American Journal of Scientific Research
ISSN 2301-2005 Issue 86 March, 2013, pp.177-187
http://www.americanjournalofscientificresearch.com

Ionic and Osmotic Equilibria of Human Red Blood Cells

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Abstract

A healthy mature human red blood cell (RBC) lacks nucleus and it is a disc-shaped under physiological conditions (Li et al., 2007). The biconcave discocyte RBC has flexible bi-layer membrane with a high surface-to-volume ratio that facilitates large reversible elastic deformation as it repeatedly passes through narrow capillaries during microcirculation which is necessary to transport oxygen and carbon dioxide though haemoglobin molecules which are contained in the RBC intracellular are essential for gas transport within the circulation. The discocyte shape of RBC is encoded in the mechanical properties of its bilayer-membranes: 7.8μm in diameter; 136μm^2 in surface area, 85.1μm^3 in volume and 1.7-2.2μm in thickness (Tse and Lux, 1999; Turgeon, 2004). Some variations in size, shape or colour of RBC may be seen on microscopic examination with Write's Romanosky – type stain (Dunphy (2010). The movement and distribution of ions across RBC membranes is greatly influenced by the presence of charged impermeable macromolecules which prompt an equivalent number of oppositely charged permeable ions to remain with them in the same compartment in which they occur (Donnan, 1911; Lang, 2007). The main basic function of the sodium pump is to maintain the Na^+/K^+ gradients across the membrane. Thus, membrane potential, nutrients uptake, intracellular pH and volume are all regulated by the integrity of functional sodium pump. Also, since solutions of extracellular and intracellular are asymmetric in concentration, diffusion of water in one direction exceeds that in the other and the net movement of water across the membrane continues until the concentrations of solute becomes the same on both sides of the membrane or until the force generated by the osmosis is balanced by some opposing pressure resulting from the tendency of one solution to increase in volume at the expense of the other. So, any change in the extracellular pH affects the intracellular solution (Lardner, 2001; Swietach et al., 2010) and the plasma barrier mechanics contributes to the explanation of stomatocyte-discocyte-echnocyte shape bending rigidity.

Keywords: Electrochemical membrane potential; Donnan equilibrium; haemoglobin; red blood cell shape; permeant ions; osmotic pressure; surface charge; K^+/Cl^- cotransport; $K^+(Na^+)/H^+$ exchanger; Na^+/K^+-ATPase; discocyte.

Introduction

Movement of molecules across red blood cell membranes is flux from one aqueous environment to another, and it is restricted to movement of solute molecules and of water (Endeward et al., 2006). Gases, such as oxygen and carbon dioxide, which are important in cellular metabolism, influxed and

effluxed through cell membranes in a dissolved state and the limiting factor in the rate of flux is the extent to which gases are soluble in the aqueous environment (Itel et al., 2012). Carbon dioxide is very soluble in water (CO_2 Absorption coefficient 1.71 at 0 ^oC and 101325 N m^{-2} (1 atm)) and fluxed freely through membranes, but oxygen has a much more limited solubility (O_2 Absorption coefficient 0.049 at 0 ^oC and 101325 N m^{-2} (1 atm)) and this becomes a limiting factor in cellular metabolism (Stark and Wallace, 1976, p.60).

Theoretically, the osmotic pressure across a perfect semipermeable membrane of 1 mol/kg solution of any non-electrolyte should be equivalent to 22.4 atmospheres at 0 ^oC (Moudgil, 2010). In fact, it deviates somewhat from this value because of finite volume of the solute molecules and their physical interactions.

Osmotic effects play a very important role in human physiological systems, for example, the flow of water into and out of cells is controlled to some extent by osmotic effects, although other effects such as active transport also are involved (Grattoni et al., 2007; Lang, 2007).

Wilhelm Pfeffer (1845-1920) was the first to measure osmotic pressure in 1877 with a semipermeable membrane and a sugar solution. Pfeffer showed that the osmotic pressure depended on the size of the solute molecules, but he was unable to find a mathematical relationship to predict osmotic pressure. Jacobus Henricus van't Hoff (1852-1911) was to carry this work much further and he derived the osmotic pressure law in 1901 on the basis of purely thermodynamic reasoning that was empirically modified by Harmon Northrop Morse (1914):

$$\pi = (n / V) R T = \acute{\imath} M R T \quad (1)$$

where π is the osmotic pressure (kPa) of the solution, R is the universal gas constant, T is absolute temperature (K), M is molarity of n moles of dissolved salt per liter solution, and $\acute{\imath}$ is the van't Hoff factor of "2" for sodium chloride.

Gibbs energy (also referred to as G) that was developed by Josiah Willard Gibbs (1839-1903) is the chemical potential that is minimized when a system reaches equilibrium at constant pressure and temperature (Atkins and de Paula, 2006). Its derivative with respect to the reaction coordinate of the system vanishes at the equilibrium point (Golestanian, 2009). As such, it is a convenient criterion of spontaneity for processes with constant pressure and temperature (Greiner et al., 1995).

The role of semipermeable membrane is to allow the solvent in the solution to come to equilibrium with the pure solvent; thus the equilibrium is reached when the molar Gibbs energy of the solvent in the solution, G_1, is equal to the molar Gibbs energy of the pure solvent, G^0_1:

$$G_1 = G^0_1 \quad (2)$$

If no pressure is imposed on the solution, the Gibbs energy of the solvent in the solution is given by:

$$G_1 = G^0_1 + RT \operatorname{Ln} x_1 \quad (3)$$

where x_1 is a mole fraction.

The effect of hydrostatic pressure P on the Gibbs energy, at constant temperature is given by:

$$\partial G = V_1 \partial P \quad (4)$$

where V_1 is the molar volume of the solvent. If V_1 is assumed to be constant, the effect of a pressure π on the Gibbs energy is given by:

$$\Delta G = V1 \int_0^{\pi} \partial P = \pi V1 \quad (5)$$

The Gibbs energy of the solution of mole fraction x_1, subjected to a pressure π , is thus:

$$G_1 = G^0_1 + RT \operatorname{Ln} x_1 + \pi V_1 \quad (6)$$

However, since $G_1 = G^0_1$ at this pressure, so that:

$$\pi V_1 = - RT \operatorname{Ln} x_1 \quad (7)$$

Substituting $x_2 = 1 - x_1 \rightarrow - x_1 = x_2 - 1 \rightarrow x_1 = 1 - x_2 \rightarrow \ln x_1 = \ln 1 - \ln x_2$ gives:

$$\pi V_1 = RT \operatorname{Ln} x_2 \quad (8)$$

If the solution is dilute, $x_2 \approx n_2 / n_1$ and $V_1 = V / n_1$ where V is the total volume. Thus,

$$\pi = (R T / V). n_2 = M R T \quad (9)$$

where M that equates to n_2 / V, is the molar concentration of the solution.

It is interesting to see the equivalence between Equation 9 (π = M R T) and the ideal gas law (PV = n R T), though there is no direct significance can be attached to this similarity. Solute molecules do not bombard the semipermrable membrane in contrast to gas pressure that is due to bombardment of gas molecules. Osmotic pressure phenomenon is about the flow of solvent molecules and it is interpreted in terms of thermodynamics as it has been done above. Also, human RBC membrane is highly permeable to H_2O. Cell intracellular water content and cell volume are thus determined by the cellular content of osmotic active compounds and by the extracellular tonicity (Hoffmann et al., 2009). Under normal physiological conditions, the osmolarity of the extracellular fluid is kept constant by body fluid homeostasis (~285 mosmol/kgH_2O), and cell volume is most commonly perturbed by changes in intracellular, rather than extracellular, osmolarity (Armstrong, et al., 2004).

The existence of a concentration gradient of soluble molecules across a membrane tends to cause a net movement of solute molecules in the direction of this concentration gradient (Alberts et al., 2007). Fluxes occur in both directions and that the net flux is the sum these two movements. The rate of flow, [the flux], of uncharged molecules in the direction of the gradient can be described by the law of simple diffusion (Fick, 1855) which may be expressed:

$$J = -D\,(\partial C/\partial x) \qquad (10)$$

where D is a simple diffusion coefficient, negative sign signifies that solute moves in direction of decreasing concentration, $\partial C/\partial x$ is the concentration gradient, chemical gradient. D can also be expressed as ωRT where ω is a mobility and RT the kinetic energy expression (Atkins and de Paula, 2006).

Kirby (2010) argued that movements of ionized solutes are influenced also by electrical gradients, but the rate of flow of the solute may still be described in simple terms by the Nernst Planck equation:

$$J_+ = -\omega C \left(\frac{dC}{C} \frac{RT}{dX} + \frac{zF\, d\psi}{dX} \right) \qquad (11)$$

where the expression in brackets is the combined force due to concentration and electrical gradients, i.e. electrochemical gradient.

The movement and distribution of ions across cell membrane is greatly influenced by the presence of charged macromolecules which cannot cross membrane barriers. On each side of the membrane the total number of positive charges must always equal the total number of negative charges (Berhardt and Ellory, 2003). In case of a simple situation involving only sodium chloride solution, any movement of sodium or chloride ions across the dividing membrane must maintain the condition (Hajjawi, 2012a; 2012b):

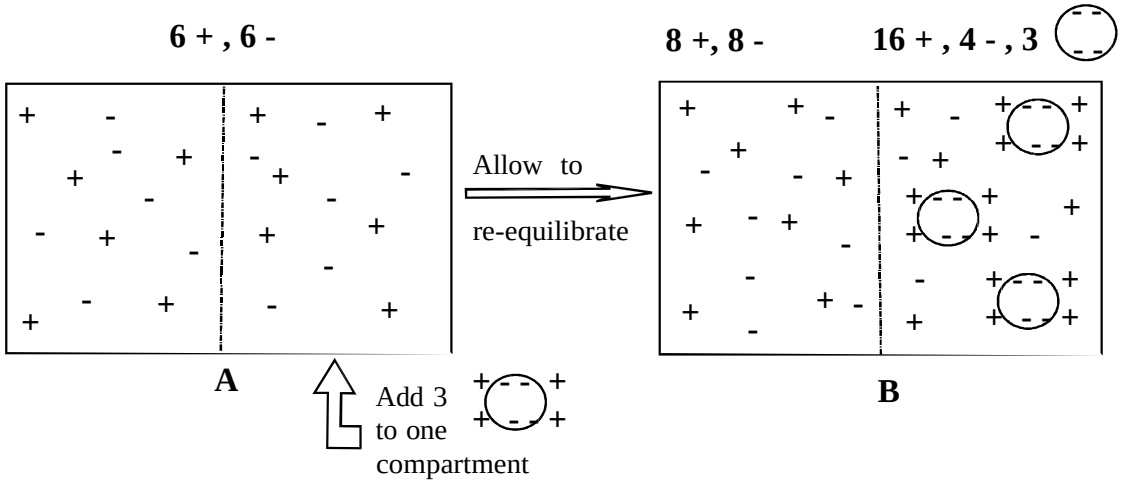
$$[Na^+_{intracellular}].\,[Cl^-_{intracellular}] = [Na^+_{extracellular}].\,[Cl^-_{extracellular}] \qquad (12)$$

If charged macromolecules are present then an equivalent number of oppositely charged permeable ions must remain with them in the compartment in which they occur. This leads to an unequal distribution of permeable ions across the membrane and it is widely referred to as a Donnan equilibrium (Figure 1) after Fredrick George Donnan (1870-1956).

Donnan Equilibrium

If two ionic solutions of deferent concentrations are put in contact at constant temperature and pressure, thermodynamic equilibrium is achieved by di usion of ionic species until any concentration gradients are extinguished. If, on the other hand, only some of the species are allowed to cross the border of the two solutions, e.g. due to a semi permeable membrane, concentration gradients will still prevail across the border also at equilibrium (Donnan, 1911).

Figure 1: The Donnan Equilibrium.



Solutions of sodium chloride are separated by a permeable membrane that allows all ions to diffuse through it and macromolecules cannot cross membrane barrier. (A) At equilibrium, equal concentrations are established $[Na^+]_{A1}$ and $[Cl^-]_{A1}$ on the left-hand side, and $[Na^+]_{A2}$ and $[Cl^-]_{A2}$ on the right-hand side. We add macromolecules $3[4Na^+.\ Protein^{4-}]$ to one $compartment_{A2}$ and allow ions to reach equilibrium. (B) At equilibrium, a new unequal distribution of diffusible ions across the membrane is established where $[Na^+]_{B1}$ and $[Cl^-]_{B1}$ and $[Na^+]_{B2}$ and $[Cl^-]_{B2}$ are unequal, i.e. 8 and 16 ions, respectively.

Human RBCs contain a significant amount of large-molecular-weight anionic colloids (mostly proteins and organic phosphates) to which the plasma membrane is impermeable, and they have a negative net charge at physiological pH (Armstrong et al., 2004; Hajjawi, 2012a). In contrast, the extracellular fluid has a low concentration of nondiffusible anion(s). If the concentration of these 'fixed' anions is $A^-_{intracellular}$ and the diffusible ions are entirely Na^+ and Cl^- then at equilibrium:

$$[Na^+_{intracellular}] = [Cl^-_{intracellular}] + [A^-_{intracellular}]$$

and

$$[Na^+_{extracellular}] = [Cl^-_{extracellular}]$$

as in Equation 12,

$$[Na^+_{intracellular}].\ [Cl^-_{intracellular}] = [Na^+_{extracellular}].\ [Cl^-_{extracellular}] \qquad (12)$$

i.e.

$$\frac{[Na^+_{intracellular}]}{[Na^+_{extracellular}]} = \frac{[Cl^-_{extracellular}]}{[Cl^-_{extracellular}]}$$

By substituting for both intracellular and extracellular sodium,

$$\frac{[Cl^-_{intracellular}] + [A^-_{intracellular}]}{[Cl^-_{extracellular}]} = \frac{[Cl^-_{extracellular}]}{[Cl^-_{intracellular}]}$$

$$[Cl^-_{extracellular}]^2 = [Cl^-_{intracellular}]^2 + [Cl^-_{intracellular}].[A^-_{intracellular}]$$

$$\frac{[Cl^-_{extracellular}]^2}{[Cl^-_{intracellular}]^2} = \frac{[Cl^-_{extracellular}].([Cl^-_{intracellular}] + [A^-_{intracellular}])}{[Cl^-_{intracellular}]^2}$$

Therefore,

$$\frac{[Cl^-_{extracellular}]}{[Cl^-_{intracellular}]} = \sqrt{\frac{[Cl^-_{extracellular}] + [A^-_{intracellular}])}{[Cl^-_{intracellular}]^2}} \qquad (13)$$

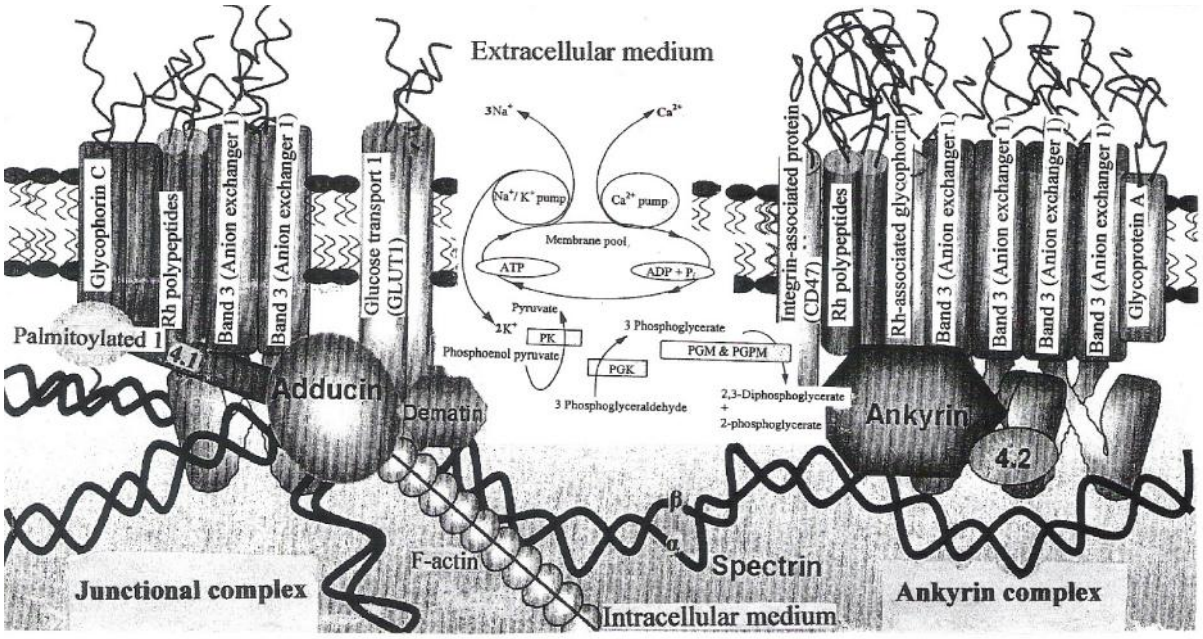
The activities of the diffusible ions on either side of the membrane are therefore unequal at equilibrium and they set up an electrical transmembrane potential, Donnan potential (Donnan, 1911; Kurbel, 2008). By changing human RBCs incubated in an isotonic NaCl solution to an isotonic sucrose

medium with only 1mM Cl^-, the membrane potential of the RBCs is drastically changed from -10mV to ~+122mV at 37 ^oC . A new Donnan equilibrium equilibrium is immediately obtained, and an alkalinization of the intracellular medium is induced (Bernhardt and Ellory, 2003, p.87). It is more likely that the positive membrane potential acts as a driving force for opening one or more cation (K^+ and N^+) channels in RBCs and Band 3 is responsible for an equimolar net efflux of anions (Denner et al., 1993). The equilibrium volume is evidently determined by the Donnan ratio per se and Band 3 that primarily controls the osmotic haemolysis (Wong, 2006). RBCs play a basic role in regulating the acid-base balance of extracellular fluids in which haemoglobin [7mmoles] the main H^+ buffer and Band 3 [10^6 copies.RBC^{-1}] mediating Cl^-/HCO_3^- are the key molecular elements for this role (Swietach et al., 2010).

The major RBC membrane-cytoskeleton complexes are of two types: (1) a junctional complex and (2) ankyrin complex (Campanella et al., 2005; Anong et al., 2009) with some overlap in the constituents of the two (Baines, 2010). The difference between them lie in their constituents (Figure 2). Also, phosphoglycerate kinase and pyruvate kinase that provide ATP for the pool, bind to Band 3 like many other glycolytic enzymes (Lewis et al., 2009). Hence, enzymes act to seclude synthesis of ATP directly into a membrane pool that fules the Na^+/K^+ and Ca^{2+} pumps directly (Skou, 1957; Hajjawi, 2012b). Band 3 is thus found in three distinct protein complexes within the RBC membrane: an ankyrin-dependant tertrameric Band 3 complex, a dimeric Band 3 complex bound to the protein 4.1-glycoprotein C junctional complex , and as freely diffusing dimeric Band 3 complex (Figure 2) (van den Akker et al., 2010). RBC membrane ATP pool might be associated with glucose transport, energizing flip-flop and the deoxygenation-promoted ATP release pathway (Lewis et al., 2009).

However, the entrapped ATP can not be highly bound, because strongly immobilized ATP would not be able to be a catalyst for glycolytic enzymes: phosphoglycerate kinase [PGK 2.7.2.3], phosphoglycerate phosphatase [PGP 5.4.2.1], phosphoglyceromutase [PGM 2.7.5.3] and pyruvate kinase [PK 2.7.1.40], or the Na^+ /K^+ and Ca^{2+} pumps. Proverbio and Hoffman (1977) estimated the entrapped ATP as 100-600 molecules per membrane pool (Figure 2). ATP sysnthesis in RBC is inhibited by acidic pH, and as the ATP concentration continues to decline, RBC is concurrently consumed to maintain function and survival (Veale et al., 2011).

Figure 2: Compartmentalization of ATP in human RBC membranes.



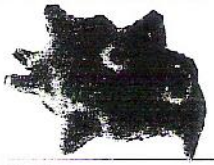
ATP membrane pool and it relation to the cytoskeletal complexes together with the bound glycolytic enzymes phosphoglycerate kinase [PGK 2.7.2.3], phosphoglycerate phosphatase [PGP

5.4.2.1], phosphoglyceromutase [PGM 2.7.5.3] and pyruvate kinase [PK 2.7.1.40]; the *Adapted from:* Anong,W.A., Franco,T. Chu,H.,Weis,T.L.,Devlin, E.E., Bodine, D.M., An,X., Mohandas, N. and Low,P.S. (2009)"Adducin forms a bridge between erythrocyte membrane and its cytoskeleton and regulates membrane cohesion", Blood, vol.114 (9), pp.1904-1912; Chu,H., Puchulu-Campanella, E., Galan, J.A., Tao, W.A., Low, P.S. and Hoffman, J.F. (2012) "Identification of cytoskeletal elements enclosing the ATP pools that fuel human red blood cell membrane cation pumps", PNAS. Retrieved December 22, 2012 from, ww.pnas.org/cgi/doi/10.1073/pnas.120914109.

Since human RBC houses substantial concentrations of charged macromolecules, mainly proteins (Figure 2), Donnan effects may be relatively important in these situations (Dahl, 2004; Blodgett, et al., 2007; Swietach et al., 2010). Also, proteins in any solution with a pH value that doffers from their isoelectric point exert both an electric Donnan effect and colloid osmotic pressure (Ganong, 2005; Swietach et al., 2010). Donnan effect alters the distribution of ions, whereas colloid osmotic pressure forces water diffusion from protein-free compartment and dilute the protein-containing fluid (Baumgarten and Feher, 2001). In other words, water tends to flow into the intracellular medium and this would, in the absence of appropriate protective mechanisms, ultimately lead to swelling and bursting of the RBCs (Satchwell, et al., 2011). Thus, the expression of a wide variety of genes is sensitive to cell volume, especially cell volume regulation genes (Ferraris and Burg, 2006). The cell shrinkage (echinocyte : speculated RBCs) stimulates expression of Na^+-K^+-$2Cl^-$ cotransporter and of the ATPase, whereas cell swelling (stomatocyte:concave RBCs) stimulates the expression of the extracellular signal regulated kinases (Waldegger et al. 1997;Lang et al., 1998; Alexander and Grinstein, 2006) (Table 1). Jacobs and Stewart (1947) proposed equations with the osmotic coefficients of haemoglobin and of salts in which a nonideal thermodynamic model predicts equilibrium Donnan ratios and red cell volume from the composition of the extracellular solution and from certain parameters of the cells (Wooten, 2003).

Table 1: Effect on human RBC shape of equilibration in altered extracellular pH buffers of constant osmolality that contain 100mM potassium chloride, 20mM potassium gluconate, and 50mM 2-[N-morphilino]ethansulphonic acid (MES), or 4-[hydroxyethyl]-1-piperazineethanesulphonic acid (HEPES), or 2-[N-cyclohexylamino]-ethanosulphonic acid (CHES) for an average pH 5.5, 7.5 and 9.5 respectively. Jacobs and Stewart (1947) and Gedde and Huestis (1997) studied physiological changes in intracellular pH, intracellular water, and membrane potential.

Adapted from: Larkin, T.J. and Kuchel, P.W. (2010) "Mathematical models of naturally "morphed" human erythrocytes: stomatocytes and echinocytes", Bull Math. Biol., vol. 72 (6), pp.1323-1333.

Shape	Stomatocyte	Discocyte	Echinocyte
Scanning electron Microscopy (x 5500)			
Buffer $pH_{Extracellular}$	Low	7.4	High
$pH_{Intracellular}$	Low	7.2	High
$[Cl^-]_{Intracellular}$	High	85mM	Low
$[H_2O]_{Intracellular}$ » osmotic solution effect	High Hypotonic	67pg Isotonic	Low Hypertonic
Membrane potential	>0	-10mV	<0

Gimsa et al. (1995) , Glaser (1995) and Swietach et al. (2010) have also established correlations between the stomatocyte-echinocyte transition and the effect of inhibition of the anion transport on the

conformation of the anion-exchange protein band 3 as well as the effect of pH on transmembrane potential (Veale et al., 2011). Hence, the appearance of a tension difference between the two leaflets of the cell membrane could be attributed to (1) different transmembrane potential of bilayer-couple on the two asymmetric sides of the cell membrane, and (2) different adsorption of counter ions at the two asymmetric surfaces (Lim et al., 2002; Tachev et al., 2004). Therefore, the maintenance of adequate cell volume is one of the most obvious prerequisites for cell survival, as excessive alterations of cell volume interferes with the integrity of cell membrane and cytoskeletal architecture (Hoffmann and Pedersen, 2006), and the discocyte shape is in fact the minimum energy configuration (Li et al., 2005).

Human circulatory system is an intricate of network of veins and arteries that distributes blood throughout the body and human blood consists of two parts, i.e., fluid and cellular. The straw coloured fluid part is known as plasma that constitutes 55% of total volume and it is composed of 92% water and the rest 8% is made up of plasma proteins (Browning et al. 2006). It is mostly composed of dissolved proteins, lipoprotein particles, serum albumin, immunoglobulins, hormones, mineral ions, glucose, clotting factors, carbon dioxide and electrolytes. Albumen regulates the colloidal pressure of blood and it accounts for 70% of colloidal osmotic pressure (Draffehn et al., 1991).Plasma circulates dissolved nutrients (such as: amino acids, fatty acids and glucose) and it removes body waste products (such as: carbon dioxide, lactic acid and urea). The remaining 45% of total volume comprises the cellular components or the formed elements such as RBCs, leukocytes and thrombocytes (Krebs, 1950; Ganong, 2005).

The features of human RBCs can be critically affected by genetic or acquired pathological conditions that impair deformity through shape and mechanical property modification (Kayden and Bessis, 1970; Bernhardt and Ellory, 2003; An and Mohandas, 2008).For example, spherocytosis is characterized by hereditary spherical RBCs that have a reduced diameter (Perrotta et al.,2008). Elliptocytosis is membrane disorders that cause elliptical, oval or elongated RBCs (Walensky et al., 2003). Sickle cell disease is an RBC sickle-shaped inherited blood disorder that affects haemoglobin (Pauling et al, 1949; Higgins, et al., 2007). Greenwood and Mutabengwa (2002) reported that malaria (*Plasmodium falciparum*) affects 500 million people and it causes more than I million deaths per year. This parasite affects RBC membrane mechanical properties and the characteristics of the biconcave shape to exhibit new cytoadherence properties (Glenister et al., 2009). Thalassemias are forms of inherited autosomal recessive blood disorders in which RBCs are destroyed at a faster rate than usual leading to anemia . The gene that controls the production of alpha or beta haemoglobin proteins is missing or mutated for α- and β- thalassemia types (Forget, 2000; Veno et al., 2006). Also, RBC morphology in Alzheimer's disease was altered as > 15% of the RBCs were elongated as compared to 5.9% in normal controls (Mohanty et al., 2008). RBC membrane architecture in Alzheimer subjects, possibly due to RBC-β-amyloid interactions and/or changes in the expression of membrane proteins (Low et al., 2002). Pasini et al. (2006) have reported about 340 membrane proteins Many of the identified proteins were shown to play a role in regulating the shape and stability of the RBC : (1) RBC shape changes in Alzheimer patients are possibly attributed primarily to the changes (elevation or decrease) in the level of a series of cytoskeleton proteins involved in regulating the stability and elasticity of the RBC membrane, and (2) changes (elevation or decrease) in the level of a second series of proteins in the RBC membrane proteome reflect similar changes reported earlier by various investigators in Alzheimer (Goodman et al., 2008; Hajjawi, 2012c).

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American Journal of Scientific Research
ISSN 2301-2005 Issue 86 March, 2013, pp.188-193
http://www.americanjournalofscientificresearch.com

The Effect of Replacing Fishmeal with *Spirulina* on Growth and Productivity of Common Carp *Cyprinus carpio* L

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Abstruct

The use of blue green algae *Spirulina* in aquaculture has several potential advantages over the production of fish. This study was designed to investigate the effect of different replacement levels of fishmeal with *Spirulina* on growth performance and some blood parameters of common carp *Cyprinus carpio* L., the trail was conducted for 105 days and for this purpose 200 fingerlings common carp. Mean initial weight was (32.7g). The fish were acclimated to laboratory conditions and fed with control pellets (31% protein) prior to the feeding trials for 21 days. Five experimental diets were used and *Spirulina* replaced fishmeal protein from the standard diet at 0% (T1), 5% (T2), 10% (T3), 15% (T4), and 20% (T5) levels. There was significant difference in the final weight attained by common carp at all levels of *Spirulina* incorporation as compared to the fish-meal-based control diet. However, the replacement of fishmeal by 10% *Spirulina* resulted in significantly superior growth of carp. The specific and relative growth rate recorded in carp improved with higher levels of *Spirulina* inclusion.

Keywords: Growth, *Spirulina,* weight gain, specific growth rate, relative growth rate

Introduction

One of the biggest problems facing the utilization of fish nutrition, in many aquaculture operations today, feed accounts more than half of the variable operating cost (NRC, 1993). Therefore, the potential use of unconventional foodstuffs such as algae, for substitution the high cost food stuffs such as fishmeal is very important. Algae have attention as a possible alternative protein source for cultured fish, particular in tropical and subtropical developing countries where algae production rates are high and their higher protein, vitamins and essential fatty acids contents (El-Hindawy, et al., 2006; Badawy, et al., 2008).

Spirulina is a cyanobacterium that has been commercially cultivated for more than 10 years due to its high nutritional content; e.g. protein, amino acid, vitamin, minerals, essential fatty acid and b-carotene (Vonshak, 1997). *Spirulina* can be considered a nutritional supplement that has various health benefits for humans, and a feed supplement for animals having economic benefits. As an example, it can be a suitable food supplement when fed to trout, sea bass, fancy carp, red tilapia, shrimp and mollusk. It has been found that the alga can be used as an alternative source of protein and can also be used to improve the color, flavor and quality of meat. Nowadays, *Spirulina* can be used to establish immune-potentiating functions in carp (Watanuki, et al., 2006; Tongsiri, et al., 2010).

(Mu, et al., 2000) and (Nandeesha, et al., 2001) indicated that *Spirulina* could be used as an effective partially or completely replacement for fishmeal in formulated aqua feeds.

However, there has been no clear data to indicate whether the effects of *Spirulina* additives for nutrient utilization can be beneficial for growth and whether there is an accumulation of carotenoids in flesh color and stomach content. As a result, the present study should provide information for the preparation of the pellet feed to maximize the productivity and growth of the common carp.

Materials and Methods

This experiment work of this study was conducted in the Fish Laboratory for the department of Animal Production, Faculty of Agricultural Sciences, University of Sulaimaniya, Iraq.

Experimental Diet

Five practical diets were formulated based on the proximate composition of the feed ingredients. Diet 1 (Control diet contained no *Spirulina*), diets 2, 3, 4 and 5 contained 5, 10, 15 and 20% dried *Spirulina* respectively by the replacement of fish meal on an equivalent protein basis. Composition and proximate analysis of algae and different experimental diet diets were shown in table 1.

Table 1: The structure of experimental diet:

Item	**100%**				
Spirulina	0	5	10	15	20
Fishmeal	24.2	21.7	19.2	16.8	14.2
wheat bran	35	35	35	35	35
Soybean	20	20	20	20	20
Broken rice	20.3	17.8	15.3	12.7	10.3
Vitamin	0.5	0.5	0.5	0.5	0.5
protein %	**31**	**31**	**31**	**31**	**31**

Moreover, the chemical composition of the used *Spirulina* showed in table (2).

Table 2: The structure of *Spirulina* used as labeled: Suitable for all herbivorous fish such as pleco's & catfish as well as shrimps & snails

Composition	**Percent**
Crude Protein	34
Crude Fat & Oils	6
Fiber Ash	5
Vitamin A	10
Vitamin D	24000lU(Per KG)
Vitamin E	2600lU
Vitamin C	280lU
	550mg/kg

Fish and Feeding Regime

Common carp (*Cyprinus carpio*) fingerlings with an average weight 32.7g were brought from a local aquarium fish supplier located in kuit, in mid of Iraq and acclimatized in plastic aquaria for three weeks before to be used in the experiment. Fish were randomly allocated on the aquaria (7/aquarium). Each treatment was represented in four aquariums (4 replicates).

A feeding regime of 3% body weight per day was employed throughout the trail. The amount of food was calculated and readjusted weekly according to change in the body weight and distributed in three equal portions for 84 days.

Experimental diets: The different feeding combinations (5 formulas of isoenergy diets, (Table 1) were prepared as follows:

T1: replacing fishmeal with 0% *Spirulina*, T2: replacing fishmeal with 5% *Spirulina*, T3: replacing fishmeal with 10% *Spirulina*, T4: replacing fishmeal with 15% *Spirulina*, T5: replacing fishmeal with 20% *Spirulina*.

Experimental System

The experimental facility consisted of 20 plastic Aquaria (100 litters each). Each aquarium was supplied with aerated and dechlorinated tap water, which was stored in tanks for 24 hours and aerated by air pamp (Model-Rina 301) during the experimental period. The water level was maintained to a fixed level by the addition of new well-aerated fresh water.

Data Calculation

Body weight gain (g/fish) = Mean of weight (g) at the end of the experimental period – weight (g) at the beginning of the experimental period.

Weight gain (DWG) = Gain / experimental period

Relative weight gain (RWG %) = Gain / initial weight X 100

Specific growth rate (SGR) = (In W_1 – In W_0) / T) X 100

Feed conversion ratio (FCR) =Total feed fed (g/fish)/ total wet weight gain (g/ fish)

Statistical Analysis of Data

Statistical analysis was performed using the Analysis of variance (ANOVA) two-way classification and Duncan's multiple Range Test, to determine differences between treatments means at significance rate of $P < 0.05$. The standard errors of treatment means were also estimated. All statistics were carried out using Statistical Analysis System (SAS) program (SAS, 2000).

Results and Discussion

The growth rates of the common carp results are shown in Table 3. Total biomass increase of fish fed T1 was significantly lower than fish fed T2, T3, T4 and T5 ($p<0.05$). The present research studied the effect of replacing 0, 5, 10, 15 and 20% of fishmeal by *Spirulina*. The highest weight yielded was found among the fish that were fed with the feed that contained *Spirulina* 10%, 16.593 g. Some studies have shown that feeding *Spirulina* to fish could improve their survival rate and growth rate (Belay, et al., 1996; Hayashi, et al., 1998; Tongsiri, et al., 2010). Significant differences were found in the average daily gain, specific and relative growth rate and feed conversion rate ($p>0.05$). T3 produced a higher average daily gain and specific growth rate than fish fed with T1, T2, T4 and T5.

Previous research has shown that *Spirulina* can be used as a protein source in feeding two important fish in India, the Cata and the Rohu. *Spirulina* was mixed in the ratios of 25, 50, 75 and 100 %, respectively. It was found that the Rohu fish increased its growth, protein efficiency ratio, digestibility of dry matter, and both protein and lipid content in correlation with the amount of *Spirulina* consumed. They concluded that it was suitable to use *Spirulina* as a protein supplement source for both fish (Nandeesha, et al., 2001).

These results showed that *Spirulina* could improve growth, reduction of mortality; overall elements of fish quality, firmness of flesh, and brightness of skin color as well as improving the cost/performance ratio of the fish feed (Vonshak, 1997; Abdul-Tawwab, et al., 2008). The results from (Tongsiri, et al., 2010) experiment indicate that 5% dried *Spirulina* could be used to replace fishmeal

and it yielded the highest weight and average daily gain/day while in this research the best replacement of fishmeal was with 10% *Spirulina*; and this was disagree with (Stander, 2004) concluded that the inclusion of dietary *Spirulina* had no significant effect on weight gain of rainbow trout. A negative trend in feed intake with increasing levels of *Spirulina* inclusion became statistically significant ($P<0.05$) above 5% *Spirulina* inclusion. (Nandeesha, et al., 2001) also found no significant difference in the final weight attained by catla at all levels of *Spirulina* incorporation as compared to the fishmeal based control diet.

Fish fed diets containing *Spirulina* (5.0 - 10.0 g/kg) had significantly better growth and feed utilization as compared to fish fed the control diet. As the study of (Abdul-Tawwab, et al., 2008) proved that dietary supplementation of *Spirulina* enhanced fish growth and immunity, as brewer's yeast, which had been reported to enhance the growth and immunity of Nile tilapia (Lara-Flores, et al., 2003; Abdul-Tawwab, et al., 2008). These results may possibly due to the improved feed intake and nutrient digestibility. Moreover, *Spirulina* contains several nutrients especially vitamins and minerals that may help in fish growth promotion. These results agree with those found by (Belay, et al., 1996), (Hayashi, et al., 1998), (Hirahashi, et al., 2002) who reported that feeding *Spirulina* to fish and poultry improved survival and growth rates. In this regard, (Watanabe, et al., 1990) mentioned that feed supplemented with *Spirulina* powder improved the feed conversion ratio and growth rates for striped jack, *Pseudocaranx dentex*. Similar results were obtained when yeasts were added to fish diet (Tovar, et al., 2002; Lara-Flores, et al., 2003; Abdul-Tawwab, et al., 2008).

From the presented results in Table 4. the average values of FCR did not show any significant ($P<0.05$) differences but numerical the T3 obtained the lowest value 0.079, FER increased significantly ($P<0.05$) with increasing of the algae replacement from 0 to 20% algae, this agree with the finding of Badwy *et al*., 2008 the incorporation 50% algae replacement resulted in the significant greater value of FCR (2.03 ± 0.08 and 1.76 ± 0.05) respectively. These results are agree with those obtained by (Dawah, et al., 2002) who found that food conversion ratio and PER were better when the fish were maintained on artificial diets with 10% and 20% dried algae. In addition, (Zeinhom, 2004) found that, Inclusion of algae in fish diets insignificantly ($P<0.05$) improved the FCR (2.33), PER (1.34) and PPV (43.10), whereas feed intake was significantly increased. However, these results are good in agreement with those obtained by (Hayashi, et al., 1998) and (Abu-Zead, 2001) who found that the protein efficiency ratio ranged from 1.1 to 1.7 for Nile tilapia and common carp fed on diets containing aquatic plant and algae, while (Ibrahim, et al., 2007) reported that, feed conversion ratio gradually increased with increasing Azolla meal percentage in the diets without significant differences until 31.8% inclusion level after that, significantly decreased, they added that, economical feed efficiency improved as the level of the dietary Azolla meal increased from 10.6 to 31.8% of the diet.

Table 3: Effect of replacing fishmeal with Spirulina on fish growth

Treatments	Initials weight	Weight gain	Daily growth rate	Specific growth rate	Relative growth rate
T1	37	8.375 ^{b}	0.100 ^{b}	23.133 ^{bc}	0.105 ^{c}
T2	37.25	12.663 ^{ab}	0.151 ^{ab}	34.431 ^{ab}	0.150 ^{b}
T3	34	16.593 ^{a}	0.198 ^{a}	48.964 ^{a}	0.205 ^{a}
T4	36.25	13.000 ^{a}	0.155 ^{a}	35.967 ^{ab}	0.155 ^{b}
T5	37.25	15.033 ^{a}	0.179 ^{a}	40.534 ^{ab}	0.175 ^{ab}

Mean values with different superscripts within a row differ significantly ($P<0.05$).

Table 4: Effect of replacing fishmeal with Spirulina on fish productivity

Treatments	Food conversion ratio	Food efficiency ratio
T1	0.190 ^{a}	674.179 ^{c}
T2	0.122 ^{a}	960.464 ^{b}
T3	0.118 ^{a}	1286.056 ^{a}

Table 4: Effect of replacing fishmeal with Spirulina on fish productivity - continued

T4	0.092 [a]	971.416 [b]
T5	0.079 [a]	1117.524 [ab]

Mean values with different superscripts within a row differ significantly (P<0.05).

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