI for the 1400, 700, 350 and 175 mM ATP solutions before (0 s) and after exposure to 514 nm laser light. Changes in the RI are proportional to the concentration.

5. DISCUSSION

5.1. LIGHT INTERACTION WITH ATP

ATP is a molecule that absorbs electromagnetic energy in the UV and in the middle-infrared part of the spectrum. This is well known by chemists and biochemists [30]. Response at visible and near-infrared light was considered impossible, even though it was noticed in the fifties that commercial ATP provided by Sigma got contaminated with some ADP molecules when the compound was exposed to light. Sigma then advised that ATP be "kept cool, in the dark". Nowadays the compound is presented in an opaque container.

After it was noticed that some effects in the molecule were obtained after a solution containing ATP was exposed to light, electrical currents, sound and centrifugation [34], it was decided to explore whether visible and near-infrared light could alter the biochemical behavior of ATP.

5.2. ABSORPTION OF ATP

The absorption of ATP in the visible and near-IR part of the spectrum raised the first problem regarding the response to light of ATP. The negligible absorption values could not be considered responsible for all the biochemical changes we observe when irradiating the molecule with visible and near-infrared light. Therefore, another explanation must be found. These exceedingly low values may be due to a Fresnel reflection that always occurs in the spectrophotometer measurements from the surfaces of the cuvette and the solution or to the lack of a perfect homogeneity of the medium [1, chap. II; 18, chap. XIV].

5.3. THERMAL EFFECT IN ATP DUE TO IRRADIATION

The temperature of an ATP solution after laser irradiation was to discard any other effect of light and confirm that the experimental results were caused by ATP interaction with electromagnetic energy.

A possible thermal effect was discarded because the effects of the temperature for visible and near-infrared wavelengths on ATP solution were not significant. This is in agreement with the effect of light in a non-magnetic medium, where absorption means the conversion of light into heath.

5.4. IMPURITIES IN ATP COMPOUND

The ATP compound used in these experiments (Sigma) was not pure but contaminated by several atoms at very small concentrations. The relatively large amount of phosphate may be due to the spontaneous breakdown of the terminal phosphate bond. Some ADP molecules also contaminate the compound. The weight of the contaminating atoms that absorb visible and near-IR light was 0.0575 % of the total. For each ATP molecule that may bind to a metal, 181.15 molecules of ATP will be free of metal. Any absorption of light by an ATP-metal complex is therefore quantitatively very low and would not significantly change the experimental results. Also, ATP has a greater affinity for atoms that do not absorb light, such as Mg^{2+} or Ca^{2+}, than for those that absorb light, such as Fe^{2+} or Cu^{2+}. In the experiments in which Mg^{2+} was used, an even smaller proportion of ATP would be bound to light-absorbing atoms. A cocktail of the contaminating

atoms (at concentrations equal to those in the ATP compound) was made and absorption was measured at 655 and 830 nm. Absorption at both wavelengths was negligible (data not shown).

5.5. KINETIC MEASUREMENTS FOR THE TWO REACTIONS

The kinetics parameter was measured differently in the two reactions (luciferine-luciferase and hexokinase) because the devices used to monitor the reactions were different. Luciferine-luciferase was measured with a luminometer that had only a graphical output (see Fig. 1) and did not record velocity. We therefore measured the curves and deducted the kinetic parameter by considering the peak V_0 and the decay of the luminescence k (see Material and Method). With the spectrophotometer used to measure the hexokinase reaction, velocity (OD340/min) was our experimental parameter.

5.5.1 The LUCIFERINE-LUCIFERASE REACTION

The firefly reaction is a standard model for quantifying ATP concentration based on the reaction described in Material and Method.

Most studies only use the firefly reaction to *quantify* the amount of ATP present in a medium or cell culture. In our study we also used it to *qualify* the behavior of ATP.

Our results show that the intensity of the initial peak of light V_0 increased at the two studied wavelengths. This means that more molecules of ATP reacted *at the very beginning of the reaction*.

The higher k values provide information about the kinetics of the reaction, which also increased when ATP was irradiated with 635 and 830 nm laser light. What it actually means is that the rate of transformation of substrates into products is higher and the reaction is faster.

The area under the V (t) curve quantifies the amount of substrates that are reacting (ATP and luciferine) with the enzyme luciferase. Since the only substrate that is irradiated is ATP, more ATP molecules must be binding some free molecules of enzyme that are in the medium, which increases the effective concentration of enzyme in the solution.

The energy of 635 nm light is much higher that that of 830 nm light and a greater effect with red light was therefore expected, but this was not the case. The power density of the near-infrared laser is double that of the red laser, but the dose was kept constant in both cases. This means that the intensity of the field (which will have a greater or lesser effect on a single molecule) is stronger when the red light is used, but that the density of the field (which will have an effect on a higher or lower number of molecules) for the infrared wavelength is twice that of the red wavelength. This may explain why in this reaction the effect was greater with the 830 nm light.

The reciprocity rule of photochemistry states that the photochemical response to light is independent of the intensity of light and the irradiation time. In this study this rule was not obeyed, but it is also not obeyed in other cases, for example in rapidly growing cells [7] in which the rate of energy parameter is more important than the total dose parameter. If we consider light as electromagnetic energy,

these results can be explained more easily in terms of intensities and densities of the electric field than in terms of total dose.

5.5.2 **The hexokinase reaction**

5.5.2.1 Introduction

The phosphorylation of glucose mediated by hexokinase is an important cellular reaction that initiates the metabolic pathway for glycolysis and ATP synthesis outside the mitochondria. Hexokinase IV is also called glucokinase and is specific for liver (non-liver hexokinases are I, II and III) or high k_m hexokinase, because its affinity for glucose is about 10-20 times less than other hexokinases [35] and results in a higher free glucose concentration in the liver. The values of k_m for ATP in the different families of hexokinases differ less than two-fold [36].

The glycolysis process, in which one molecule of glucose is metabolized into two molecules of pyruvate and two molecules of ATP, is started by the hexokinase reaction [37]. It is an anaerobic process that evolved before the accumulation of substantial amounts of oxygen in the atmosphere as a way of using the energy contained in carbohydrates, perhaps before mitochondria were incorporated into cells. Pyruvate can be further processed anaerobically to lactate by lactic acid fermentation [38]. This reaction is a source of ATP when mitochondria are unable to synthesize ATP and when the cell has a low oxygen concentration [39].

If the results of this experiment were reproduced in the cell after light irradiation, glycolysis would be increased. It seems unlikely that this could happen in aerobic conditions, but when a cell has a low oxygen concentration and mitochondrial

ATP synthesis is not active, this pathway could be used by the cell to produce ATP [40]. Most cells actually switch from aerobic to anaerobic metabolism depending on the environmental conditions [41]. The hexokinase reaction is therefore essential for cellular function. The clinical results obtained in light therapy with stressed tissues could be partially explained by the activation of glycolysis.

5.5.2.2. Light threshold

In the hexokinase experiment there were a number of variables, such pH, temperature and Mg^{2+} concentration. All except one, light irradiation, were kept constant in control and experimental groups. The different results between the control and the irradiated groups were therefore due to irradiation.

We studied the dose-effect of the light and found an activation threshold at 6 J/cm^2 and saturation at 12 J/cm^2 (Fig.14).

For the k_m and v_{max} experiment, the radiant exposure used was 12 J/cm^2, since this was the minimum dose that had maximum effects on the velocity of the hexokinase reaction. The activation threshold may correspond to the minimum energy needed to produce significant effects in ATP. The saturation observed in the reaction above 12 J/cm^2 corresponds to the point at which all available ATP molecules have interacted with the light and any additional energy will have no effect.

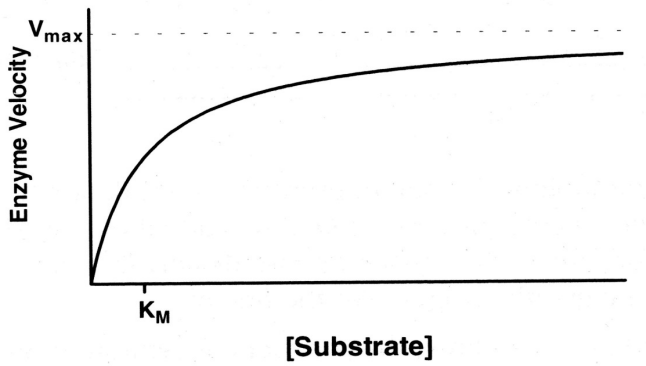

Figure 21. A graph of Michaelis-Menten kinetics becomes asymptotic when v_{max} is approached. The velocity is analyzed against different concentrations of substrate. k_m is the concentration of substrate when the velocity is $v_{max}/2$ [43].

5.5.2.3. Kinetic measurements

The hexokinase reaction follows Michaelis-Menten kinetics for ATP concentrations. The graph (Fig. 21) is defined by the Michaelis-Menten equation

$$v = v_{max} [S] / (k_m + [S])$$

where (v) is the velocity and [S] is the substrate (ATP-Mg^{2+} and glucose) concentration. For many years, Lineweaver-Burke, Hanes-Wolff and Eadie-Hofstee plots (linear regression) were used to infer the kinetic parameters and k_m and v_{max} were extrapolated from the resulting straight line. In all of these plots data are weighted very differently at high and low substrate concentrations, and the results may be misleading. To accurately determine k_m and v_{max}, the rate measurements should be treated statistically with appropriate weighting for the uncertainty of the data at different substrate concentrations. The best way to infer the kinetic parameter is to use a computer program and a non-linear regression fit,

i.e. to fit a previously defined curve directly to the experimental results. The values for R^2 (0.9865, 0.9907 and 0.9316 for the control, 655 group and 830 nm group, respectively) showed the goodness of fit. The Lineweaver-Burke plot was therefore used because it is an intuitive way to see the slope of the reaction and changes in k_m and v_{max} [42].

In Michaelis-Menten kinetics, the reaction takes place in different steps. First, the enzyme hexokinase (H) and the substrate (ATP-Mg^{2+}-G) are converted into the enzyme-substrate complex (H- ATP-Mg^{2+}-G). k_1 is the transformation rate from H + ATP-Mg^{2+}-G to H-ATP-Mg^{2+}-G, and k_{-1} is the transformation rate in the opposite direction, when H- ATP-Mg^{2+}-G again dissociates to H + ATP-Mg^{2+}-G. In the second step, H- ATP-Mg^{2+}-G is transformed into H + ADP-Mg^{2+}-G6P (products) with a rate of k_2.

$$\text{H + ATP-Mg}^{2+}\text{-G} \underset{k_{-1}(\leftarrow)}{\overset{k_1(\rightarrow)}{\leftrightarrow}} \text{H- ATP-Mg}^{2+}\text{-G} \xrightarrow{k_2} \text{H + ADP-Mg}^{2+}\text{-G6P}$$

k_m is defined by the relation

$$k_m = (k_{-1} + k_2) / k_1$$

Because of this definition, k_m is the rate of dissociation of the H- ATP-Mg^{2+}-G complex in both directions.

This model can be extended to consider all possible steps from H- ATP-Mg^{2+}-G to H + ADP-Mg^{2+}-G6P by defining the enzyme product complex (H- ADP-Mg^{2+}-

G6P), where the substrate (ATP-Mg^{2+} and glucose) has been transformed into product but the product (ADP-Mg^{2+} and G6P) is still attached to hexokinase. The complete reaction steps are then

$$H + ATP\text{-}Mg^{2+}\text{-}G \underset{k_{-1}(\leftarrow)}{\overset{k_1(\rightarrow)}{\leftrightarrow}} H\text{-}ATP\text{-}Mg^{2+}\text{-}G \xrightarrow{k_2} H\text{-}ADP\text{-}Mg^{2+}\text{-}G6P \xrightarrow{k_3} H + ADP\text{-}Mg^{2+}\text{-}G6P$$

The constant defined by a combination of k_2 and k_3 is the catalytic constant $k_{cat.}$ When the enzyme is saturated (high substrate concentrations), k_{cat} gives a measurement of the catalytic production (H + ADP-Mg^{2+}-G6P formation). At saturation, the velocity of product formation is maximum, and the total concentration of hexokinase (H_t) multiplied by the catalytic constant (k_{cat}) defines this maximum velocity (v_{max})

$$v_{max} = k_{cat}\,[H_t]$$

k_{cat} is also known as the turnover of an enzyme because it gives the number of catalytic cycles completed by the enzyme per unit of time under the assay conditions. Since $[H_t]$ is constant during the experiment, changes in the k_{cat} produce parallel changes in the v_{max} in the same direction.

When the rate of product formation is low ($k_{-1} >> k_2$), k_m is sometimes an inverse measure of the strength of substrate binding to the enzyme, but when the reaction has two substrates, as in this case, k_m is not related to the affinity of H- ATP-Mg^{2+}-G.

A very large value of k_2 can also lead to a large value of k_m. In fact, k_m indicates the rate of H- ATP-Mg^{2+}-G dissociating in any direction (H+ ATP-Mg^{2+}-G or H+ ADP-Mg^{2+}-G6P) relative to its propensity to be formed. k_m has another meaning that arises from the Michaelis-Menten equation.

When k_m = [ATP], then

$$V = v_{max} / 2$$

Then, k_m is numerically equal to the ATP concentration at which the reaction velocity has reached half the maximum velocity v_{max}. When k_m is greater, hexokinase needs a greater concentration of ATP to achieve a given reaction velocity if k_{cat} does not change.

When ATP concentrations are very low, [ATP] << k_m, most of the hexokinase is free and $[H_t] \approx [H]$. The Michaelis-Menten equation becomes

$$V \approx (k_{cat} / k_m) [H] [ATP]$$

At low concentrations, the reaction is second-order because it depends on the concentration of two parameters, H and ATP, and the ratio k_{cat}/k_m is defined as the second-order constant (k_{cat} is the first order constant because at saturation of ATP it only depends on one factor, the concentration of H). This ratio has a maximum possible value, which is determined by the frequency with which enzyme and substrate molecules can collide. An enzymatic curve is characterized by any two of these three variables: k_{cat} (v_{max}), k_m and k_{cat}/k_m.

When the rate of dissociation of H- ATP-Mg^{2+}-G to H+ ATP-Mg^{2+}-G is very low ($k_{-1} << k_2$), every molecule of H- ATP-Mg^{2+}-G formed will go into H + ADP-

Mg^{2+}-G6P. This occurs when the binding of enzyme and product is almost irreversible, which is the case for ATP-Mg^{2+} and H. In the hexokinase reaction, the kinetic model then reduces to

$$H + ATP\text{-}Mg^{2+}\text{-}G \xrightarrow{k_1} H\text{-}ATP\text{-}Mg^{2+}\text{-}G \xrightarrow{k_2} H + ADP\text{-}Mg^{2+}\text{-}G6P$$

Then, $k_{cat} = k_2$, $k_m = k_2/k_1$ and $k_{cat}/k_m = k_1$. At a very low ATP concentration, every molecule of H- ATP-Mg^{2+}-G will go rapidly into H + ADP-Mg^{2+}-G6P. As the ATP concentration increases, the rate of formation of H- ATP-Mg^{2+}-G increases until it becomes faster than the breakdown of the H- ATP-Mg^{2+}-G complex into H + ADP-Mg^{2+}-G6P. The H- ATP-Mg^{2+}-G complex then begins to pile up and at a very high ATP concentration the entire hexokinase will exist as H- ATP-Mg^{2+}-G. When this happens**,** the rate of product formation is entirely determined by the rate of breakdown of the H- ATP-Mg^{2+}-G complex (k_m), and the reaction has reached its maximum velocity (v_{max}). Then, k_m and v_{max} change in the same direction. An increase in v_{max} is the result of an increase in k_m [43 - 45, 46 (pp. 80-91), 47 (pp.375-383)].

The higher values of k_m and v_{max} after laser irradiation may be explained as velocities or rates that define concrete steps in the hexokinase reaction. We have just seen that an increase in k_m leads to an increase in v_{max}. We could not explain an increase in the maximum velocity of the system if the H-ATP-Mg^{2+}-G dissociates slowly. The higher values of v_{max} correspond to a higher k_{cat}, since these parameters are directly proportional. A higher k_{cat} is caused by a higher rate

of H- ATP-Mg^{2+}-G transformation into H + ADP-Mg^{2+}-G6P, which is in agreement with the higher directly measured velocities after laser irradiation. Since the ATP concentration did not change, irradiated ATP must have different characteristics from those of non-irradiated ATP. A conformational change in the molecule due to a polarization of the molecule can promote a better fit with the active center of the enzyme. Also, light may alter the stability of the phosphate bond. Somehow the reaction is different because one of the substrates (ATP) is different. This is confirmed by the change in the refractive index after laser irradiation. Moreover, a structural change in ATP may produce a structural change also in the H-ATP-Mg^{2+}-G and H- ADP-Mg^{2+}-G6P complexes. Structural differences in these complexes for non-irradiated and irradiated groups mean that the reaction is different when irradiated ATP is used, and a different v_{max} must be expected.

The increase in k_m for irradiated ATP groups may be also explained by the reaction rates. In the initial moments of the reaction, before the linear part of the reaction is reached, light-excited ATP reacts faster than non-irradiated ATP and more H + ATP-Mg^{2+}-G goes into H-ATP-Mg^{2+}-G at both low and high ATP concentrations. This is in agreement with the measured velocities for irradiated ATP. At a certain point, irradiated groups will have a greater H-ATP-Mg^{2+}-G concentration and less free H + ATP-Mg^{2+}-G, so the H- ATP-Mg^{2+}-G pool will increase. Then, in the linear part of the reaction, more H- ATP-Mg^{2+}-G can be converted into H + ADP-Mg^{2+}-G6P because experimental groups depart from a privileged situation i.e. a higher H- ATP-Mg^{2+}-G complex concentration. The

faster decrease of the H- ATP-Mg^{2+}-G pool into H + ADP-Mg^{2+}-G6P, because of the structural differences in ATP and the complex of ATP with hexokinase, may be the mechanism by which irradiated ATP alters the kinetics of the reaction. The H- ATP-Mg^{2+}-G complex will dissociate faster ($\uparrow k_m$) and this will produce a higher rate of H + ADP-Mg^{2+}-G6P formation ($\uparrow k_{cat} \rightarrow \uparrow v_{max}$). If we extend this argument, there will be a certain point in the reaction time that irradiated groups will have less free H and ATP-Mg^{2+}-G concentrations than non-irradiated groups and the rate of H + ATP-Mg^{2+}-G to H- ATP-Mg^{2+}-G (k_1 will decrease). Since $k_m=k_2/k_1$, even if there were no change in k_2 (or k_{cat}), in the linear part of the reaction the values of k_m would be higher due to this decrease in k_1 ($k_2/\downarrow k_1 = \uparrow k_m$) Another point about the increase in k_m concerns the possible structural change in the ATP molecule. When ATP binds ions such as H^+, Mg^{2+}, Mn^{2+} or Ca^{2+}, the molecule experiences conformational changes due to the interaction of different electrical charges [48]. The displacement of charges in ATP due to interaction with the electromagnetic disturbance may also lead to structural changes in the molecule. These structural changes would then produce a different fit in the active center of hexokinase after ATP irradiation. If irradiated ATP fits the centre of an enzyme better, more enzyme molecules can be active. If for some reason the solution contains enzymatic molecules that did not reach the ATP molecule, structural changes in ATP may lead these hexokinase molecules to bind to irradiated ATP. An "effective" increased enzyme concentration would then be observed. The hexokinase reaction would then admit more ATP molecules and a higher ATP concentration would be needed to fill half of the active centers of the

enzyme. As a result, k_m would increase. Only if k_{cat} does not change does an enzyme with a higher k_m require a higher substrate concentration to achieve a given velocity than an enzyme with a lower k_m. In the hexokinase reaction experiment, irradiated ATP groups showed that k_m and k_{cat} both increase, which indicates that more substrate molecules would be transformed into products.

k_m is also the dissociation rate of the H- ATP-Mg^{2+}-G complex, both into H + ATP-Mg^{2+}-G and H + ADP-Mg^{2+}-G 6P. Assuming that the enzymatic reaction is irreversible and that the H- ATP-Mg^{2+}-G complex does not dissociate back into H + ATP-Mg^{2+}-G, k_m will reveal the rate of transformation of H- ATP-Mg^{2+}-G into H + ADP-Mg^{2+}-G6P. A larger k_m would then mean a faster rate of H-ATP-Mg^{2+}-G transformation into H + ADP-Mg^{2+}-G6P. k_{cat} and v_{max} would then be increased. Increased k_m values for irradiated ATP could indicate that the components of H-ATP-Mg^{2+}-G bind more weakly, that H- ATP-Mg^{2+}-G dissociates more quickly and that the reaction is faster.

A way for an enzyme to increase its efficiency is to reach maximum velocity with less substrate concentration, which will decrease k_m and keep v_{max} constant .This is not the mechanism used by light to improve the hexokinase reaction with irradiated ATP because in this reaction the maximum velocity also increases, so the whole transformation rates in the reaction steps change. A lower k_m would lead to a lower dissociation rate of the H- ATP-Mg^{2+}-G complex, which would slow the whole reaction down.

In the hexokinase reaction system, an increase in v_{max} may be the actual effect of the light in the reaction, and the changes in the k_m will show how the increase in

velocity is produced: increasing the rate of catalysis of the H- ATP-Mg^{2+}-G complex, decreasing its binding forces or increasing the effective enzyme or substrate concentration.

k_m and v_{max} were calculated with the experimental data on velocity obtained for each group and these measures are treated independently. The values of the kinetic parameter were extrapolated directly from the curve by the software Graph Pad Prism (see Table 8). These values were different for the control and 655 nm and 830 nm irradiated groups, so there is ample scope for discussing the differences between the two wavelengths.

Since temperature, pH and the concentrations of the cofactor (Mg^{2+}), enzymes and substrates were kept constant during the experiment, the measured differences were due to the effect of light. The data showed an increase in v_{max} for ATP irradiated at 655 nm and 830 nm, which indicates that the turnover of the enzyme was higher. One unit of hexokinase will therefore convert 0.07862 mM of ATP-Mg^{2+}-G into ADP-Mg^{2+}-G6P in a unit of time for the control group, against 0.1166 mM/unit of time for the 655 nm group and 0.1044 mM/unit of time for the 830 nm group. The increase in the efficiency of the system therefore depends on the energy of light used.

For k_m values, the dissociation rate of the H-ATP-Mg^{2+}-G complex is 0.385 mM per unit of time for control groups (non-irradiated ATP) and 0.732 and 0.482 for the 655 and 830 nm groups, respectively. In this case the rate again depends on the energy (wavelength) of light used. The high value of k_m for the 655 nm laser (0.732 mM) compared to that of the control (0.385 mM), indicates that not all this

change in the dissociation rate of H-ATP-Mg^{2+}-G is transformed effectively into a v_{max} value (0.1166 for 655 nm and 0.07862 for control). Somewhere between H-ATP-Mg^{2+}-G and H + ADP-Mg^{2+}-G6P we may find different pool concentrations of intermediate compounds and/or a limiting rate or factor in hexokinase that has reached its maximum.

5.6. FLUORESCENCE OF ADENOSINE N-PHOSPHATE MOLECULES

The fluorescence observed in ATP, the less fluorescence for ADP and the weak fluorescence in AMP (Fig. 18) can be explained by the number of phosphates. Light emission appears to be proportional to the number of phosphor atoms that are present in the molecule.

Magnesium ion binds to two of the ATP oxygen atoms in the phosphate group to form the ATP-Mg^{2+} complex, which is the true substrate for all ATPase reactions (Fig. 22) [49].

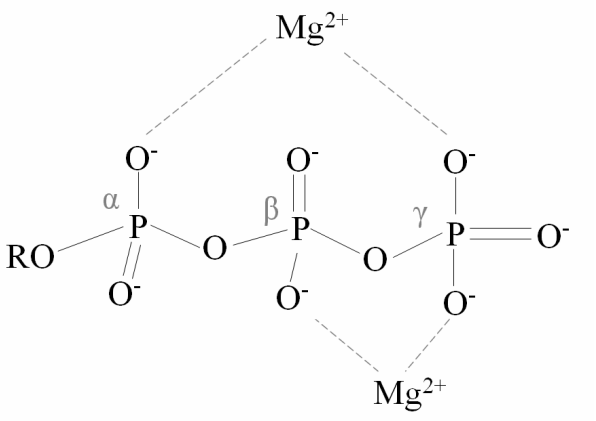

Figure 22. This diagram shows where Mg^{2+} binds to oxygen atoms of ATP's phosphates. The terminal phosphate (γ) is always involved and the other phosphate can be α o β.

To explain why ATP has less fluorescence when Mg^{2+} is added, we must discuss the quenching and shielding processes.

There are three types of *Quenching*: chemical quenching, where other substances compete for the energy of the radiation; color quenching, where substances absorb the primary or secondary emission of light; and dilution quenching, where adding a large volume of sample to the fluorescent molecule reduces the efficiency [50]. In our case, chemical quenching is not possible because Mg^{2+} does not compete for the excitation photon (260 nm); Mg^{2+} has no absorption at this wavelength, while ATP has a strong absorption peak at 260. Color quenching is also not possible because Mg^{2+} does not absorb at the wavelengths that ATP emits fluorescence (300 to 500 nm). Dilution quenching is not responsible for the decreased fluorescence because the volume of ATP solution and the effective ATP concentration were kept constant.

A shielding takes place when the substance added to the solution blocks the light emission and the detector cannot read the signal [50]. When Mg^{2+} was added to ATP, the signal was never completely extinguished even at very high concentrations of Mg^{2+}. Rather, the decay of fluorescence when Mg^{2+} was added showed a binding associative process between Mg^{2+} and the phosphate in which the plateau was not reached, except at very high Mg^{2+} concentrations, and the asymptotic curve was parallel to the x-axis but never crossed it. Therefore, even if the highest Mg^{2+} concentration were added, light emission would never be zero. We must therefore discard a shielding effect for the decreasing fluorescence.

We should also bear in mind that the greater fluorescence in ATP-Mg^{2+} after irradiating with 655 and 830 nm light could be due to the emission of light by the magnesium ion. However, the fluorescence for the magnesium ion measured at 655 and 830 nm was zero.

The most likely explanation for the lower fluorescence of ATP when Mg^{2+} is added is that the ion binds to the phosphates, which seem to be the structures that modify the light emission. The binding of Mg^{2+} to the molecule is by a weak non-covalent bond [51]. When ATP is in a solution, the –OH terminals in the phosphate are ionized to O^- [47]. The phosphate chain is a negatively charged structure that permits the binding of a positively charged ion such as Mg^{2+} [48].

When ATP was irradiated at 655 and 830 nm, the fluorescence did not change. When ATP-Mg^{2+} (which showed lower fluorescence than ATP) was irradiated, the fluorescence increased. The magnesium ion binds to the phosphates more weakly after light irradiation and permits an increase in fluorescence, since previously the fluorescence was decreased by magnesium. The mechanism of a different interaction of ATP with Mg^{2+} may be related to the dipole radiation. The electrons behaving as dipole radiators will create a local electric field that will affect to the electrical charges in the vicinity of the molecule, which is the Mg^{2+} bounded by weak ionic forces, and may displace the ion from the phosphates.

5.7. INSTABILITY OF ATP'S TERMINAL BOND

The ATP hydrolysis to ADP plus inorganic phosphate depends on three important factors: resonance stabilization, electrostatic repulsion and stabilization due to

hydration. ADP and P_i have greater resonance stabilization than ATP [21, pp.202-206], so the terminal bond will tend to break down with minimum disturbance. Orthophosphate (PO_4^{3-}) has a number of resonance forms of similar energy. Resonance stabilization means that both $-O^-$ (or OH) and =O can "travel" around the phosphate or, more precisely, that electrons are moving around the phosphorus atom. These multiple forms of the orthophosphate contribute to the high entropy of the structure [47, pp. 73-78] (Fig. 23).

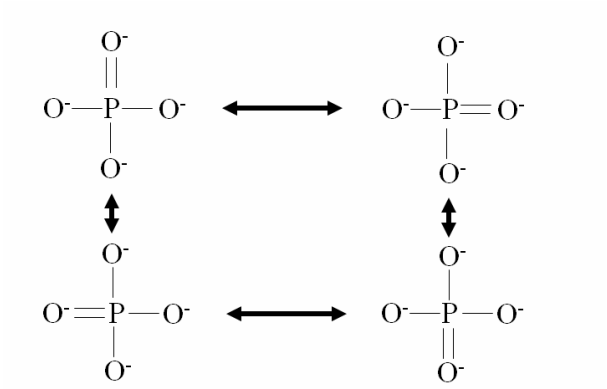

Figure 23. Probable resonance structures of orthophosphate, where two negative charges are not in adjacent atoms.

Other forms of orthophosphate (Fig. 24) are more unstable because a positively charged oxygen atom is adjacent to a positively charged phosphorus atom, which creates a repulsion force between them. Moreover, at physiological pH the triphosphate unit of ATP carries four negative charges. These charges repel one another because they are in close proximity. The repulsion between them is reduced when ATP is hydrolyzed [48,52]. Again, the phosphate bond tends to be broken because the products, ADP and P_i, are more stable than ATP itself.

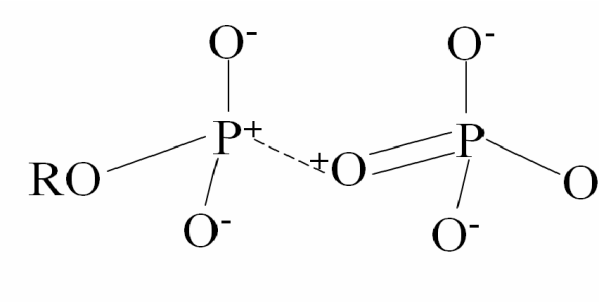

Figure 24. Improbable resonance structure of orthophosphate, which contributes to the breakdown of ATP's terminal bond.

Releasing the phosphate residue from its bonded state allows greater opportunities for hydration, especially, as in this case, when both products are charged. Since hydration is an energetically favored state, the terminal phosphate bond will tend to break down.

These three factors (resonance stabilization, electrostatic repulsion and stabilization due to hydration) are responsible for the instability of ATP's terminal bond, which favors the transfer of its energy in a metabolic reaction.

The breakdown of ATP requires different amounts of activation energy when the molecule is bond to Mg^{2+} or H^{+} or other metallic or non-metallic atoms. Different atoms bound to ATP represent variations in the number of protons interacting with the molecule and a different electric interaction [48, 52, 53]. These observations indicate that the breakdown of ATP's terminal bond is clearly influenced by electrical changes. It has been reported that the instability of ATP's terminal bond increases after exposure to visible light and electrical currents, but these energies are not enough to split the bond ([34], and unpublished work by

Hillman, H., in the 1960s). The light induced dipole radiation in the electrons may explain the effect of light altering the kinetics of the enzymatic reactions. When the electrons are accelerated by the electric field, the phosphate bond that contains the electrons will be displaced from its thermally equilibrated level. As a result, the bond will become unstable. Cleavage of the phosphate by the enzyme, hexokinase, will be then much easier in a wavelength dependent manner.

5.8. OPTICAL PROPERTIES OF AN ATP SOLUTION

The Michelson interferometer was set up to show an interactive mechanism between light and ATP. For this reason, we used a high ATP solution (1.4 M) in order to demonstrate measurable changes in the molecule. Interaction is mechanism-dependant and not concentration-dependant. However, solutions used in the hexokinase experiment were in the normal cellular range or even smaller and a light response was clearly observed. The results of the interference pattern analysis show that air, water and an ATP solution interact differently with light. The thicknesses and densities of the bright and dark fringes of the three materials were different.

The measurements of RI for water, NaCl and ATP solutions confirmed that RI does not depend on the amount of substance but on its characteristics, since NaCl and ATP were used at the same concentration. In this case, refraction depends on the electrical permittivity (ε) of the medium because all the media are non-conducting. The refractive index (n) can be expressed by

$$n = c/v = (\varepsilon/\varepsilon_0)^{1/2}$$

Therefore, since c (≈3 x 10^8 m/s) and the electrical permittivity of the vacuum ε_0 (8.854 x 10^{-12} F/m) are constants [1], the RI will be an indirect measure of both the speed of light in the medium (v) and the electrical permittivity (ε) of the medium. These values are shown in Table 10.

	RI (n) at 589 nm (nD)	Permittivity (ε) at 589 nm (F/m)	Speed of light (v) at 589 nm (m/s)
Water	1.3327	15.725x10^{-12}	2.25107 x 10^8
NaCl (1.4 M)	1.3423	15.952x10^{-12}	2.23497 x 10^8
ATP (1.4 M)	1.4115	17.787x10^{-12}	2.11655 x 10^8

Table 10. Measured RI (at 589 nm) of water, 1.4 M NaCl and ATP solutions, and inferred permittivity values (ε) and speed of light in the media at the same wavelength. Water and NaCl solution have very similar values, but ATP differs significantly.

When water and NaCl were irradiated with various wavelengths, their RI did not change. For ATP, there was a clear increase in RI at the studied wavelengths except for 969 nm light that has a very weak intrinsic energy. This means that the light will be slowed down from 2.1254 to 2.12149 x 10^8 m/s after ten minutes irradiation with 632.8 nm, and to 2.11655 x 10^8 m/s after 405 nm irradiation. This slowing down of the light is due to the electrical interaction of the

electromagnetic field with the molecule, because the electrical permittivity will be increased from 17.64 to 17.663 x 10^{-12} F/m after irradiation with 632.8 nm and to 17.787 x 10^{-12} F/m after irradiation with 405 nm. The effects are perfectly wavelength dependent, and according to the theory of dispersion of the refractive index [18, 19]. When light at different frequencies is shown to an ATP solution, the acceleration of the electrons is proportional to the frequency. When the refractive index is measured at 589 nm, the incoming wave of the refractometer will encounter electrons that are highly accelerated by the previous energy, and thus, loosely tightened to the molecule. Since electrons are loosely tightened they will have a greater interaction with the incoming light. The new electric field produce by these electrons emit will have greater interference with the electric field of 589 nm light. The result of this interference will be a wavelength dependant increase of the refractive index.

5.9. LIGHT CAN CONTRIBUTE TO THE INSTABILITY OF THE BOND

The electromagnetic disturbance caused by 655 nm and 830 nm light in an ATP solution produces a displacement of charges in the phosphate bond and a polarization of ATP. The acceleration of the electrons produced by the electric field if the light may increase the instability of the bond. Our data show that ATP's terminal bond is more easily split by hexokinase after irradiation, which indicates that, effectively, light increases the instability of the bond, thus allowing the energy to be used by ATP to improve its physiological function: energy donation.

With regard to the kinetic parameters in the hexokinase reaction after irradiating ATP, shorter wavelengths (655 nm) have more effect in a dielectric medium (dispersion effect) containing ATP than longer ones (830 nm) after interacting with the electrons in the phosphate bonds. This wavelength-dependence of the effects is characteristic of the interactive mechanism of light in a dielectric medium. A stronger interaction will have a greater effect on the terminal bond and less activation energy by the enzyme hexokinase will be needed to produce its breakdown. A possible conformational change in the ATP molecule after irradiation could change the affinity of the molecule for the active center of the enzyme. When the reaction occurs, the ATP-Mg^{2+}-glucose complex enters the active center of hexokinase, and this binding produces a conformational change in the structure of the protein [47, pp. 450-459]. The conformational change in hexokinase is the mechanism that causes the breakdown of the terminal phosphate in ATP. If ATP is more unstable in that bond due to the electromagnetic disturbance, possibly produced by the new electric field radiated by electrons, less time and less energy are needed to produce its hydrolysis by the enzyme. The v_{max} and k_m would therefore increase.

5.10. EFFECT OF LIGHT IN THE TWO PHOSPHOR-OXYGEN BONDS OF ATP

We should bear in mind that ATP has two phosphor-oxygen bonds in its molecule, and that the energy-donor function is only related to the terminal one. A characteristic of Biology is that it creates the simplest things to produce a

function: "*Nature does nothing in vain and more in vain when less will serve; for Nature is pleased with simplicity, and affects not the pomp of superfluous causes*"*. We can ask, then, why the phosphate is not obtained from ADP, which stores the same amount of energy in its second bond (31 kJ/mol)?. A possible explanation is that when the end of the molecule is farther from the adenine-ribose part, it will easily increase its vibrational energy and the molecule will be more negative (ADP has three negative charges and ATP has four). Consequently, the third phosphate will be more unstable that the second one, which will help to break down the bond.

This can explain very well why the effects of the luciferine-luciferase reaction and the glucokinase reaction are quantitatively very different. We can relate the velocities, V for the glucokinase reaction and k for the luciferine-luciferase reaction by

$$\frac{V_{irr} / V_{control}}{k_{irr} / k_{control}}$$

This formula can be also expressed by

$$\frac{Effect\ on\ the\ \gamma\ bond}{Effect\ on\ the\ \beta\ bond}$$

of ATP, because one reaction, that of glucokinase, affects the γ bond and the other reaction, that of luciferine-luciferase, affects the β bond. The values were 7.72 for the red laser and 5.63 for the IR laser. This shows that the terminal bond is more affected by the electromagnetic field than the second bond. This is reasonable because the second bond, due to of its physically location, is a much more stable

* Isaac Newton, *The Mathematical Principles of Natural Philosophy*. Book III, Rule I.

structure. However, it is a double bond and it can still be affected and its charges displaced by an electromagnetic field.

We must remember that ATP is a translator of energies, and that the economic nature of Biology has made it sensitive to the greatest forms of energy, such as light. The efficiency of this interaction in the ATP molecule is therefore greater than if the mechanism were related to electronic excitation, because with EM interaction all the wavelengths (with a greater or lesser effect) can play a role. If the mechanism were electronic excitation, ATP would have to be a black molecule to be excited by any wavelength.

5.11. CELLULAR INDUCED EFFECTS BY ELECTRICAL CURRENTS AND A COMPARISON WITH LIGHT EFFECTS

A fundamental mechanism that converts light energy into chemical energy (ATP) is photosynthesis. If light is seen as an electromagnetic radiation and its effects due to the electrical field it produces, a functional comparison between light and electrical currents may be considered. This assimilation of effects between light and electricity has been demonstrated by exposing chloroplasts to light pulses and electrical pulses: the observed effects on ATP synthesis are exactly the same [54].

ATP synthesis in cells has been reported after light exposure to visible (633 nm) wavelengths [9]. The proposed mechanism is related to light absorption in the mitochondrial cytochrome c oxidase molecule due to metallic atoms located inside the protein. However, ATP synthesis has also been reported for *in vivo* mitochondria after the solution containing the organelle is exposed to electric

pulses [55]. The effect is not due to an absorption of the current (Joule heating) but to a transmembrane potential.

The Na^+/K^+ ATPase pump that exchanges sodium and potassium from outside to inside the cytoplasm against the concentration gradient of Na^+ and K^+ requires the breakdown of a molecule of ATP. This pump can be activated without ATP if cells are exposed to electrical currents [56, 57]. These effects are also due to transmembrane potentials and not to absorption. More than a third of ATP in a resting animal is used to pump these ions [21], so if the pump runs without ATP this will affect the cellular ATP concentration and/or ATP synthesis. If we continue the analogy between light and electrical currents, light irradiation may also affect the Na^+/K^+ ATPase pump, and ATP levels in the cell after light irradiation may increase. Moreover, some enzymes can operate in reverse mode after certain conditions, and can either break or synthesize a molecule. This is the case of F_1F_0 ATP synthase in the mitochondria, which can also act as ATPase [58]. Electrical currents do not have this effect in Na^+/K^+ ATPase. However, the electric field applied was in the range of KHz (10^3 Hz), and visible and near-infrared light have an intrinsic frequency of 1-4 THz (10^{14} Hz) [1]. Differences in the biological response due to differences in frequencies can be expected and extra mitochondrial ATP synthesis due to light, both in the glycolysis and in the reverse mode of ATPases, need to be considered. Moreover, ATP synthesis in light-irradiated cells in which oxygen is diminished, which increased ATP synthesis due to light absorption of cytochrome c oxidase fails to explain, can be directly explained by this extra mitochondrial ATP synthesis.

5.12. MITOCHONDRIAL ACTIVITY IS INFLUENCED BY ATP HYDROLYSIS

Mitochondrial activity, and particularly cytochrome c oxidase activity, are controlled by the free ADP concentration [59, 60] and the ATP/ADP ratios [61]. ATP synthesis is enhanced when the ADP cytoplasmic levels are increased by the simple mechanism of varying the amount of substrate available. Moreover, cytochrome c oxidase, which has a fundamental regulatory effect on ATP synthesis, is especially sensitive to the ADP concentration levels and the ATP/ADP ratio changes. This enzyme is known to be allosterically inhibited by ATP and activated by ADP [62, 63]. A higher ATP/ADP ratio and a lower ADP concentration are important stimuli for mitochondrial ATP synthesis [60].

In our experiment in the hexokinase reaction, ATP converted to ADP as time progressed, which means that the ATP/ADP ratios would also be decreasing over time. Since in irradiated groups the ATP conversion to ADP increases and since this process is irreversible, the instantaneous ATP/ADP ratios will decrease and the ADP concentration will increase in the experimental groups. At the end of the process, the free ADP concentration and the ATP/ADP ratios of non-irradiated and irradiated ATP groups will be significantly different.

The fact that a cell is not normally exposed to light does not mean that the interacting mechanism does not exist. It only means that it is not used. This electromagnetic effect on ATP may have been used earlier in evolution when cells were exposed to light and other environmental energies.

The mechanism proposed here can be applied to the effects seen in the photomodulation (Low Level Laser Therapy) of cells or tissues. This mechanism may not have an effect in normal conditions, but it will improve cellular metabolism in injured and diseased tissues, which require more energy to sustain cell functions and repair cell or tissue structures. When cells suffer from lack of nutrients, oxygen or glucose, and mitochondrial ATP synthesis is not significant, light excitation of the ATP located in cytoplasm increases cellular metabolism. If ATP light interaction is reproduced when living systems are irradiated, irradiated ATP reacts faster with cellular substrates and enzymes and cellular metabolism and/or activity increases. As a result, the ATP/ADP ratio inside the cytoplasm decreases and the free ADP concentration increases. That would be the stimulus for starting mitochondrial ATP synthesis.

6. CONCLUSIONS

1. **Light can interact with biological non-chromophores such as ATP, and change its physical properties and chemical behavior. The molecule is polarized and the electromagnetic wave is as well affected by the medium. The interacting mechanism is non-absorptive and is produced by the electric field induced in the ATP solution.**

2. **The two phosphor-oxygen bonds of ATP can be affected by light, but the greater effects are found in the terminal phosphate bond due to its physiological instability. The enzyme is ultimately responsible for the breakdown of the phosphate bonds, and this occurs easily when ATP has been exposed to light. The kinetic parameter of the enzymatic reactions in which irradiated ATP is involved change significantly: the reaction occurs faster and a greater number of molecules react.**

3. **This interacting mechanism may have an important biological significance when cells or tissues are exposed to light. The effects of this mechanism depend on the wavelength and intensity of the light.**

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Effect of Visible and Near-Infrared Light on Adenosine Triphosphate (ATP)

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ABBREVIATIONS AND SYMBOLS

ADP, Adenosine diphosphate.

AMP, Adenosine monophosphate.

ATP, Adenosine triphosphate.

β-NADPH, Beta-nicotine amine dinucleotide phosphate (reduced form).

C, coulomb, unit of electric charge.

c, *celeritas*, speed of the light in the vacuum (2.99792457×10^{8} m/s).

E, intensity of the electric field.

e, elementary charge of the electron (1.60210×10^{-19} C).

EM, electromagnetic.

ε, *epsilon*, permittivity (electric constant) of a medium.

ε_0, permittivity of the vacuum (8.854×10^{-12} F/m).

F, faraday, unit of electric charge quantity (96,500 coulomb = 1 faraday).

G6PDH, glucose 6 phosphate dehydrogenase.

h, Planck's constant (6.6256 x 10^{-34} Joules. second).

IR, infrared

J/cm^2, joules per square centimeter (radiant exposure units).

k, constant of the light -luminescence- decay (in the Luciferine-luciferase reaction).

k, restoring-force constant acting on bound electrons (after a polarization of a medium due to electromagnetic fields).

k_m, Michaelis constant of a Michaelis-Menten enzymatic reaction.

λ, *lambda*, wavelength of electromagnetic waves (light).

μ, *mu*, permeability (magnetic constant) of a medium.

N, number of displaced electrons in a chemical bond by an electrical field.

n, refractive index of a medium.

ν, *nu*, frequency of electromagnetic waves (light).

P, macroscopic polarization of a medium due to an electrical field.

π, *pi*, the ratio of the circumference to the diameter of a circle ($\pi \approx 3.1416$).

RI, refractive index of a medium.

UV, ultraviolet.

V, volt, unit of electric potential.

v, velocity of electromagnetic waves (light) in a medium.

$\mathbf{v_{max}}$, maximum velocity of a Michaelis-Menten enzymatic reaction.

$\mathbf{V_0}$, peak voltage of the luminescent signal in the Luciferine-luciferase reaction.

V(t), exponential function (voltage vs. time) of the luminescent signal (Luciferine-luciferase reaction).

ω, *omega*, angular frequency of electromagnetic waves (light).

$\boldsymbol{\omega_0}$, resonance angular frequency of electromagnetic waves (light).

ABSTRACT

ATP is a key molecule in cellular metabolism. In this thesis, I examined the effects of visible (635 and 655 nm) and near-infrared (810 and 830 nm) light on ATP in solution. I also examined were the biochemical behavior of light-exposed ATP in the luciferine-luciferase reaction and hexokinase reaction, the initial step in glycolysis that begins extra mithocondrial ATP synthesis. Irradiated groups in the luciferine-luciferase reaction showed an improvement in the kinetic parameters V_0 and k, and more ATP molecules reacted with the enzyme when they were excited by light. When irradiated ATP was added to the hexokinase reaction, the experimental groups showed significant differences in the Michaelis-Menten kinetic parameters (k_m for ATP and v_{max}) and the rate of product synthesis was greater. Changes in both reactions were wavelength and dose dependant.

When ATP was excited with UV photons, it fluoresced. This fluorescence decreased when Mg^{2+} was added, probably because the ion binds the phosphates, which are the part of the molecule responsible for light emission. Irradiating the ATP-Mg^{2+} solution with 655 nm and 830 nm light increased the fluorescence resulting from a displacement of charges in the phosphor-oxygen bond that repels Mg^{2+}.

The refraction of light in an ATP solution was observed by the Michelson interferometer and by directly measuring the refractive index. The refractive index changed after red and near-infrared light interaction due to a change in the electrical permittivity of the medium.

Since ATP in water is transparent to visible and near-infrared light, and is therefore not a chromophore for those wavelengths, I conclude that the observed light interaction with ATP is not due to photon absorption but to the electromagnetic disturbance produced by the light, which leads to a polarization of the dielectric molecule that is ATP.

This interaction of visible and near-infrared electromagnetic energy with ATP offers new perspectives for explaining light interaction at subcellular level.

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