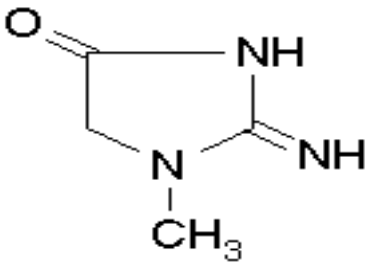
Creatinine (2-Amino-1-methyl-5H-imidazol-4-one) is a heterocyclic compound with an empirical formula of $C_4H_7N_3O$ and the following chemical structure:



It has a unique chemical structure, compared to the basic twenty ‘‘standard’’ amino acids, consisting of a five-member ring, a guanidine group and a peptide bond that helps to maintain the wellness of skeletal muscles and facilitates in mechanisms for the supply of energy to them (Wyss and Kaddurah-Daouk, 2000). Creatinine is a natural by-product of muscles doing work in your body and is usually produced at a fairly constant rate by the body (depending on muscle mass). In the body, creatinine is formed by a spontaneous and irreversible conversion from creatine and creatine phosphate, which is the source of high-energy phosphate bonds for the immediate reformation of ATP during muscular contraction. It starts out as creatine phosphate, and it ends up as a waste product in our blood, which is then eliminated in urine. In vivo, creatine is irreversibly and non-enzymatically transformed to creatinine with a steady rate of approximately 2.0 % per day (Wyss and Kaddurah-Daouk, 2000). Thus, creatinine production essentially reflects lean body mass. There is direct relationship between plasma creatinine level and muscle mass. Because this mass changes little from day to day, the production rate of creatinine is fairly constant. In normal subjects, sex and age appear to be the most important factors affecting total muscle mass and thereby the generation of creatinine. Males have a greater muscle mass than females, which accounts for their greater creatinine generation (Whelton *et al.,* 1994). Muscular young or middle-aged adults may have more creatinine in their blood than that for the general population. Older people tend to have decreased muscle mass and therefore have lower creatinine levels. The decline in muscle mass associated with aging accounts for the reduced creatinine generation in the elderly people (Cockcroft and Gault, 1976). Pathophysiologic states associated with muscle wasting and reduced muscle mass also feature reduced creatinine generation. Such conditions include chronic glucocorticoid therapy (Horber *et al.,* 1985), hyperthyroidism, dermatomyositis and polymyositis (Friedman *et al.,* 1980), muscular dystrophy (Fitch and Shields, 1966), muscular paralysis (Monler *et al.,* 1986), and all causes of negative nitrogen balance. In

people with malnutrition, severe weight loss, and long standing illnesses, the muscle mass tends to diminish over time and, therefore, their creatinine level may be lower than the expected for their age. Levels may be slightly lower during pregnancy due to increased kidney filtration rates. Vegetarians have been shown to have lower creatinine levels (Delanghe *et al.*, 1989). In addition to the total muscle mass, the size of the creatine pool is influenced by the dietary intake of creatine, largely in the form of meat. It has been demonstrated that elimination or reduction of meat intake reduces creatinine generation by 10-30 %. Cooking converts creatine in meat to creatinine; for example, when ground beef is baked for 45 mins in dry heat, the creatinine content increases from 0.2-0.4 to 3.2 mg per g. Ingestion of this "preformed" creatinine results in rapid gastrointestinal absorption and renal excretion. Restricting intake of cooked ground beef by 4 ounces per day could potentially decrease creatinine generation and excretion by 350 mg per day. However, the ingestion of cooked meat can raise the serum creatinine because cooking converts the creatine to creatinine in meat. Certain drugs, notably the psychoactive phenacemide, can increase the production rate. Creatinine levels may increase when ACE inhibitors (ACEI) or angiotensin-II receptor blockers (ARBs) are used in the treatment of chronic heart failure (CHF). The use of both ACEI & ARB concomitantly tend to increase creatinine levels to a greater degree than either of the two drugs would individually. An increase of <30 % is expected with ACEI or ARB use.

Like urea, creatinine distributes throughout total body water. Its concentration in serum is a function of the usually constant production and excretion rates. It may be slightly higher in the evening than in the morning, due to dietary meat intake during the day. In normal subjects, creatinine is excreted primarily by the kidneys. There is minimal extrarenal disposal or demonstrable metabolism. Creatinine, a low-molecular-weight (113 daltons) cation, is distributed throughout total body water, is not bound to plasma proteins, and is freely filtered by the renal glomerulus. This waste product can be easily measured in both blood and urine, and, because it is released at a steady rate by our skeletal muscles. Creatinine is chiefly filtered out of the blood by the kidneys, though a small amount is actively secreted by the kidneys into the urine. There is little-to-no tubular reabsorption of creatinine. The relationship of urinary creatinine excretion to active tissue mass was described by Folin (1905). Many investigators have since reported that the quantity of daily urinary creatinine in humans and in animals is significantly correlated with lean body mass and/or muscle mass as measured by a variety of methods

(Van-Nierkerk *et al.*, 1963; Chinn, 1967). Unlike urea, creatinine is largely unaffected by gastrointestinal bleeding or by catabolic factors such as fever and steroids.

2.1 CREATININE LEVEL IN PATHOLOGICAL CONDITIONS

The occurrence of creatine and creatinine in the blood has been investigated by Bohn & Hahn (1933). The measurement of creatinine levels in human blood or urine is clinically essential because the levels partially reflect the state of renal and muscle function. Creatinine is naturally produced by the body and is filtered from the bloodstream by the kidneys in relatively constant amounts every day. The typical human reference ranges of creatinine are 0.3-1.2 mg/dl (22.9-91.5 µmol/L) for new-borns, 0.2-0.4 mg/dl (15.3-30.5 µmol/L) for infants, 0.3-0.7 mg/dl (22.9-53.4 µmol/L) for children, 0.5-1.0 mg/dl (about 45-90 µmol/L) for women and 0.7-1.5 mg/dl (60-110 µmol/L) for men. In clinical practice, there are two significant decision levels: Values above 140 µM indicates the necessity of performing other tests for assessment of renal function such as creatinine clearance test. Values above 530 µM are almost invariably associated with severe renal impairment. Serum creatinine level of 0.6 - 1.5 mg/dl (45.8-114.4 µmol/L) means we can have up to 50 % kidney nephron loss, serum creatinine level of 1.6 to 4.6 mg/dl (77.9-350.8 µmol/L) means we can have over 50 % kidney nephron loss & therefore impaired kidney function, serum creatinine level of 4.7-9.9 mg/dl (358.4-755.0 µmol/L) means we can have 75 % kidney nephron loss & seriously impaired kidney function and serum creatinine level of 10 mg/dl and over (762.6 µmol/L and over) means we can have 90 % kidney nephron loss & end-stage kidney disease (Tietz, 1986). Levels of 24 hr urine creatinine are evaluated with blood levels as part of a creatinine clearance test. Random urine creatinine levels have no standard reference ranges. They are usually used with other tests to reference levels of other substances measured in the urine. Some examples include the microalbumin test and urine protein test. The total creatinine excretion in a normal man averages 14 to 26 mg/kg/day, and in a normal woman 11 to 20 mg/kg/day. Excretion declines with age, and is about 10 mg/kg/day in a 90 year old man. However, it should not vary more than 10 to 15 % in a given individual. The amount excreted has been used as a rough index of the completeness of daily urine collection. Creatinine is present not only in serum and erythrocytes but in all body secretions, such as sweat, bile, and gastrointestinal fluids (Schumann, 1931). Being ultra filtrable, it is present in the cerebrospinal fluid (Myers and Fine, 1919).

2.2 CLINICAL SIGNIFICANCE OF CREATININE DETERMINATION

Measurement of creatinine in plasma or serum and urine is essential in the evaluation of renal functions. The creatinine blood test is used along with blood urea nitrogen (BUN) test to assess kidney function. Both are frequently ordered as part of a basic or comprehensive metabolic panel (BMP or CMP), groups of tests that are performed to evaluate the function of the body's major organs. Urine creatinine may also be used with a variety of other urine tests as a correction factor. Creatinine is among the most commonly found nitrogen based compounds in urine. Since it is produced and removed at a relatively constant rate, the amount of urine creatinine can be compared to the amount of another substance being measured e.g. when creatinine is measured with protein to calculate a urine protein/creatinine ratio (UP/CR) and when it is measured with microalbumin to calculate microalbumin/creatinine ratio (also known as albumin/creatinine ratio, ACR). These tests are used to evaluate kidney function as well as to detect other urinary tract disorders. The creatinine diffuses out of the muscle cells and is excreted by the kidneys to urine. Creatinine cannot be reutilized and thus is a waste product. It is freely filtered by the glomeruli and there is no significant tubular reabsorption. A small amount of creatinine is excreted by active tubular secretion, the amount increasing with raised plasma creatinine concentrations (Bauer *et al.,* 1982). The plasma creatinine level depends on the creatinine production rate and the rate of elimination in the glomerular filtrate. In the steady state, the rates of creatinine production and elimination are equal and can be estimated as the product of urine creatinine concentration and urine flow rate. If the production of creatinine is constant the plasma creatinine level increases, when creatinine elimination via the kidneys decreases. The kidneys maintain the blood creatinine in a normal range. There are two kinds of tests involving creatinine:

Serum creatinine: This is obtained from a simple blood test. Serum creatinine is the major component in the various equations used to estimate GFR, and is used as an index of renal function in clinical practice. The use of serum creatinine as a marker of the GFR originated from the work of Rehberg (1926), studied the renal clearance of exogenously administered creatinine. Glomerular filtration of creatinine, however, is only one of the variables that determine its concentration in serum. Alterations in renal handling and metabolism of creatinine and methodological interferences in its measurement may have a profound impact on the serum concentration of creatinine. Irregularities in the function

of kidneys or any condition that impair the function of the kidneys cause improper filtering of the urine, raising the creatinine level in the blood and its retention in the blood (uremia). Abnormally high levels of creatinine thus warn of possible malfunction or failure of the kidneys. High concentrations of creatinine in the serum indicate inadequate function of the kidneys also known as acute/chronic renal failure (ARF/CRF) (Yokozawa *et al.*, 1997). It is important to recognize whether the process leading to kidney dysfunction (kidney failure, azotemia) is long-standing or recent. Plasma creatinine, however, is not usually measurably increased until there is at least 50 % loss of renal function (Renkin and Robinson, 1974). Scientific interest in creatinine was intensified after its role in kidney failure was understood. It is for this reason that standard blood tests routinely check the amount of creatinine in the blood. The use of serum creatinine as an index of the GFR depends on the constancy of creatinine metabolism, with production being equal to the renal excretion rate. Differences in production or extra renal elimination among individuals or changes over time in an individual may significantly affect the serum creatinine concentration, even if renal function remains the same.

Creatinine clearance: A more precise measure of the kidney function can be estimated by calculating how much creatinine is cleared from the body by the kidneys and it is referred to creatinine clearance. This is obtained from a measured 24 hr urine collection. It measures the actual amount of creatinine which has been excreted, and as such, it is roughly the equivalent of GFR, which is the percentage of kidney function that most kidney patients are familiar with. Creatinine clearance calculated from measured creatinine concentrations in plasma and in timed urine collections might thus be expected to indicate early dysfunction more sensitively (Bowers and Wong, 1980). It is an excellent indicator of kidney function. The reproducibility of measurements is an important aspect in the assessment of the glomerular function (Morgan *et al.,* 1978).

The concentration of plasma or serum creatinine is often considered sufficient to describe the GFR. For an accurate interpretation, reference values from healthy individuals should be collected separately for different age and gender groups to adjust with differences in muscle mass, otherwise the reference values will disguise small but significance impairment of renal function. To overcome this problem numerous formulas and nomograms have been developed with more satisfactory results for estimating creatinine clearance from the serum creatinine concentration, thereby passing the need for urine collection (Jelliffe, 1971, 1973; Kampmann *et al.*, 1971, 1974; Cockcroft and Gault, 1976; Bjornsaon *et al.,* 1983; Schwartz *et al.,* 1987; Levey *et al.,* 1999, 2007). These

formulas and nomograms are based on the principle that creatinine excretion is constant and equal to creatinine production, which, in turn, is proportional to muscle mass and can be estimated from an individual's age, sex, and weight. Thus, based on these measurements and the serum creatinine concentration, creatinine clearance can be predicted. Furthermore, these formulas and nomograms also assume stability of renal function, namely, that the serum creatinine has attained its steady-state value for the prevailing renal function. Meaningless results will be obtained in situations in which the serum creatinine concentration is rising or falling.

Unlike urea, which also measures kidney function to some extent, creatinine is only slightly affected by the meat proteins we eat. As a result, creatinine is a more precise, more specific measure of our kidney function than urea. Although it is a waste, creatinine serves a vital diagnostic function. The creatinine blood test may be ordered, along with BUN test and microalbumin, at regular intervals when we have a known kidney disorder or have a disease that may affect kidney function or be exacerbated by dysfunction. Both BUN and creatinine may be ordered when a CT scan is planned, prior to and during certain drug therapies, and before and after dialysis to monitor the effectiveness of treatments. Increased/ decreased creatinine levels are associated with a number of physiological conditions. The diseases associated with either high concentration of creatinine or low concentration of creatinine in blood serum are classified in two categories as given below:

2.2.1 Diseases associated with high creatinine concentration in serum

In renal disease, serum creatinine values do not increase significantly until renal function has been considerably impaired. Determination of creatinine clearance ratios, however, may more sensitively indicate renal impairment and thus can be useful for following the effect of a course of treatment in cases of acute glomerulonephritis, to identify a primarily gbomerular dysfunction, and to assess overall renal function (Fink *et al.,* 1999). A creatinine clearance value that is 20-40 % of normal indicates severe renal disease, values 40-60 % of normal suggest moderate impairment, and values between 60-80 % of normal reflect mild dysfunction (Ravel, 1978). Increased creatinine levels in the blood suggest diseases or conditions that affect kidney function (Perrone *et al.,* 1992). These can include:

Glomerulonephritis: It is also known as glomerular nephritis, is a renal disease (usually of both kidneys) characterized by inflammation of the glomeruli, or small blood vessels in

the kidneys. It may present with isolated hematuria and/or proteinuria (blood or protein in the urine); or as a nephrotic syndrome, a nephritic syndrome, ARF or CRF. These are categorized into several different pathological patterns, which are broadly grouped into non-proliferative or proliferative types. Diagnosing the pattern of glomerulonephritis is important because the outcome and treatment differs in different types. Primary causes are ones which are intrinsic to the kidney, whilst secondary causes are associated with certain infections (bacterial, viral or parasitic pathogens), drugs, systemic disorders (vasculitis) or diabetes.

Acute tubular necrosis: It is a medical condition involving the death of tubular cells that form the tubule that transports urine to the ureters, while reabsorbing 99 % of the water (and highly concentrating the salts and metabolic byproducts). Acute tubular necrosis presents with ARF and is one of the most common causes of ARF. The presence of "muddy brown casts" of epithelial cells found in the urine during urinalysis is pathognomonic for acute tubular necrosis.

Nephritis: It is a bacterial infection of one or both kidneys. Nephritis is inflammation of the nephrons in the kidneys (Rudnick *et al.*, 1983). Nephritis is the most common producer of glomerular injury. It is a disturbance of the glomerular structure with inflammatory cell proliferation. This can lead to reduced glomerular blood flow, leading to reduced urine output (oliguria) and retention of waste products (uremia). In experimental nephritis, the increases in creatinine are less regular. In general, no change is observed during the first few days, after which a gradual rise takes place. Obviously, the rapidity of the increase in creatinine depends upon the extent and severity of the renal lesion.

Rhabdomyolysis: It is a condition in which damaged skeletal muscle tissue breaks down rapidly. Breakdown products of damaged muscle cells are released into the bloodstream; some of these, such as the protein myoglobin, are harmful to the kidneys and may lead to kidney failure. The severity of the symptoms, which may include muscle pains, vomiting and confusion, depends on the extent of muscle damage and whether kidney failure develops. The muscle damage may be caused by physical factors (e.g. crush injury, strenuous exercise), medications, drug abuse, and infections. Some people have a hereditary muscle condition that increases the risk of rhabdomyolysis (Rudnick *et al.*, 1983).

Pre-eclampsia: It is a medical condition in which the hypertension arises in pregnancy (pregnancy-induced hypertension) in association with significant amounts of protein in

the urine. It refers to a set of symptoms rather than any causative factor, and there are many different causes for the condition. It appears likely that there are substances from the placenta that can cause endothelial dysfunction in the maternal blood vessels of susceptible women. While blood pressure elevation is the most visible sign of the disease, it involves generalized damage to the maternal endothelium, kidneys, and liver, with the release of vasoconstrictive factors being secondary to the original damage (Sims and Krantz, 1958).

Diabetic nephropathy: It is also known as Kimmelstiel-Wilson syndrome, or nodular diabetic glomerulosclerosis and intercapillary glomerulonephritis, is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is characterized by nephrotic syndrome and diffuse glomerulosclerosis. It is due to long-standing diabetes mellitus.

Bilateral nephrectomy: It is seldom performed routinely nowadays but it may be indicated for some patients with end stage renal failure who are having maintenance dialysis. After bilateral nephrectomy, the creatinine of the blood rises quite rapidly.

Hemolytic-uremic syndrome or haemolytic-uraemic syndrome (HUS): It is a disease characterized by hemolytic anemia (anemia caused by destruction of red blood cells), ARF (uremia) and a low platelet count (thrombocytopenia). It predominantly, but not exclusively, affects children. It is a medical emergency and carries 5–10 % mortality; of the remainder, the majorities recover without major consequences but a small proportion develop chronic kidney disease (CKD) and become reliant on renal replacement therapy. However, eleven months post-transplant another sudden, biopsy-proven recurrence of HUS occurred, with a rise in serum creatinine to approximately 700 μmol/L (Besbas *et al.* 2006).

Cardio vascular disease: It is a class of diseases that involve the heart or blood vessels (arteries and veins). Cardiovascular disease is the main cause of death in patients with kidney failure. Moreover, the presence of impaired renal function is an important prognostic factor in patients with heart disease, and is a determinant of outcome during follow-up. Creatinine level at admission is one of the most important covariates in early prognostic stratification in these patients. A high serum creatinine level (or a low glomerular filtration rate) increases the probability of death (Facila *et al.*, 2006).

Hypoxic-ischemic encephalopathy (HIE): It is associated in most cases with oxygen deprivation in the neonate due to birth asphyxia, it can occur in all age groups, and is often a complication of cardiac arrest. It is an important disease in the asphyxiated

newborn infants, usually leads to neonatal death and neurological sequelae. HIE caused by perinatal asphyxia is the main cause of neonatal death and neurological sequelae. Thus, early identification of HIE is of great importance in preventing neonatal death and neurological sequelae. The urinary L/C was significantly higher in the newborns who were identified with HIE later, showing satisfactory sensitivity and specificity in predicting HIE development (Liu *et al.,* 2006).

Acute lymphoblastic leukemia (ALL): It is a form of leukemia, or cancer of the white blood cells characterized by excess lymphoblasts. Malignant, immature white blood cells continuously multiply and overproduction occurs in the bone marrow. ALL causes damage and death by crowding out normal cells in the bone marrow, and by spreading (infiltrating) to other organs. Protein energy malnutrition is well-recognized in children with acute leukemia and may result in loss of lean body mass (LBM) with attendant morbidities. Much of the LBM consists of skeletal muscle, the mass of which is reflected in urinary creatinine excretion. Serum creatinine concentration provides a surrogate measure of LBM in children with acute lymphoblastic leukemia. Clearly, a simple and accurate measure of LBM would be a useful contribution to the care of children with cancer (Atkinson *et al.,* 1998; Morrison *et al.,* 2011).

Acute Pancreatitis: It is a sudden inflammation of the pancreas. It can have severe complications and high mortality despite treatment. Amylase/creatinine clearance ratios are useful as an indicator of acute pancreatitis (Murray and Mackay, 1977). The specificity of amylase/creatinine clearance ratio as a marker for acute pancreatitis has been questioned because it increases in disorders other than acute pancreatitis e.g. acute duodenal perforation (Berger *et al.,* 1976) and chronic renal insufficiency (Tedesco *et al.,* 1976).

Type 1 diabetes mellitus: Increase in the GFR, creatinine clearance is also increased in early type 1 diabetes (Puig *et al.,* 1981). A variety of changes in renal function and creatinine metabolism occur during episodes of diabetic ketoacidosis and nonketotic hyperosmolar coma. Extreme hyperglycemia, osmotic diuresis, and depletion of extracellular fluid volume may cause a decline in GFR and an increase in serum creatinine. Long-standing diabetes mellitus is associated with the development of renal failure in ~30-50 % of type 1 patients (Krolewski *et al.,*, 1985).

The maturity of a baby and its ability to breathe could be estimated by measuring the amount of creatinine in the amniotic fluid. If the baby has mature kidneys and lungs it

will have more creatinine. This knowledge can be invaluable in a decision as to whether to deliver a baby prematurely due to complications in the mother.

2.2.2 Diseases associated with low creatinine concentration in serum:

Low blood levels of creatinine are not common, but they are also usually a cause for concern. They can be seen with conditions that result in decreased muscle mass. The low concentration of creatinine associated with some diseases as described below:

Muscular dystrophy: It is a group of muscle diseases that weaken the musculoskeletal system and hamper locomotion. Muscular dystrophies are characterized by progressive skeletal muscle weakness, defects in muscle proteins, and the death of muscle cells and tissue. These diseases predominately affect males, although females may be carriers of the disease gene. Most types of muscular dystrophy are multi-system disorders with manifestations in body systems including the heart, gastrointestinal system, nervous system, endocrine glands, eyes and brain. As early as 1909, it was recognized that urinary excretion of creatinine was low in muscle disease, especially in muscular dystrophy (Levene and Kristeller, 1909). It is well known that in certain conditions associated with marked atrophy of the muscular system, the creatinine output is generally lowered, and creatine makes its appearance in the urine in excessive amounts as compared with normal individuals of the same age, weight, and sex.

Type 2 diabetes mellitus (T2DM): Diabetes mellitus type 2, formerly known as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency. Long-term complications from high blood sugar can include heart attacks, strokes, diabetic retinopathy where eye sight is affected, kidney failure which may require dialysis, and poor circulation of limbs leading to amputations. Serum creatinine may serve as a surrogate marker of muscle mass, and a possible relationship between low serum creatinine and type 2 diabetes has recently been demonstrated (Hjelmeseth *et al.*, 2010). Low serum creatinine levels were associated with a higher risk of T2DM in a recent study of non-obese middle aged Japanese men (Harita *et al.*, 2009), leading to speculate that low creatinine might reflect low muscle mass volume. The serum creatinine concentrations indicating renal insufficiency in the Korean patients with type 2 diabetes were considerably lower than those in Caucasians, and the serum creatinine concentration alone exhibited a limited diagnostic value (Lee *et al.*, 2009).

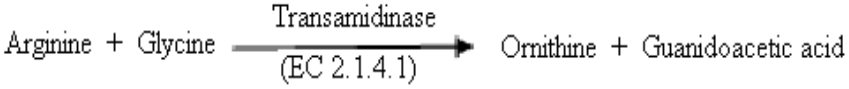
Myasthenia gravis: It is an autoimmune neuromuscular disease leading to fluctuating muscle weakness and fatiguability. It is an autoimmune disorder, in which weakness is caused by circulating antibodies that block acetylcholine receptors at the postsynaptic neuromuscular junction, inhibiting the excitatory effects of the neurotransmitter acetylcholine on nicotinic receptors throughout neuromuscular junctions. The hallmark of myasthenia gravis is fatigability. Muscles become progressively weaker during periods of activity and improve after periods of rest. Muscles that control eye and eyelid movement, facial expressions, chewing, talking, and swallowing are especially susceptible. The muscles that control breathing and neck and limb movements could also be affected.

2.3 METABOLISM OF CREATININE

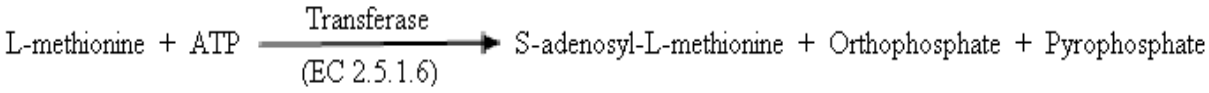
The history of creatinine dates back to 1847, when Liebig (1847) so named a substance he obtained by heating creatine with mineral acids. Creatinine was synthesized by Horbaczewski (1885). His synthesis was confirmed by Paulmann (1894), also furnished proof that creatine was methylguanidoacetic acid and that creatinine was its internal anhydride. The synthesis of creatine, the precursor of creatinine, takes place primarily in the liver. Creatinine formation begins with the transamidination from arginine to glycine to form glycocyamine or guanido acetic acid (GAA). This reaction occurs primarily in the kidneys, but also in the mucosa of the small intestine and the pancreas. The GAA is transported to the liver, where it is methylated by S-adenosyl methionine (SAM) to form creatine. After its release into the circulation, creatine is actively taken up by the muscles and other tissues**.** In a reaction catalyzed by creatine kinase (CK, EC 2.7.3.2), most of this muscle creatine is phosphorylated to creatine phosphate. Muscle contains ~98 % of the total body creatine pool (i.e., 120 g in a 70 kg man), of which 60-70 % exists as phosphocreatine, and the remainder as free creatine. Small amounts of creatine are measurable in liver, kidney, brain, and body fluids. The serum concentration in adults ranges from 1.6 to 7.9 mg/L (Geigy, 1971). Creatine is freely filtered and actively reabsorbed by the kidney, and urinary creatine excretion is usually <100 mg/24hr. Urinary excretion of creatine increases in diseases associated with abnormal muscle metabolism ((Fitch and Shields, 1966)). Creatinine is formed as a result of the nonenzymatic dehydration of muscle creatine (Hahn and Meyer, 1928; Borsook and Dubnoff, 1947) **(Fig. 1).** The reaction rate may vary with changes in intracellular pH and temperature. Both the rate of phosphorylcreatine hydrolysis and the ratio of creatine to

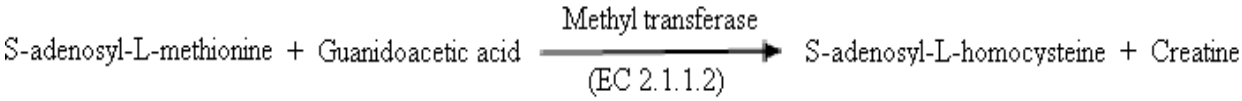
creatinine formed depend on temperature & pH and could additionally be influenced in a concentration-dependent manner by molybdate (Ennor and Morrison, 1958; Morrison and Ennor, 1960). Creatine level is favored at high pH and low temperature, whereas creatinine level is favored at elevated temperatures and in acidic solutions (Lempert, 1959). In contrast to these in vitro studies, experiments with ^{15}N-labeled compounds clearly showed that the conversion of creatine into creatinine in vivo is an irreversible process (Bloch and Schoenheimer, 1939). Because creatinine is a very poor substrate of the creatine transporter, no other specific saturable uptake mechanism exists for creatinine and creatinine, most likely due to its nonionic nature, and being membrane permeable, creatinine constantly diffuses out of the tissues into the blood and is excreted by the kidneys into the urine. Twenty to twenty-five percent of the in vivo conversion of phosphorylcreatine into creatinine may proceed via phosphorylcreatinine as an intermediate (Iyenger *et al.,* 1985). The reactions involved in the metabolism of creatine and creatinine at various sites in the body are summarized as follows:

In the kidneys

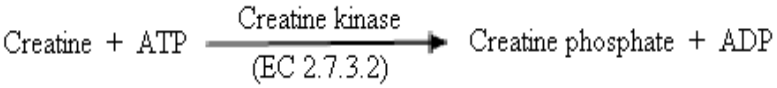


In the liver





In muscle



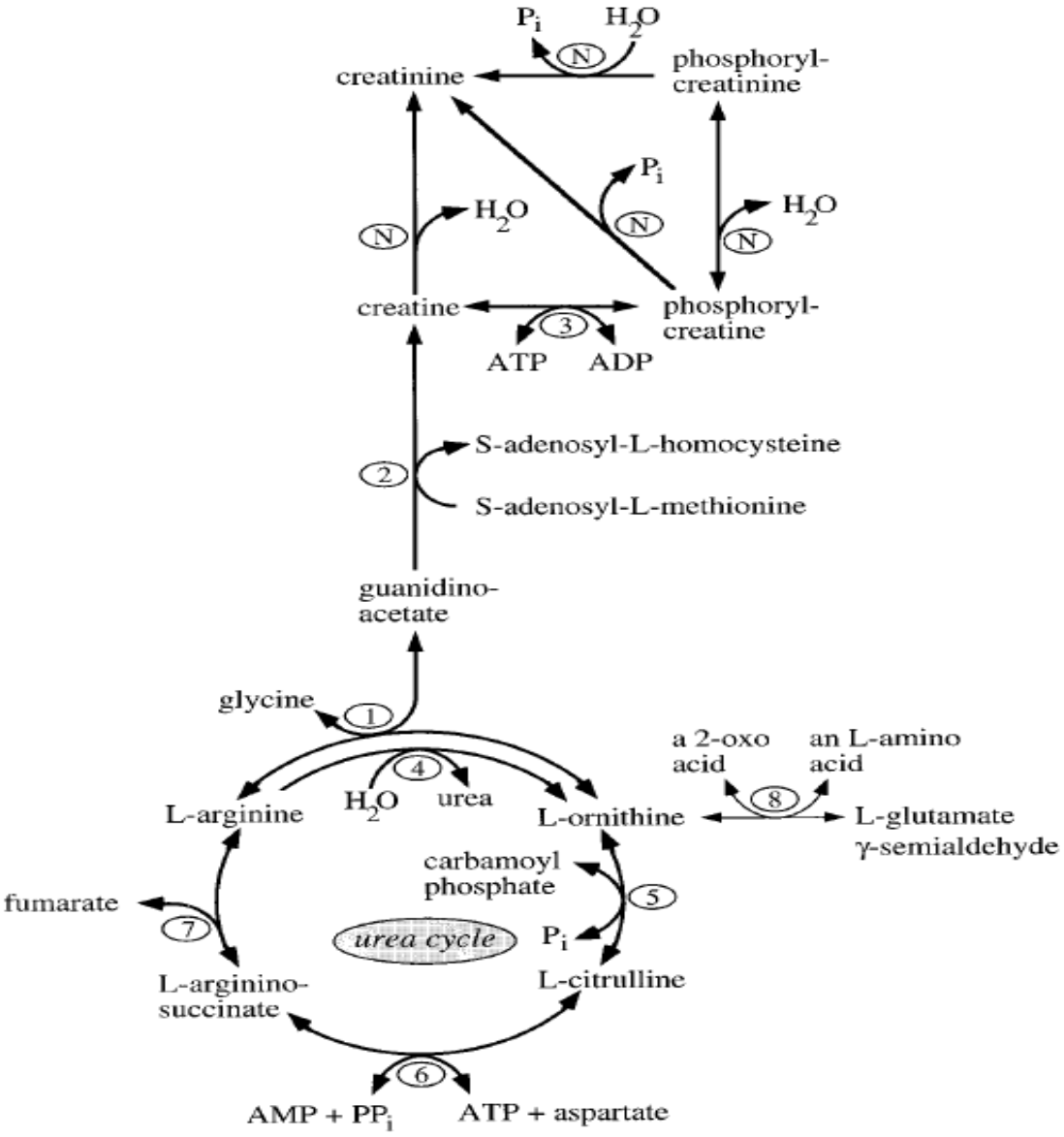


Fig. 1. Schematic representation of the reactions and enzymes involved in creatine and creatinine metabolism. *The respected enzymes are denoted by numbers: 1) L-arginine: glycine amidinotransferase (AGAT; EC 2.1.4.1); 2) S-adenosyl-L-methionine: N-guanidino acetate methyltransferase (GAMT; EC 2.1.1.2); 3)Creatine kinase (CK; EC 2.7.3.2); 4) Arginase (L-arginine amidinohydrolase; EC 3.5.3.1); 5) Ornithine carbamoyltranferase (EC 2.1.3.3); 6) Argininosuccinate synthase (EC 6.3.4.5); 7) Argininosuccinate lyase (EC 4.3.2.1); 8) L-ornithine: 2-oxo-acid aminotransferase (OAT; EC 2.6.1.13); N) Non enzymatic reactions.* [Source: Wyss and Kaddurah-Daouk, 2000]

2.4 ENZYMES USED IN CREATININE DETERMINATION

2.4.1 Creatininase (Creatinine amidohydrolase, CA) and creatinase (creatine amidinohydrolase, CI)

Both creatininase (CA, EC 3.5.2.10) and creatinase (CI, EC 3.5.3.3) are inducible enzymes, being expressed in bacteria only when creatinine or creatine is provided as main source of carbon or nitrogen (Shimizu *et al.,* 1986). CA activity has been detected in *Alcaligenes, Pseudomonas, Artheobacter, and Flavobacterium* species and CI activity has been detected in *Alcaligenes, Artheobacter, Bacillus, Flavobacterium, Micrococcus* and *Pseudomonas* species. CA and CI were found intracellularly in *Pseudomonas* while, the same enzymes from *Alcaligenes* seem to be located extracellularly (Akamatsu and Kanai, 1951; Kaplan and Naugler, 1974; Tsuru *et al.,* 1976; Inouye *et al.,* 1986; Chang *et al.,* 1992; Suzuki *et al.,* 1993). CA from *Artheobacter* and *Pseudomonas* were most likely octameric molecules with a subunit molecular weight of ~30 KDa, whereas *Alcaligenes* CA was a dimer composed of two identical 80 KDa subunits.

CA was stable over quite a broad pH and temperature range and display pH optima between 7 & 9 in both directions of the reaction (Akamatsu and Kanai, 1951; Tsuru *et al.,* 1976; Inouye *et al.,* 1986). The K_m values for creatine and creatinine were 80–162 and 26–66 mM, respectively. The V_{max} values of *Pseudomonas* CA were 390–1,400 and 1,510 µmol/min/(mg protein) respectively, in the direction of creatine and creatinine formation (at pH 7 or 8 and 30 °C). Atomic absorption spectrophotometry revealed that *Pseudomonas* CA contains one zinc atom per subunit (Rikitake *et al.,* 1979), and it was highly probable that the CA of *Alcaligenes* and *Artheobacter* were metal-containing enzymes as well (Kaplan and Szabo, 1974; Inouye *et al.,* 1986). Accordingly, CA was inactivated by EDTA, with the metal-free enzyme being completely inactive. In addition, the native (metal containing) CA was inactivated to different degrees by Co^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+} and Hg^{2+}. Reactivation of metal-free *Pseudomonas* CA, on the other hand, was reached, in order of decreasing effectiveness, with Mn^{2+}, Co^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+}. Sulfhydryl reagents inhibited the enzymatic activity by not more than 40 %, while N-bromosuccinimide, o-phenanthroline, ethoxyformic anhydride, and photooxidation caused strong or even complete inactivation.

CI has been purified partially from *Artheobacter ureafaciens* (Kaplan and Szabo, 1974) and to apparent homogeneity from *Pseudomonas putida* (Yoshimoto *et al.,* 1976),

Bacillus (Suzuki *et al.,* 1993), *Artheobacter* sp. TE1826 (Nishiya *et al.,* 1998) and *Alcaligenes* (Matsuda *et al.,* 1986). The molecular weight of native *Artheobacter ureafaciens* CI was 100 KDa while the subunit molecular weight of CI from *Artheobacter* sp. TE1826 determined by SDS-PAGE is 48 KDa. *Pseudomonas* and *Bacillus* CI were composed of two identical 43-47 KDa subunits each, while *Alcaligenes* CI was a monomer with a molecular weight of 51 KDa. The CI of *Alcaligenes* and *Pseudomonas* displayed a pI of 4.7–4.8, a pH optimum of 7.5–8.0 (in the direction of creatine breakdown), and were inactivated by sulfhydryl reagents like p-chloromercuribenzoate. Complete inactivation of *Pseudomonas* CI was achieved upon modification of a single sulfhydryl group per protomer. *Alcaligenes* CI has a 65 times higher specific activity than its *Pseudomonas* counterpart, whereas the K_m values for creatine were in a similar range (17.2 vs 1.3–25 mM). Creatine was the preferred substrate of *Pseudomonas* CI, but N-acetimidoylsarcosine was also hydrolyzed readily (Roberts and Walker, 1985). In contrast, guanidinoacetate, GPA, N-ethylguanidinoacetate, N-propylguanidinoacetate, N-methyl-3-guanidinopropionate and cyclocreatine do not or only poorly serve as substrates. *Pseudomonas* CI also catalyzed the degradation of pseudothiohydantoic acid to urea and thioglycolic acid, this reaction is inhibited by carbamoyl sarcosine, succinic acid, sarcosine, succinamic acid, creatine and some other compounds. Crystal structure determination of CI from *Pseudomonas putida* further corroborated the biochemically determined size of the molecule and showed that the two identical subunits were linked in the dimer by more than 20 hydrogen bonds and four salt bridges (Hoeffken *et al.,* 1988; Coll *et al.,* 1990). Each subunit was composed of two domains: a smaller -NH_2 terminal domain (residues 1–160) and a larger -COOH terminal domain (161– 402). Residues 155–160 form a hinge region connecting the two domains and allows the movements of the two domains relative to each other. Co-crystallization of CI with creatine, carbamoylsarcosine, succinamic acid, or sarcosine revealed that the active site was buried in the interior of the large domain and is partly covered by the small domain of the neighboring subunit. The entrance to the cavity was blocked by two arginine residues. Pocket opening and closure was most likely brought about by a rotation and/or translation of the two domains relative to each other. When bound to the enzyme, the guanidinium and carbamoyl groups of creatine and carbamoylsarcosine, respectively, were not planar but display a distorted geometry that was characterized by disrupted electron delocalization. His-B232 was close to the guanidinium group of creatine and probably plays a central role in catalysis by serving as a proton donor and acceptor. All residues of

Pseudomonas CI interacting with the substrate creatine, the product sarcosine, and the competitive inhibitor carbamoylsarcosine were fully conserved in the *Bacillus*, *Flavobacterium*, *Artheobacter*, and *Alcaligenes* CI sequences. Alkylation of Cys-298 causes inactivation of the enzyme. Because this residue was far from the active site, it was tempting to speculate that inhibition was due to an impairment of domain (or subunit) motion, with the enzyme being locked in either an open or closed conformation (Coll *et al.,* 1990). Comparison of the amino acid sequences and three dimensional structures revealed that the –COOH terminal domain of *Pseudomonas putida* CI was related to methionine aminopeptidase (AMPM; EC 3.4.11.18) and proline-specific aminopeptidase (AMPP; EC 3.4.11.9) from E. coli (Murzin, 1993, Wilce *et al.,* 1998). Within an ~260 residue chain segment, 218 C^{α} atoms of the CI and AMPM structures superimpose within 2.5 Å, but only 41 of these overlapping positions (19 %) feature identical amino acids. AMPM (AMPP) was activated by Co^{2+} (Mn^{2+}), which was coordinated by Asp-97, Asp-108, His-171, Glu-204 and Glu-235 (Asp-260, Asp-271, His-354, Glu-383, and Glu-406). The structurally equivalent residues of CI, Asn-249, Ala-260, His-324, Glu-358, and His-376, were substantially different. Accordingly, CI is not a metal dependent enzyme. Searches of protein data banks using sequence and structure-based profiles revealed other enzymes, including amino peptidase P (EC 3.4.11.9), prolidase (proline dipeptidase, EC 3.4.13.9), eIF-2-associated p67 factors, and agropine synthase, that likely share the same "pita-bread" fold common to CI and AMPM. In very preliminary studies, Miyoshi *et al.* (1980a,b) reported on the detection and partial characterization of CI from human skeletal muscle. The enzyme displayed a native molecular weight of ~50,000 and a pH optimum of 6.2. Whereas CI from non myopathic patients revealed normal Michaelis-Menten behavior with a K_m (creatine) of 80 mM, the enzymatic activity of creatinase from patients with Duchenne muscular dystrophy depended in a sigmoidal manner on creatine concentration, with half-maximal velocity at 360 mM. In the light of the widespread belief that nonenzymatic conversion to creatinine represents the only pathway for creatine degradation in vertebrates, these findings clearly await confirmation.

2.4.2 Sarcosine oxidase (SO)

In various microorganisms, sarcosine was metabolized further to glycine. In most bacteria belonging to the genera *Alcaligenes*, *Artheobacter*, *Bacillus*, *Corynebacterium*, and *Pseudomonas* as well as in fungi from the genera *Cylindrocarpon* and *Streptomyces*, this degradation was achieved by a sarcosine oxidase (SO, EC 1.5.3.1). SO and sarcosine

dehydrogenase activities were induced in these microorganisms only when grown on a medium containing creatinine, creatine, 1-methylhydantoin, sarcosine, dimethylglycine, choline, or betaine as main source of carbon and/or nitrogen. At least two classes of microbial SO have to be discriminated, all of which were flavoproteins with a K_m for sarcosine of 0.9–12.2 mM. *Artheobacter*, *Bacillus*, *Cylindrocarpon* and *Streptomyces* species express monomeric SO with ~390 amino acid residues and a molecular weight of 42–45 KDa. On the other hand, heterotetrameric SO with a molecular weight of 160–185 KDa have been identified in *Corynebacterium*, *Pseudomonas* and *Artheobacter denitrificans*. The *corynebacterial* enzyme was composed of α-subunit (molecular weight 103 KDa), β-subunit (44 KDa), γ-subunit (21 KDa) and δ-subunit (11 KDa), with the covalent flavin being attached to the β-subunit. In addition to the covalent flavin, a noncovalent flavin and an NAD^+ molecule have been identified as coenzymes of *corynebacterial* SO. While the flavins were involved in catalysis, NAD^+ was not reduced by sarsocine and seems not to be in redox equilibrium with the flavins. Finally, the SO of *Alcaligenes denitrificans* was suggested to be a 190 KDa molecular weight heterodimer or heterotrimer composed of an α-subunit (molecular weight 100 KDa) and one or two β-subunits (molecular weight 55 KDa). However, it cannot be ruled out at present that this protein also is a heterotetrameric enzyme, with the γ and δ subunits having escaped detection so far. All bacterial SO catalyze the oxidative demethylation of sarcosine to yield glycine, H_2O_2, and formaldehyde. The heterotetrameric SO, in contrast to the monomeric ones, also use tetrahydrofolates as substrates, giving rise to 5,10-methylenetetrahydrofolates instead of formaldehyde as reaction products. In this regard, heterotetrameric SO resemble mammalian sarcosine and dimethylglycine dehydrogenases (EC 1.5.99.2) that catalyze the last two steps in the consecutive degradation of choline to betaine, dimethylglycine, sarcosine (methylglycine), and glycine. Interestingly, sequence comparisons revealed pronounced homology between the β-subunit of heterotetrameric corynebacterial SO, monomeric bacterial SO, rabbit peroxisomal SO, pipecolic acid oxidase from mammalian liver, amino acid deaminase from *Proteus mirabilis*, a (duplicated) protein with unknown function from *Caenorhabditis elegans*, and the -NH_2 terminal half of mammalian dimethylglycine dehydrogenase (Wyss and Kaddurah-Daouk, 2000). Likewise, the α-subunit of heterotetrameric *corynebacterial* SO displays sequence homology to the A subunits of octopine and nopaline oxidases from *Agrobacterium tumefaciens*, to T proteins from *E. coli* and several eukaryotes (components of the multienzyme glycine cleavage system), and to the –COOH terminal

half of dimethylglycine dehydrogenase. These results therefore point to common evolutionary ancestors for all of these (flavo) proteins.

2.5 VARIOUS METHODS FOR DETERMINATION OF CREATININE

The significance of the creatinine level in serum or urine, due to its presumed relationship to renal diseases, thyroid malfunction & muscular disorder (Julia *et al.*, 2002), led to the development of numerous methods for its quantitative determination. A variety of methods including chemical (Jaffe, 1886), enzymic colorimetric methods (Miller and Dubos, 1937), high performance thin layer hromatography (HPTLC) (Brown, 1966), high performance liquid chromatography (HPLC) (Sadilek, 1965), liquid chromatography-mass spectrometry (LC-MS) (Takatsu and Nishi, 1993), liquid chromatography-tandem mass spectrometry (LC-TMS) (Keevil *et al.*, 2002), liquid chromatography-isotope dilution mass spectroscopy (LC-IDMS) (Harlen *et al.*, 2010), gas chromatography-mass spectrometry (GC-MS) (Siekmann, 1985), capillary electrophoresis (Guzman, 1990), isotope dilution mass spectroscopy (IDMS) (Bergman and Ohman, 1980), molecular imprinted polymer methods (Tsai and Syu, 1995) and optical methods with UV-absorbance (Fridolin *et al.*, 2010; Tomson *et al.*, 2011) are available for the analysis of creatinine. Most of these methods are precise and suitable for many applications. However, these methods do not satisfy the requirements for a fast, accurate and specific analysis as these are complicated, time-consuming, complex and usually required complicated sample pretreatment of collected samples, relatively large amounts of biological samples, delicate, bulky & expensive instrumental set-up and expertise handling specifically in case of chromatographic methods. The following methods were used for creatinine determination:

2.5.1 Chemical Methods

Several different color reactions have been used in attempts to develop a satisfactory method for direct determination of creatinine. Among the many color reactions for creatinine, the most commonly used method for the determination of creatinine is based on the Jaffe reaction (1886) involving alkaline sodium picrate. Creatinine reacts with picric acid in alkaline conditions to form a color complex which absorbs at 510 nm. The rate of formation of color is proportional to the creatinine concentration in the sample. In the endpoint method, the difference in absorbance measurements after color formation yields a creatinine value corrected for interfering substances.

Creatinine + Sodium Picrate $\xrightarrow{\text{Alkali}}$ Creatinine - Picrate complex (yellow-orange)

Creatinine is also determined colorimetrically after reacting with 3,5-dinitrobenzoic acid in alkaline solution (Greenwald and Gross, 1924; Benedict and Behre, 1936), $Ba(OH)_2$-$ZnSO_4$ (Van-Pilsum et al, 1956) and 1,4-naphthoquinone-2-potassium sulfonate (Sullivan and Irrecere, 1958). The Jaffe reaction for creatinine measurement has been known for more than a century but Folin (1914) developed the first application of this method for the determination of the so-called total creatinine in blood, milk and exudates, the preliminary precipitation with picric acid. In this method the advantage of using known creatinine solutions instead of 0.5 N potassium dichromate as standards is nowhere else so decisive as in the determination of creatinine and creatine in blood and milk, where the preformed creatinine amounts to only l-2 mg per 100 cc. The reaction of picric acid under the conditions used is not specific for creatinine. Jaffe himself reported that creatinine solution and glucose reacted with the reagent and gave similar colors (Jaffe, 1886). Since then numerous metabolites and drugs such as glucose (Viraraghavan and Blass, 1990), cycloketones (Kroll *et al.,* 1987), albumin (Pardue *et al.,* 1987) and carbonyl compounds (Weber and Zanten, 1991) have been shown to interfere with this creatinine determination method. Therefore, that much effort has been expended in attempts to improve the specificity of the method **(Table 1).**

Table 1. A summary of different approaches used to improve the specificity of the Jaffe reaction with creatinine (Source: Spencer 1986 with modification)

Approaches	References
1. Adsorption of creatinine by	
i. Kaolin	Wilson and Plass, 1917; Greenwald and McGuire, 1918
ii. Lloyd Reagent (Fuller's Earth)	Edward and Whyte, 1958; Husdan and Rapoporf, 1968; Haeckel, 1981
iii. Tungstic acid	Roscoe, 1953
iv. Ion Exchange	Mitchell, 1973; Bowers and Wong, 1980
2. Oxidation of interferents by	
i. Ceric Sulphate	Kostir and Rabek, 1950; Kostir and Sonka, 1952
ii. Iodine	Taussky, 1954

iii.	$K_4Fe(CN)_6$	Knapp and Mayne, 1987
3. Solvent extraction of interferents		Taussky, 1954, 1956
4. pH adujustment to decolorize the Jaffe creatinine chromogen		Slot, 1965; Yatzidis, 1974; Cook, 1975; Lolekha and Taksinamanee, 1980
5. Continuous flow dialysis		Chasson *et al.,* 1961; Rapoport and Husdan, 1968; Sakai *et al.,* 1995
6. Kinetic measurement of reaction with		
i.	Reaction interval measurement	Cook, 1971; Lustgarten and Wenk, 1972; Romer, 1975
ii.	Modified assay conditions	Spencer and Price, 1980
iii.	Diode array spectrophotometers	Antunes *et al.,* 1999; Quinn *et al.,* 1999; Juan *et al.,* 2000
iv.	Second-order calibration and multivariate	Guterres *et al.,* 2004
v.	Two-point, fixed-time kinetic procedure	Bowers and Wong, 1980
7. Flow-through microsystem with optical detection		Grabowska *et al.,* 2005
8. Fully automated zone fluidics (ZF)		Ohira *et al.,* 2009

Efforts to develop a method for both practical and accurate separation of creatinine from noncreatinine chromogens have not been completely successful. Adsorption and extraction techniques don't lend themselves to automation and popularity of such procedures has dimished in recent decades, largely due to the need for automated assays with large throughput.

The Jaffe creatinine test was usually configured to a two point, fix time kinetic measurement with or without a correction algorithm (Blijenberg *et al.,* 1994). Cook (1971) reported that the rate of color development was initially fast, followed by a much slower phase, the slope of which was similar to that obtained when measuring aqueous creatinine solutions. He and other (Lustgarten and Wenk, 1972; Romer, 1975) concluded that signals caused by slow and fast reacting interfering substances could be avoided by choosing a correct time window for measurement. In the seventies, numerous applications of the kinetic creatinine method, with wide variation in the reaction conditions used,

especially in picrate and hydroxide concentrations and measurement intervals, have been published (Cook, 1971; Lustgarten and Wenk, 1972; Schoucri and Pouliot, 1977; Soldin *et al.,* 1978; Van Stekelenburg *et al.,* 1978; Vuorinen *et al.,* 1987). Non-enzymatic kinetic assays were highly sensitive to reaction conditions and wide variations in these conditions has produced much conflicting data on assay precision and the extent of interference (Spencer and Price, 1980; Spencer, 1986).

With the advances in instrumentation for simultaneous multivariate detection, such as diode array spectrophotometers, multivariate kinetic methods could be investigated with the goal of improving the specificity of creatinine assay. Second-order calibration techniques have been demonstrated to be powerful methods for extracting qualitative and quantitative information from three-way data arrays such as multivariate spectroscopic-kinetic measurements with diode array spectrophotometers (Antunes *et al.,* 1999; Quinn *et al.,* 1999; Juan *et al.,* 2000; Guterres *et al.,* 2004). The advantage of second-order calibration is that quantitative analysis could be performed in the presence of unknown interferents (second-order advantage), because mathematical methods like parallel factor anaylsis (PARAFAC) or direct trilinear decompostion (TLD), used for second order calibration, are capable of resolving the underlying profiles and the relative concentrations of each component in the system.

The era of automation began in 1957 with the introduction of the Technicon continuous flow AutoAnalyzer invented by Skeggs. This system provided the first opportunity to efficiently process increasing workloads, and incorporated on-line dialysis to remove protein, (Chasson *et al.,* 1961) an important interfering substance in the Jaffe assay, which was further used in different methods such as auto analyzer methods of measuring serum and urine creatinine by the Jaffe reaction. The Technicon AutoAnalyzer method, the Beckman Creatinine Analyzer II, DuPont aca, Electro-Nucleonics GEMSAEC and Instrumentation Laboratory Model 919 kinetic methods were evaluated for determination of serum creatinine (Bowers and Wong, 1980), flow-injection analysis of creatinine with use of the Jaffe reaction (Sakai *et al.*, 1995), flow-through microsystem with optical detection for measurement of creatinine concentration in urine and postdialysate fluid (Grabowska *et al.,* 2005) and a fully automated zone fluidics (ZF)-based urinary creatinine determination system **(Fig. 2)** relying on a kinetic adaptation of the Jaffe reaction (Ohira *et al.,* 2009).

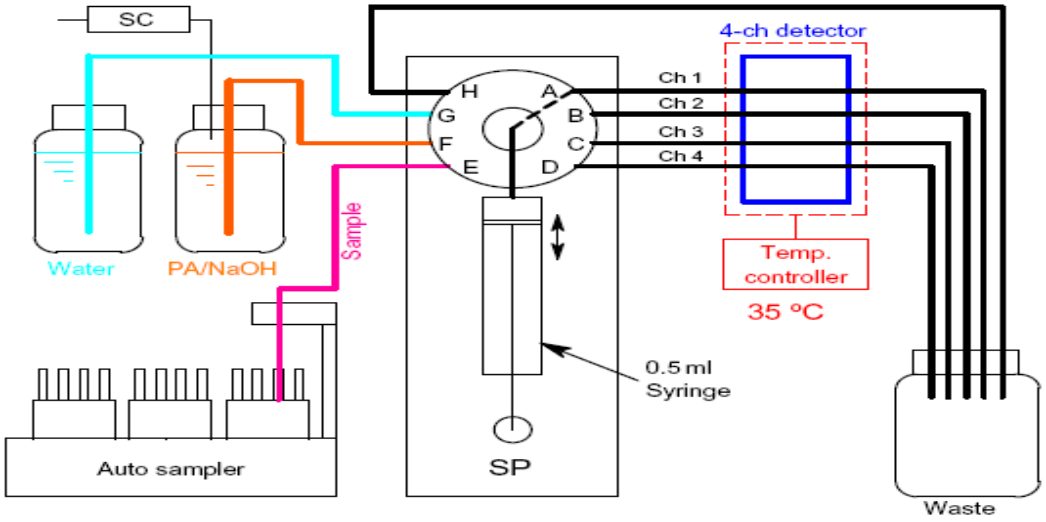


Fig. 2. Zone fluidics based creatinine analyzer (Source: Ohira *et al.*, 2009)

Alternative colorimetric method: Various alkaline picrate methods have dominated creatinine determinations but alternative chemical methods have also been proposed over the years (Benedict and Behre, 1936; Con Jr, 1960; Cooper and Biggs, 1961; Parekh *et al.*, 1976; Sims and Parekh, 1977; Parekh and Sim, 1977). They have not, however, been decisively superior to alkaline picrate, since sensitivity was not improved and specificity was achieved only by means of protein precipitation procedures (Cooper and Biggs, 1961; Parekh and Sims, 1977). Nevertheless, the creatinine reaction with dinitrobenzoates (Benedict and Behre, 1936) has found its uses in solid phase methods (Spotchem, Kyoto Daiichi Kagaku, Tokyo, Japan; Ames Seralyzer, Ames Division, Miles Labs., Elkhart IN, USA).

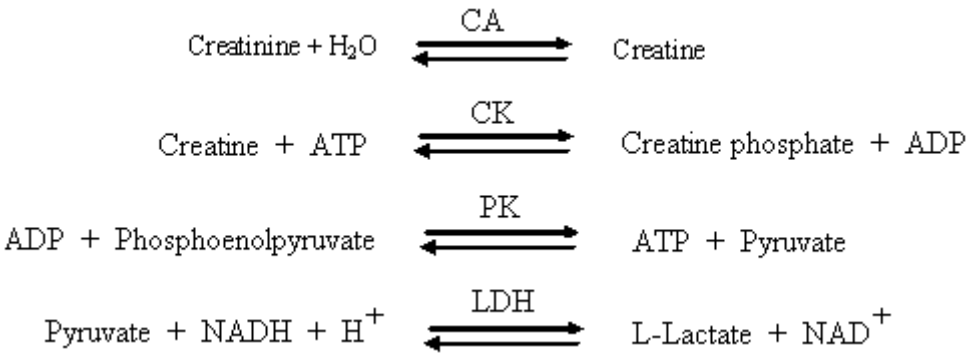
Merit: The advantages of the kinetic picrate method are its simplicity, low reagent costs, rapidity and small sample size necessary for analysis (Fossati *et al.*, 1994). The method also yields results, which are on the average close to true creatinine concentrations, but displays significant variability in the lower range of plasma creatinine concentrations (Hicks *et al.*, 1979; Soldin *et al.*, 1981)

Demerit: The introduction of a dialysis step with continous-flow automation in the early 1960s improved the specificity of the Jaffe reaction, although dialyzable interfering substances like glucose, ketoacids and pyruvate still contributed to the non-creatinine Jaffe chromogens. Other disadvantages of this method were large sample volume and poor precision at low creatinine level (Scwartz *et al.*, 1987).

2.5.2 Enzymatic Methods

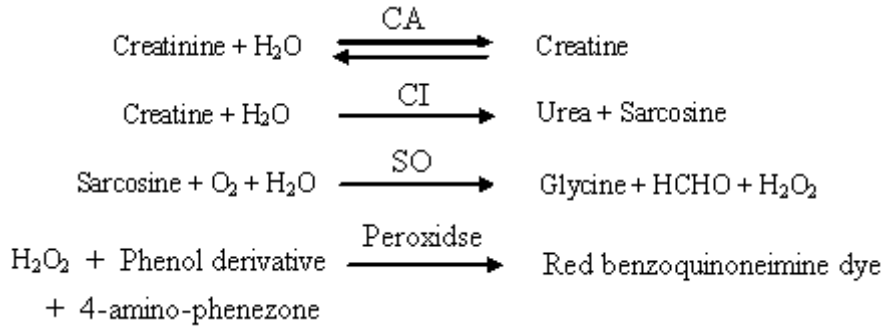
Methods employing enzymatic breakdown of creatinine represents the alternatives to the alkaline picrate type creatinine assay. Miller and Dubos (1937) isolated bacteria, which were capable of decomposing creatinine. Using crude extract of these bacteria, they developed an enzymatic procedure to estimate the creatinine concentration in plasma and urine. They treated one portion of plasma or urine with enzyme extract and then compared the colors produced by Jaffe reaction in the enzyme treated and untreated samples. This difference was attributed to the true creatinine content of the sample. Since then a number of authors have studied and isolated creatinine-degrading enzymes from a variety of bacterial sources (Cook, 1975). Studies of these enzymes have revealed the involvement of two enzymes, CA and creatinine iminohydrolase (CIH, EC 3.5.4.21). Enzymatic methods for creatinine determination have been developed in an attempt to overcome some of the problems in the Jaffe-based method.

Creatininase: CA, catalyses the hydrolytic conversion of creatinine to creatine and production of creatine was monitored with a multienzyme series of indicator reactions. Their method involved CA plus following enzymes: CK, pyruvate kinase (PK, EC 2.7.1.40) and lactate dehydrogenase (LDH, EC 1.1.1.27) (Bonvicini *et al.,* 1982; Perakis and Wolff, 1984; Beyer *et al.,* 1984; Wisser and Knoll, 1987; Sonntag, 1991).



The rate of change in absorbance at 340 nm due to oxidation of NADH was directly proportional to the amount of creatinine in the sample. Endogenous creatine and pyruvate were removed during pre-incubation with indicator enzymes before the addition of CA. This method was specific, giving almost identical values with a mass fragment graphic method (Bjorkhem *et al.,* 1979), but its precision was poor at low creatinine levels. It also proved expensive and impractical for routine use of many reagents and required large sample volume.

In the second reaction scheme, the transformed creatine was converted to sarcosine with CI. Sarcosine was then oxidized with SO to form formaldehyde, glycine and hydrogen peroxide. The amount of the formaldehyde formed was directly proportional to the creatinine concentration in the sample and could be measured with formaldehyde dehydrogenase (E.C. 1.2.1.1) (Sugita *et al.,* 1992). A more common approach was to measure the hydrogen peroxide formed using the peroxidase (Guder *et al.,* 1986). The hydrogen peroxide was measured at 510 nm in a reaction catalyzed by horseradish peroxidase, with 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone (Fossati *et al.,* 1983) as the chromogen.



Other color reagents/chromogens also have been used (Guder *et al.,* 1986; Lindback Bergman, 1989; Pieroni *et al.,* 2011). Bilirubin (Witte *et al.,* 1978) and ascorbic acid (White-Stevens, 1982) may, however, interfere with peroxidase detection systems and to avoid such disturbance Guder and co-workers (1986) added potassium hexacyanoferrate and ascorbate oxidase to the assay system.

CA was also used in combination with the Jaffe reaction (Miller and Dubos, 1937; McLlean *et al.,* 1973). However, the enzyme used was a crude preparation, which actually contained a mixture of CA, and CI. When the purified CA combines with the Jaffe reaction, the creatinine concentrations obtained were falsely low. This effect was eliminated by converting creatine, the product of the enzymic reaction, to creatine phosphate (Masson *et al.,* 1981). The combined enzymic-Jaffe method was therefore modified to include CA, CK, ATP, and Mg^{2+} in the reaction mixture. The modified method had good precision. Serum creatinine concentrations were assayed in the Central Biochemistry Laboratory of the National Institutes of Health (NIH)-funded Chronic Kidney Disease in Children (CKiD) study utilizing an enzymatic assay (Siemens Advia 2400) against a method traceable to reference isotope dilution mass spectroscopy (IDMS) developed by the National Institute of Standards and Technology (NIST) (Schwartz *et al.,* 2009).

Creatinine iminohydrolase: Creatinine iminohydrolase or creatinine deaminase (CIH or CD, E.C. 3.5.4.21), catalysed the conversion of creatinine to methylhydantoin and ammonia. Most methods based on this approach have monitored the production of ammonia by pH change (Toffaletti *et al.,* 1983; Kanakam and Raj, 2011), a specific electrode (Thompson and Rechnitz, 1974; Mayerhoff and Rechnitz, 1976; Ripamonti *et al.,* 1984; Suzuki and Matsugi, 2004), the Berthelot reaction (Moss *et al.,* 1975; Tabata *et al.,* 1983) or colorimetric titration of ammonia with hypobromite (He *et al.,* 2000). Glutamate dehydrogenase (GLDH, E.C. 1.4.1.4) was also to remove endogenous ammonia (Tangatelli *et al.,* 1982; Pieroni *et al.,* 2011**)**, but this enzyme has also been used as a part of the detection system. The reaction scheme was then as follows:

$$\text{Creatinine} + H_2O \xrightarrow{\text{CD}} NH_4^+ + \text{N-methylhydantoin}$$

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NAD(P)H} \xrightarrow{\text{GLDH}} \text{L-glutamate} + \text{NAD(P)}^+ + H_2O_2$$

The reaction was monitored by following the decrease in absorbance at 340 nm as NADPH is consumed. Endogenous ammonia was eliminated during pre-incubation with GLDH. Especially old urine samples contained high ammonia concentration and the pre-incubation step then consumed substantial portion of NADPH and narrows the assay range. The α-oxoglutarate and NADPH consumed could be restored via a reaction catalyzed by isocitrate dehydrogenase (IDH, E.C. 1.1.1.42) (Fossati *et al.,* 1994). The activity of isocitrate dehydrogenase was strictly dependent on magnesium ion and could be completely blocked by magnesium-complexing agent (e.g. trans-1,2-cyclohexanediamine-N,N,N,N-tetraacetic acid) when the creatinine assay was started with CIH.

Merit: Enzymatic methods offer greater specificity and sensitivity than the chemical method (Fossati *et al.,* 1994). Enzymatic techniques are also very selective

Demerit: Enzymatic methods are more specific than those based on Jaffe procedure, but some interference has also been reported e.g. bilirubin (Witte *et al.,* 1978) ascorbic acid (White-Stevens, 1982), dopamine and dobutamine (Karon *et al.,* 1998) disturbed peroxide detection system. Some drugs also hampered enzymatic approach such as calcium dobesilate (Goren *et al.,* 1986), N-ethylglycine, a metabolite of lidocaine (Roberts *et al.,* 1988) and metamizol (Bagnoud and Reymond, 1993) interfered the enzyme assay system. Enzymatic methods also suffer from relatively long analysis time (Radomska *et al.,* 2004a,b) and short enzyme lifetime (Ramanavicius, 2007).

2.5.3 Chromatographic methods

The various chromatographic methods were used to determine creatinine in serum or urine. These are as follows:

2.5.3.1 High performance thin-layer chromatography (HPTLC)

Principle: It involves a stationary phase of a thin layer of adsorbent like silica gel, alumina or cellulose on a flat, inert substrate. Selection of layer material is often not only determined by chromatographic requirements, but also by need to carry out in-situ derivatizations, if there is no direct method of detection available. The layer materials should be chromatically inert towards the reagent. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and for quantification, HPTLC could be used.

Creatine and creatinine were separated on 300MN cellulose in a chilled propanol-acetone-ammonia-water (5:2:4:1) buffer (Brown, 1966). In this method visualization involved treatment with picric acid or α-naphthol. Urinary creatine and creatinine along with uric acid and carbohydrates were also analyzed on an amino-modified HPTLC plate without sample preparation (Klaus *et al.*, 1991). In this method, almost identical chromatographic properties of the system used here as non modified silica gel, the in-situ treatment required to convert the separated components into visually recognizable compounds, which could be carried out in one step by heating with an IR radiator, heating plate or drying cupboard. In this method no sample preparation was required for determination of creatinine in serum and urine.

Merits: The method was low cost assay and highly reproducible and could be used for routine creatinine analysis, due to its higher sensitivity than enzymatic method.

Demerits: The method is time consuming.

2.5.3.2 High performance liquid chromatography (HPLC) methods

High-performance liquid chromatography is a chromatographic technique that can separate a mixture of compounds and used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of a mixture. HPLC is a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. It also allows packing material for column, which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past

it. HPLC is a form of liquid chromatography that utilizes smaller column size, smaller media inside the column, and higher mobile phase pressures compared to ordinary liquid chromatography.

Principle: HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector to provide a characteristic retention time for the analyte. The detector may also provide additional information related to the analyte, (i.e. UV/Vis spectroscopic data for analyte if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase.

HPLC is the most popular detection method for the creatinine. HPLC has been proposed as a reference method and a number of procedures have been developed based on this technique for creatinine determination. The first were based on ion exchange chromatography followed by post-column alkaline picrate reaction (Sadilek, 1965; Xue *et al.,* 1988), but subsequebtly, direct ultravoilet-detection of creatinine became more popular (Chiou *et al.,* 1978,1983; Ambrose *et al.,* 1983; Yokoyama *et al.,* 1991, 2005a,b; Scott 1992; Pacakava *et al.,* 1993; Yang, 1998). Also normal phase (Ogata and Taguchi, 1988; Kratochvıla *et al.,* 2007), reverse phase (Achari *et al.,* 1983; Zhiri *et al.,* 1985; Werner *et al.,* 1987; Linnet and Bruunshuus, 1991; Yokoyama *et al.,* 2000; Li *et al.,* 1995), paired-ion (Soldin and Hill, 1978; Paroni *et al.,* 1990; Yokoyama *et al.,* 1996; Dash and Sawhney, 2002; Tsikas *et al.,* 2004) and isocratic (Rosano *et al.,* 1990; Theinpoint *et al.,* 1995) HPLC techniques have been developed for the determination of creatinine. The mobile phase in these HPLC techniques varies from method to method, but in most cases has contained acidic or neutral buffer and methanol. The specificity of these candidate reference methods has been evaluated chiefly by analyzing solutions and samples containing possible interfering substances. HPLC methods with fluorimetric detection have also developed for the analysis of creatinine (Verhelst *et al.,* 1997; Zhao *et al.,* 2011). Two more elegant approaches have been developed, in which re-chromatography of samples done after enzymatic breakdown of creatinine (Van-Landuyt *et al.,* 1994) and analysis of the UV spectrum by a diaode-array detector was performed after chromatography (Linnet and Bruushuus, 1991). These latter investigators also compared with UV detection and enzymatic determination of creatinine after chromatography and the results were found identical. Most of these HPLC methods appeared to be fairly specific, but few have been strictly validated to such extent that they

can be regarded as reference methods (Rosano *et al.,* 1990; Linnet and Bruushuus, 1991; Thienpoint *et al.,* 1995). Most HPLC methods have been developed solely for evaluation of the authors own routine method.

Merits: These methods are highly specific i.e. these can measure true creatinine value.

Demerits: Most of these methods are time consuming, very laborious, needed costly equipments, expertise handling and some require a complicated assay system. HPLC methods required pretreatment of collected samples before injection as well as relatively large amounts of biological sample, and sometimes pre concentration is needed to fulfill the detection.

2.5.3.3 Liquid chromatography-mass spectrometric (LC-MS) methods

Principle: Liquid chromatography–mass spectrometry (LC-MS) is a hyphenated analytical technique, combining the separation power of HPLC, with the detection power of mass spectrometry (MS). Even with a very sophisticated MS instrument, HPLC is still useful to remove the interferences from the sample that would impact the ionization. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture).

Serum creatinine was analyzed by isotope dilution method based on analysis by discharge-assisted thermospray liquid chromatography–mass spectrometry incorporating stable isotope dilution using (D_3) creatinine as an internal standard (Takatsu and Nishi, 1993). The method effluents were directly introduced to the mass spectrometer and $[MH]^+$ ions were monitored during LC/MS using the selected ion monitoring method. Liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS) was developed for determination of creatinine, creatine, and guanidinoacetic acid simultaneously in human serum and urine (Yasuda *et al.,*1997). To provide results of creatinine in serum for an international measurement evaluation programme (IMEP) and the Comite Consultatif pour la Quantite de Matière (CCQM) international inter-laboratory studies a highly accurate LC–MS method was used (Stokes and O'Connor, 2003). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) were validated for the analysis of creatinine in serum (Keevil *et al.,* 2002; Owen *et al.,* 2006; Dodder *et al.,* 2007; Hetu *et al.,* 2010) and urine (Park *et al.,* 2008). An automated turbulent flow liquid chromatography–isotope dilution mass spectrometric

(LC–IDMS) method (Harlan *et al.,* 2010) was developed for analysis of serum creatinine The automated turbulent flow LC–IDMS method for serum creatinine was accurate, robust and easy to perform and may serve as a quick and inexpensive alternative to current creatinine reference methods. These LC-MS methods also compared with Jaffe reaction, UV detection and enzymatic determination of creatinine and the results were found identical. Most of these LC-MS methods appeared to be fairly specific, but few have been strictly validated to such extent that they can be regarded as reference methods (Stokes and O'Connor, 2003; Owen *et al.,* 2006). Two certified reference materials (CRM) namely Nos. GBW09170 and 09171 for creatinine in human serum were developed (Dai *et al.,* 2011). They were prepared with mixtures of several dozens of healthy people's and kidney disease patient's serum, respectively. The certified values of 8.10, 34.1 mg/kg for these two CRMs have been assigned by liquid chromatography-isotope dilution mass spectrometry (LC-IDMS) method, which was validated by using standard reference material (SRM) of SRM909b (a reference material obtained from National Institute of Standards and Technology, NIST). These CRMs were capable of validating routine clinical methods for ensuring accuracy, reliability and comparability of analytical results from different clinical laboratories. They could also be used for instrument validation, development of secondary reference materials, and evaluating the accuracy of high order clinical methods for the determination of creatinine in human serum.

Merits: Capable of analysing a much wider range of components.

Demerits: Costly, time consuming, need expertise handling, complicated assay system.

2.5.3.4 Gas chromatography-mass spectrometric (GC-MS) methods

Gas chromatography–mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample.

Principle: The Gas Chromatography/Mass Spectrometry (GC/MS) instrument separates chemical mixtures (the GC component) and identifies the components at the molecular level (the MS component). It is one of the most accurate tools for analyzing environmental samples. The GC works on the principle that a mixture will separate into individual substances when heated. The heated gases are carried through a column with an inert gas (such as helium). As the separated substances emerge from the column opening, they flow into the MS. Mass spectrometry identifies compounds by the mass of

the analyte molecule. A "library" of known mass spectra, covering several thousand compounds, is stored on a computer. Mass spectrometry is considered the only definitive analytical detector.

Several methods involving isotope dilution GC–MS have been suggested as reference methods for the determination of creatinine in serum or urine. Creatine and creatinine must be derivatized to facilitate their movement through a GC column, and as both commonly form the same derivatives, the creatinine was first separated from creatine by passage through a weakly acidic cation-exchange column in the hydrogen-form (wash with water to remove creatine, elute creatinine with 1–2 M ammonia) (Siekmann, 1985; Welch *et al.,* 1986; Thienpont *et al.,* 1996), or by HPLC (Bjorkhem *et al.,* 1977; Bjorkhem *et al.,* 1981; Hartmann *et al.,* 1983,). This initial isolation step, and the specificity of MS, means that a method developed for serum should be equally applicable to urine analysis. Derivatives which have been used include the trimethylsilyl derivative by reaction with the di-trifluoroacetate of the (2-hydroxy, 2-methyl)ethyl derivative, made by reaction with 1,2-epoxypropane then trifluoro-acetic acid (ions at m/e 294 and m/e 296) (Bjorkhem *et al.,* 1977), O-trifluoroacetylcreatinine (Lim *et al.,* 1978), N-methyl-N-trimethylsilyl-trifluoroacetic amide (MSTFA) (Siekmann, 1985), the ethyl ester of N-(4,6-dimethyl-2-pyrimidinyl)-N-methylglycine made by reaction with 2,4-pen-tanedione (Welch *et al.,* 1986) and [methyl-trideutero] creatinine with pentafluorobenzyl (PFB) bromide (Tsikas *et al.,* 2004).

Merits: One of the advantages of GC-MS is that the identification is based on both a retention time and a mass spectrum.

Demerits: The sample pretreatment prior to GC-IDMS analysis of creatinine is a very laborious multi-step procedure that includes precipitation of serum proteins, isolation of creatinine by liquid chromatography, evaporation of the creatinine fraction, and formation of creatinine derivatives. This complexity leads to higher costs per sample and to a lower throughput, which limits the applicability of definitive methods for large-scale validation of routine laboratory creatinine assays.

2.5.4 Capillary electrophoretic methods

The use of capillary electrophoresis (CE) for the analysis of biological fluids in the clinical laboratory is an alternative to many current analyses and appears to offer an optimal approach to overcome some of the limitations of conventional analytical methods.

Principle: Capillary electrophoresis (CE), also known as capillary zone electrophoresis (CZE), could be used to separate ionic species by their charge and frictional forces and hydrodynamic radius. CE encompasses a family of related separation techniques that use narrow-bore fused-silica capillaries to separate a complex array of large and small molecules. High electric field strengths are used to separate molecules based on differences in charge, size and hydrophobicity. Sample introduction is accomplished by immersing the end of the capillary into a sample vial and applying pressure, vacuum or voltage. Depending on the types of capillary and electrolytes used, the technology of CE could be segmented into several separation techniques. Separation by CE could be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. In order to obtain the identity of sample components, capillary electrophoresis could be directly coupled with mass spectrometers or Surface Enhanced Raman Spectroscopy (SERS). In most systems, the capillary outlet is introduced into an ion source that utilizes electrospray ionization (ESI). The resulting ions are then analyzed by the mass spectrometer. Micellar electrokinetic capillary chromatography (MECC) allows the resolution of uncharged molecules by adding surfactants to modify the conditions to extend the application of CE.

Separation of creatine and creatinine has been accomplished with actual electrophoresis (Fischl *et al.,* 1964), but the use of CE is much more convenient. Several CE based separation methods for serum and urine creatinine have been developed (Guzman *et al.,* 1990; Gatti *et al.,* 1999; Clark *et al.,* 2001; Paroni *et al.,* 2004; Zinellu *et al.,* 2004, 2006; Liotta *et al.,* 2009). Further to improve the CE micellar phase has been added which allows separation of neutral species that would otherwise migrate together, was called micellar electrokinetic capillary chromatographic (MECK) method. Due to this the separation of other species would also have been enhance. Most of these methods rely on sodium dodecyl sulfate (SDS) (Miyake *et al.,* 1991; Shirao *et al.,* 1997; Pobozy *et al.,* 2003), sodium tetraborate buffer with β-cyclodextrin (Fujii *et al.,* 1999) or sodium cholate (Yan *et al.,* 1999) micelles to separate zwitter ionic creatinine from other natural components in the fluid. The methods use UV or UV-Vis absorbance as their mode of detection.

An electrophoretically mediated microanalysis (EMMA) method was developed to perform online chemistry between two small molecules (Kochansky *et al.,* 2001). The

"plug-plug" type EMMA method involved electrophoretic mixing and subsequent reaction of nanoliter plugs of creatinine-containing samples and alkaline picrate (Jaffe reaction) within the confines of the capillary column, which acts as a micro-reactor. Analyses were performed by pressure injecting a plug of picrate followed by a plug of the creatinine-containing sample. CE was also integrated with the electrophoretic microchips to develope electrochemical and pulsed electrochemical detection methods (Wang and Chatrathi, 2003, Garcia and Henry, 2004) for the analysis of creatinine. In CE chip-based detection system fluid control was used for mixing the sample with the enzymes CA, CI and SO and for separating the neutral hydrogen peroxide end product from the anionic *p*-aminohippuric and urate species. Such a multianalyte microchip detection device would allow renal function testing to be performed more rapidly, easily, and economically in the point-of-care setting. Electrophoretic microchips have the potential to integrate separation power of CE with devices that are small, portable, and have the speed of conventional sensors. The microchip CE system device used the pulsed amperometric detection (PAD) to detect the nitrogen-containing compounds as well as the easily oxidizable uric acid. An automatic SPE has been coupled on-line to CE with UV detection by a transfer tube and the replenishment system of the CE instrument (Ruiz-Jimenez *et al.*, 2007). The approach allowed the target analytes (*viz.* creatinine, creatine, xanthine, hypoxanthine, uric acid, *p*-aminohippuric acid and ascorbic acid in urine samples) to be removed from the sample matrix, cleaned up, preconcentrated and injected into the capillary. Nevertheless, despite the fantastic effort of many researchers to set up different (and often complex) CE techniques, CE/CZE remains the simplest way to obtain faster and easier separations.

Merit: The features of this technique are: high analytical versatility, sensitivity, separation selectivity, minimal need of sample, neglect solvent consumption, high-speed analysis, efficiency, automation, possibility of multi component analysis and high recovery.

Demerit: One disadvantage of CE is the size, cost of the instrumentation and expertise handling limiting the long term applicability as point-of-care measurement devices.

2.5.5 Mass spectrometric methods

Principle: Isotope dilution mass spectrometry (IDMS) method is type of mass spectrometry which involves the addition of a known amount of an enriched isotope of the element of interest to the sample. The underlying principle of IDMS is that an

isotopically enriched analogue (inorganic MS) or an isotopically labelled analogue (organic MS) of the analyte compound is used as an internal standard in quantification by mass spectrometry. An accurately known amount of the isotopic analogue is added to the sample. The consequent ratio of the amounts of the two isotopes (one resulting from the analyte and the other from the isotopic 'spike') is measured on a portion of the sample using a mass spectrometer, so enabling the analyte concentration to be calculated. This technique is considered a definitive method and is well suited and established for the certification of certified reference materials.

Tandem mass spectrometry (MS/MS) another type of mass spectrometry is a very sensitive method for the identification of organic compounds in mixtures and employs two stages of mass analysis in order to examine selectively the fragmentation of particular ions in a mixture of ions. The first mass spectrometer serves to ionize all components of the mixture and to select out a major ion, most often the molecular ion of the compound(s) of interest, which is then fragmented by injection into a region containing a neutral gas at intermediate pressure and the resulting fragment ions are then separated and mass analyzed in the second mass spectrometer. This approach makes quantitative analysis more specific and reliable by monitoring a specific fragment ion from the precursor ion of interest. It also allows qualitative analysis by interpretation of the fragment ion spectrum of a particular component independent of the presence of the others.

Isotope dilution-mass spectrometry (IDMS) were used as a reference method for the determination of serum creatinine, to show that the kinetic Jaffe method for determination of serum creatinine sometimes produces results that are much too high (Bergman and Ohman, 1980; Bjorkhem *et al.,* 1981). Serum creatinine was quantified with the application of quantitative potential of FTICRMS using an exact matching isotope dilution method (Bristow *et al.,* 2005). The accuracy of the quantitation of creatinine was found to be equivalent to that obtained using LC/MS. A novel Surface Enhanced Raman Scattering (SERS) based approach for the quantitative determination of creatinine in human serum was developed (Stosch *et al.,* 2005) using isotopically labeled (2-^{13}C, 2,3-$^{15}N_2$) creatinine as internal standard, SERS acquires the character of a ratio method that works similar to the well-established isotope dilution techniques. In conjunction with multivariate data analysis, the method was successfully applied for quantifying creatinine at clinically relevant levels and below. The prediction performance of the model was thereafter validated with independent reference samples giving a standard deviation of

less than 2%. Finally, a conditioning procedure to prepare real serum samples for SERS-based creatinine analysis was worked out and validated. Serum creatinine concentrations as measured were within 3% of the values obtained from GC-IDMS on the same serum starting material. Creatinine in urine was determined by tandem mass spectrometry with direct sample infusion into an ion source (Huskova *et al.,* 2004). Electrospray tandem mass spectrometry method utilizing both approaches with and without ion-exchange column was acceptable according to CLIA criteria. In order to determine importance of matrix effects, the samples were introduced into mass spectrometer after dilution of urine sample without pretreatment and after SPE cation-exchange clean-up. Urine samples were pretreated by cation-exchange SPE or samples were solely diluted without additional pretreatment.

Merits: Need small sample size, fast responding, differentiates isotopes, can be combined with GC and LC to run mixtures, or can be run in tandem.

Demerits: Radioactive material requires expertise handling, use of costly chemical and instruments, cumbersome and time consuming.

2.5.6 Molecular imprinted methods

Molecularly imprinting methods, a specific separation technique, was developed and applied in recent years. Molecular imprinting has emerged as a simple and elegant method to impart recognition sites in synthetic polymers to interact with molecules of interest with specificity. Molecularly imprinted polymers (MIP) are an approach using an artificial material as a biomimetic antibody. They are synthesized by solidification of polymers in the presence of template molecules as the mold. They have been widely used in various fields, such as chromatographic separation, solid-phase extraction (SPE), biosensor and chemical sensor, analysis of drugs and drug delivery, monitoring of aquatic environment, clinical assay of the analytes of human serum and urine, catalysis and quartz-crystal microbalance (Kugimiya and Takeuchi, 1999; Wizeman and Kofimas, 2001).

Principle: This synthetic approach relies on using a functional monomer to form a complex with the target molecule (template) and process the polymerization with a crosslinking agent. After polymerization, the solvent was applied to wash the polymer so that the imprinted template could be removed. Thus, the specific recognition sites of template were created on the surface of the polymer.

Different artificial receptors such as poly (β-cyclodextrin) (Tsai and Syu, 1995), polymethacrylic acid (PMA), poly N-vinyl pyrrolidine (PMVP), poly 2-hydroxy ethyl methacrylate (PHEMA) (Sreenivasan and Sivakumar, 1997), polymerized hemithioacetal (Subrahmanyam *et al.,* 2001), photo-polymerized acryl amido methyl propane sulfonic acid & methylenediacrylamide (Delaney *et al.*, 2002), poly(4-vinylpyridine-co-divinylbenzene) (Tsai and Syu, 2004), poly(vinyl alcohol-co-ethylene) (Lee *et al.*, 2008), polymethacrylic acid/silicone dioxide ($PMAA/SiO_2$) (Gao *et al.*, 2010) and poly(tetraethoxysilanol) (Tsai and Syu, 2011) synthesized by the method of MIP were used for determination of creatinine. The MIPs and non imprinted polymers were used to adsorb creatinine from solutions. The imprinted factors and selectivity factors were assessed according to the results. The results also suggested that strong hydrogen binding force would decrease the specific adsorption of creatinine. With the development of MIPs, the preparation of creatinine imprinted polymer has been made not only using hydrogen-bonding (Sreenivasan and Sivakumar, 1997) as the main interaction in the complex between functional monomer and creatinine but also using hydrogen bonding and covalent bonding to form the complex (a ‘‘bite-and-switch’’ approach) (Subrahmanyam *et al.,* 2001), which is similar with the relationship between antigen and antibody.

Merit: MIPs possess many advantages such as recognition specificity, anti-interference property, physicochemical stability and so on.

Demerit: The conventional method to prepare MIPs, entrapment way, has some disadvantages, such as time consuming and complicated preparation process, less recognition sites inside matrix particles obtained via crushing and grinding the imprinted polymeric monolith, and greater diffuse barrier for the template molecules, coming from thick matrices, leading to poor binding capacity and lower binding kinetic of MIPs towards the template molecules.

2.5.7 Optical methods utilizing UV-absorbance

Optical method, utilizing UV-absorbance, has been developed for the monitoring of dialysis adequacy. Amount of creatinine removed during dialysis through dialysis was estimated by multiwavelength UV-absorbance method (Fridolin *et al.,* 2010; Tomson *et al.,* 2011), which improved the measurement accuracy for optical creatinine concentration estimation in spent dialysate in terms of relative error, compared to the algorithm, based on the single wavelength approach.

2.6 BIOSENSOR

The concept of biosensors is just five decades old and the feasibility of biosensing was first demonstrated by an American scientist Leland C. Clark in 1962. Leland C. Clark introduced the principle of the first enzyme electrode with immobilized glucose oxidase. He described how to make electrochemical sensors more intelligent by adding "enzyme transducers as membrane enclosed sandwiches" **(Fig. 3)** (Clark and Lyons, 1962). This idea was commercially exploited in 1975 with the successful launch of the Yellow Springs Instrument Company's glucose analyser based on the amperometric detection of hydrogen peroxide (H_2O_2). Since then, research communities from various fields such as very large scale integration (VLSI), physics, chemistry, and material science have come together to develop more sophisticated, reliable, and mature biosensing devices. Biosensors are portable, simple-to-use, and high specificity analytical tools, which are compatible with data-processing technologies. Biosensors have improved the performance of the conventional analytical tools, have eliminated slow sample preparation and the use of expensive reagents and have provided low cost analytical tools. Therefore, biosensors would have promising applications in various fields, such as health care, pharmacy, pollution monitoring, food, agricultural product processing biotechnology as well as the military and bioterrorism detection and prevention etc. On the other hand, biosensors have some limitations: electrochemically active interferences in the sample, weak long term stability and troublesome electron-transfer pathways. Today, a multitude of instruments referred to as biosensors could be found in labs around the world and there is a growing number of biosensors being used as diagnostic tools in point-of-care testing, but the realization of cheap handheld devices is almost limited to one well-known example: the glucose sensor. In many cases the main limitation in realizing point-of-care testing/sensing devices is the ability to miniaturize the transduction principle and the lack of a cost-effective production method. Thus, they have to be confined to expert users of high-cost equipment in a lab environment and cannot be used e.g. by patients themselves or doctors in the field.

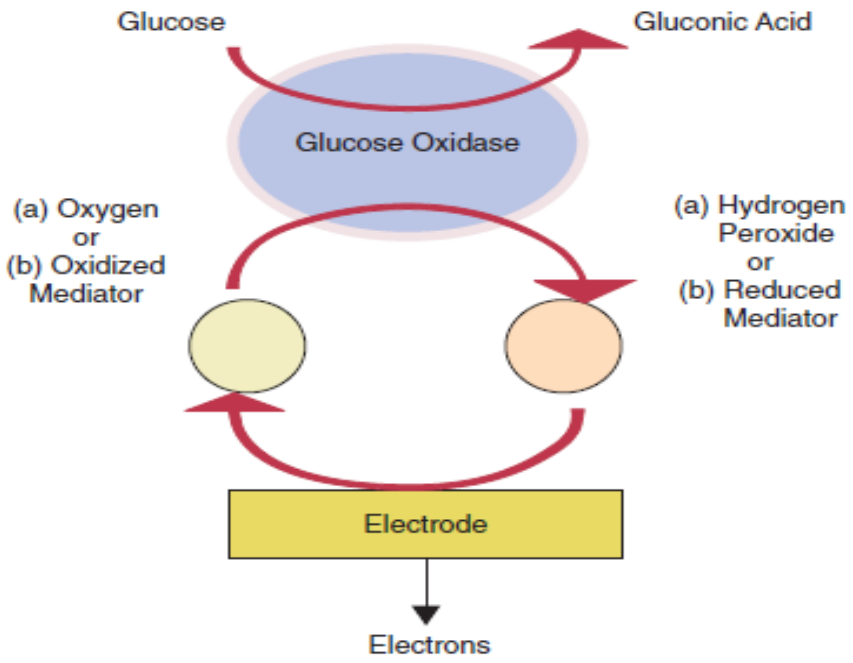


Fig. 3. Clark experiment

2.6.1 Basic principle of biosensors

Biosensors are the analytical tools that consist of a substrate and a selective interface in closed proximicity or intregrated with a transducer, therefore, the substrates and transducers are important components of the analytical tools which contain an immobilized biologically active compound that interact with specific species of interest. This reaction between the bioactive substance and the species (substrates) produces a product in the form of a biological or chemical substance electrochemical, heat, light or sound, then a transducer such as an electrode electrochemical, semiconductor, thermistor, counter or sound detector changes the product of the reaction into usable data **(Fig. 4)** (Mehrvar *et al.*, 2000; D'Souza, 2003; Zhang and Tadigadapa, 2004; Arya *et al.*, 2006; Yadav *et al.*, 2011). Biosensors—first reported in the 1960s—differ from classical chemical sensors in the following two ways: (a) the sensing element consists of a biological material such as proteins (e.g., cell receptors, enzymes, antibodies), oligo- or polynucleotides, microorganisms, or even whole biological tissues and (b) the sensor is used to monitor biological processes or for the recognition of biomolecules.

A basic biosensors assembly includes a receptor, transducer and processor (amplification and display) as shown in **Fig. 5**. Technically, it is a probe which incorporates a biological/ biologically derived sensing element (e.g. whole cells/antibodies/enzymes/nucleic acids) forming a recognition layer, that is either integrated within or intimately associated to the second major component of biosensors that is a transducer via immobilization,

adsorption, cross-linking and covalent bonding so that the close proximity of the biological component to the transducer is achieved. This is necessary so that the transducer can rapidly and easily generate the specific signals in response to the undergoing biochemical interactions, secondly the transmittance should be proportional to the reaction rate of biocatalyst with the measured analyte for a high range of linearity. The transducer critically acts like a translator, recognizes the biological/chemical event from the biological component and transforms it into another signal for interpretation by the processor that converts it into a readable/measurable out put. The transducer could take many forms depending upon the type of parameters being measured. They may be a) Amperometric (Yadav *et al.,* 2011), b) Potentiometer (Soldatkin *et al.,* 2002a,b), c) Piezoelectric (Arya *et al.,* 2006), d) Thermal (Zhang and Tadigadapa, 2004), e) Optical (Mehrvar *et al.,* 2000).

2.6.2 Classification of biosensors

Biosensors could be classified into different classes on the basis of evolution and transducing mechanism.

2.6.2.1 Classification of biosensor on the basis of evolution: There are three so-called 'generations' of biosensors:

2.6.2.1.1 1st generation biosensors

The first generation biosensors were proposed by Clark and Lyons (1962) and implemented by Updike and Hicks (1967), who coined the term enzyme electrode. In the first generation of the biosensor, the biocatalyst was entrapped between or bound to membrane and fix upon the surface of the transducer e.g. a first-generation amperometric glucose biosensor based on a Prussian Blue-modified electrode. Prussian blue was a better electrocatalyst for hydrogen peroxide oxidation than platinum. H_2O_2 was detected at the Prussian Blue-modified electrode in the presence of oxygen by both electrooxidation and electroreduction (Karyakin *et al.,* 1996). However, since this detection principle may lead to poor reproducibility of the overall sensing process due to varying O_2 concentrations in the sample under investigation, the application of artificial redox mediators has been introduced in order to avoid the interference-prone oxygen dependence.

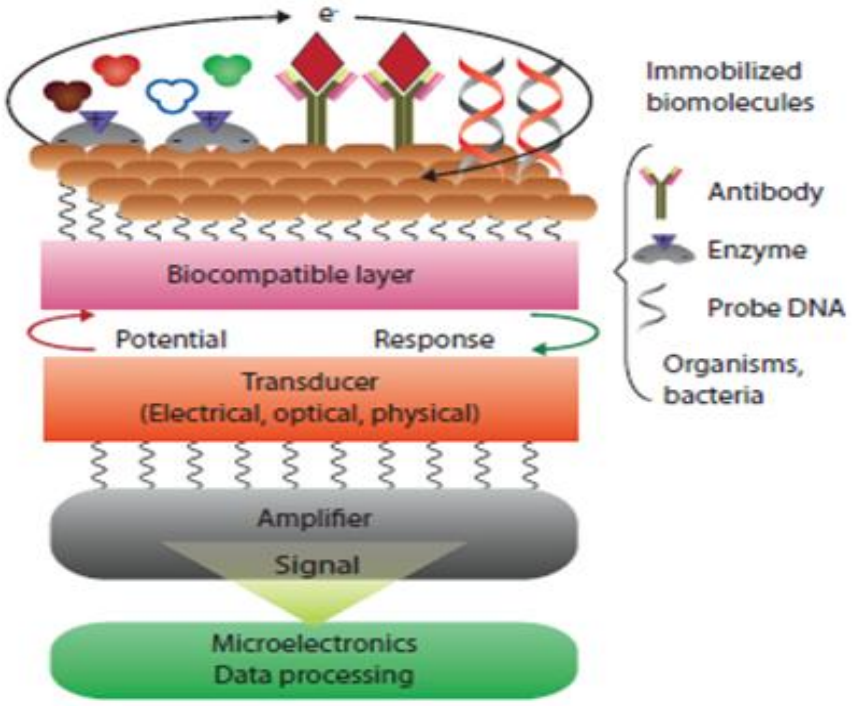


Fig. 4. Elements and selected components of a typical biosensor Schematic structure and operating principle of a biosensor

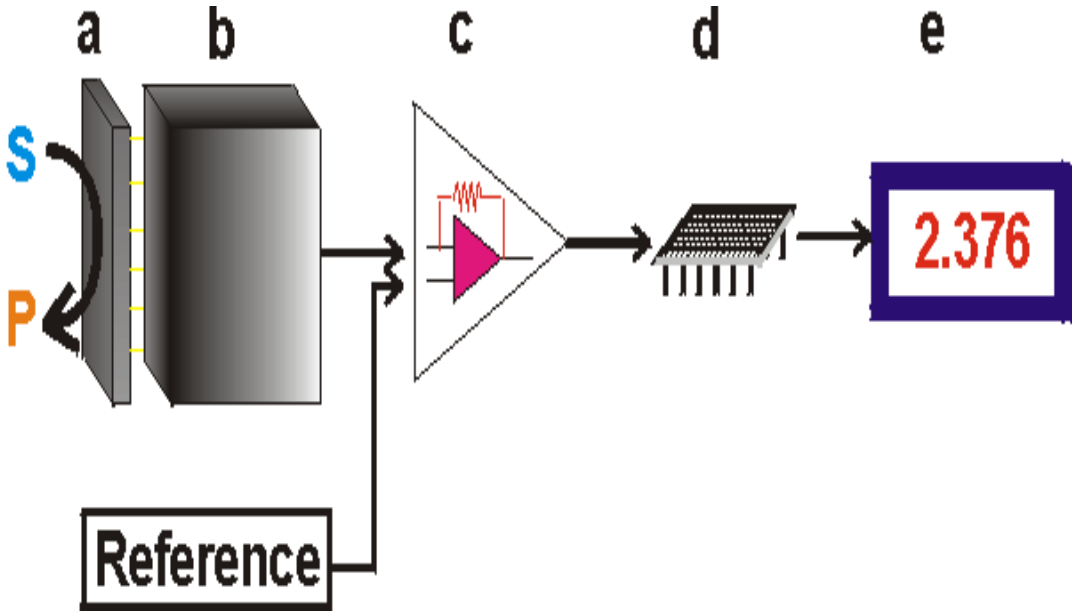


Fig. 5. Schematic diagram showing the main components of a biosensor. The biocatalyst (a) converts the substrate to product. This reaction is determined by the transducer (b) which converts it to a signal. The output from the transducer is amplified (c), processed (d) and displayed (e). (Reproduced with permission from Mart Chaplin, http://www.isbu.ac.uk/biology/enztech/biosensors)

2.6.2.1.2 IInd generation biosensors

In second-generation biosensors, redox enzymes donate or accept electrons to or from electrochemically active redox mediators having a redox potential adjusted to that of the enzyme's cofactor. Ideally, the mediator is otherwise inactive, that is, highly specific only for the desired electron-transfer process between the recognition element and the transducer. As a matter of fact, free-diffusing, low-molecular-weight redox mediators are prone to leak from the electrode surface thus imposing overall decreased long-term operation stability to this type of enzyme electrode. In second generation biosensor, there is covalent fixation of biologically active component onto the transducer's surface which eliminates the use of the semipermeable membrane e.g. enzyme-membrane electrodes using glucose oxidase in combination with peroxide detection dominate in the field of laboratory analyzers for diluted samples. Using the same indication principle, extremely fast responding glucose sensors have been fabricated by covering thin metal electrodes with a porous enzyme layer. In the second generation biosensors auxiliary enzymes and/or co-reactants were co-immobilized with the analyte, in order to improve the analytical quality and to simplify the performance (Scheller, 1991). Second-generation biosensors have been commercialized, mostly in single-use testing format. MediSense (Waltham, MA) was the first company to launch a second-generation product. Again the application was blood glucose monitoring, but this device was for home use. The mediation was provided by a ferrocene species.

2.6.2.1.3 IIIrd generation biosensors

Third generation biosensors arises from the self contained nature of the sensor, where reaction itself causes the response and no product or mediator diffusion is directly involved**.** The direct binding of the biocatalyst to an electronic device that transduces and amplifies the signals e.g. the gate of a field affect transistor, is the basis for a further miniaturization of biosensors. Third-generation biosensors have the mediator integrated with the enzyme and the electrode to ensure direct electron transfer. Mediators for this generation include conducting polymers, such as polypyrrole (Devi *et al.,* 2011a), polyaniline (Yadav *et al.,* 2011a; Devi *et al.,* 2011b) etc. Direct electron transfer has been realized with the use of carbon nanotubes (Yadav *et al.,* 2011a,b; Devi *et al.,* 2011b) or zinc oxide nanoparticles (Devi *et al.,* 2011a) etc.

2.6.2.2 Classification of biosensor on the basis of transducing mechanism (Fig. 6)

2.6.2.2.1 Thermal-detection biosensors

Thermal detection biosensors exploit one of the fundamental properties of biological reactions, namely absorption or production of heat that in turn changes the temperature of the medium in which the reaction takes place. These biosensors were constructed by combining immobilized enzyme molecules with temperature sensors e.g. calorimetric glucose biosensor (Zhang and Tadigadapa, 2004). When the analyte comes in contact with the enzyme, the heat reaction of the enzyme is measured and calibrated against the analyte concentration. The total heat produced or absorbed is proportional to the molar enthalpy and the total number of molecules in the reaction. The measurement of the temperature is typically accomplished via a thermistor, and such devices are known as enzyme thermistors. Their high sensitivity to thermal changes makes thermistors ideal for such applications. Unlike other transducers, thermal biosensors do not need frequent recalibration and are insensitive to the optical and electrochemical properties of the sample. Common applications of this type of biosensor include the detection of pesticides and pathogenic bacteria.

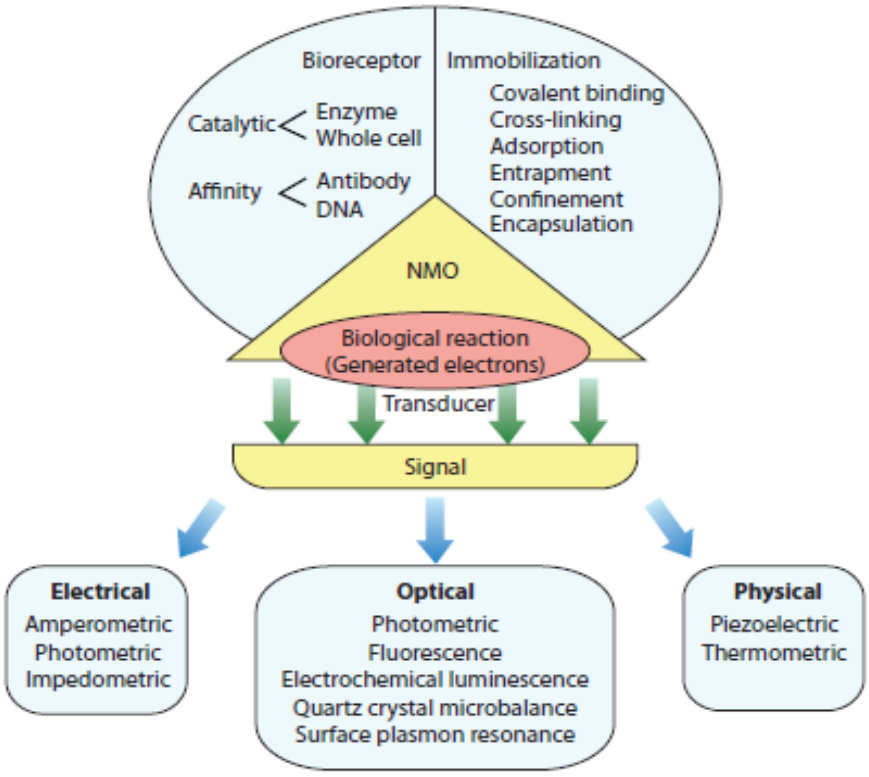


Fig. 6. Various types of transducers for biosensing

2.6.2.2.2 Optical-detection biosensors

Optical detection is usually based on the measurement of luminescent, fluorescent, colorimetric, or other optical signals produced by the interaction of microorganisms with the analytes and correlates the observed optical signal with the concentration of target compounds. Optical sensing techniques are especially attractive in high throughput screening since they enable biosensors to monitor multiple analytes simultaneously (Brogan and Walt, 2005). In such biosensors, optical fibres were used to guide the light waves to suitable detectors like an electrode or a semiconductor (Mehrvar *et al.,* 2000). The resulting signal could be measured or could be further amplified before measurement for improved sensitivity e.g. optical biosensor for dichlovos using stacked sol–gel films containing acetyl cholinesterase and a lipophilic chromoionophore (Wong *et al.,* 2006) and electrochemiluminescence biosensor for uric acid determination (Lin *et al.,* 2008). These biosensors measured the light output during the reaction or a light absorbance difference between the reactants and products. Surface Plasmon resonance (SPR), has also been shown to be an effective optical transducer mechanism for biosensor use. Fluorescence and chemiluminescence transducers are the most developed within the optical transducer class. Their limits of detection are the lowest that one can obtain using biosensors. The fluorescent sensing technique is based on the measurement of fluorescence intensity which is proportional to the concentration of the target analyte. Fluorescence could be detected at a longer wavelength after the excitation of the fluorescent substance at a shorter wavelength. Fluorescent biosensors have been widely applied in analytical chemistry due to their easy construction using standard molecular biology techniques (Ibraheem and Campbell, 2010). Chemiluminescence occurs when the electron excitation energy necessary for photon emission is supplied by a chemical reaction. Bioluminescence is a subdivision of chemiluminescence and occurs in living organism such as fireflies, glowworms and bacteria among others. Even though these biosensors are very sensitive, they cannot be used in turbid media (Chaubey and Malhotra, 2002). The main limitations of these biosensors are relatively long assay time (Polyak *et al.,* 2001; Horsburgh, 2002). The bioluminescence biosensors have the longest response time of the biosensors. SPR transducers, which measure minute changes in refractive index at and near the surface of the sensing element, have been proposed. SPR measurement is based on the detection of the attenuated total reflection of light in a prism with one side coated with a metal. When a p-polarized incident light passes through the

prism and strikes the metal at an adequate angle, it induces a resonant charge wave at the metal/dielectric interface that propagates a few microns. The total reflection is measured with a photodetector, as a function of the incident angle.

2.6.2.2.3 Piezoelectric biosensors

Piezoelectric biosensors operate on the principle of coating the surface of the biosensor with selectively binding biologically active substances (Fung and Wong, 2002; Su *et al.,* 2002). The coated surface was placed in a solution analytes that mass of the bind to the binding substance. The piezoelectric transducer allows a binding event to be converted into a measurable, for example resonance frequency change. Change in mass was sensed by variations in the frequency of oscillation in a piezoelectric crystal or surface acoustic wave e.g. Cholesterol oxidase was immobilized covalently onto 11-amino-1-undecanethiol hydrochloride (AUT) self-assembled monolayer (SAM) fabricated on gold (Au) substrates using glutaraldehyde as a crosslinker (Arya *et al.,* 2006). Piezoelectric biosensors offer a real time output, simplicity of use wider working pH range and cost effectiveness. Possible disadvantages of the biosensors include the lack of specificity or sensitivity or selectivity and interferences from the liquid media where the analysis takes place.

2.6.2.2.4 Electrochemical biosensors

Electrochemical detection is another possible means of transduction that has been used in biosensors. Electrochemical biosensors are more amenable to miniaturization have compatible instrumental sensitivity and can even operate in turbid media. Electrochemical biosensors are based on the electrochemical species consumed and/or generated during a biological and chemical interaction process of a biological active substance and substrate. In such a process, an electrochemical detector measures the electrochemical signal produced by interaction e.g. zinc oxide/polypyrrole based xanthine biosensor (Devi *et al.,* 2011) and c-MWCNT/PANI based oxalate biosensor (Yadav *et al.,* 2011a) & xanthine biosensor (Devi *et al.,* 2011b). Although biosensing devices employ a variety of recognition elements, electrochemical detection techniques use predominantly enzymes. This is mostly due to their specific binding capabilities and biocatalytic activity Electrochemical biosensors emerge as the most commonly used biosensors in monitoring and diagnosis test in clinical analyses. Poor coupling of biochemical recognition materials and electrochemical tranducers however, affect the selectivity, sensitivity, limited

dynamic range and stability of the electrochemical biosensors. Electrochemical biosensors have been the most commonly used classes of biosensors due to their faster response, greater simplicity, high sensitivity and lower cost compared to optical, calorimetric and piezoelectrical biosensors (Brahim *et al.*, 2002). Depending on the electrochemical properties measured by a detector system, electrochemical biosensors can be classified as:

2.6.2.2.4.1 Conductometric biosensors

Conductometric biosensors measure biological and chemical changes in the conductance between a pair of metal electrodes in a bulk solution. The measured parameter in conductometric sensors is the electrical conductance/resistance of the solution. When electrochemical reactions produce ions or electrons, the overall conductivity or resistivity of the solution changes. This change is measured and calibrated to a proper scale. If the electrodes are prevented from polarizing, the electrolyte shows ohmic behavior. The capability of the analyte to conduct an electrical current is monitored from Ohm's law (E = IR) it is apparent that the electric current (I) is inversely proportional to the resistance (R), where E represents potential difference. The inverse of the resistance is the conductance (G = 1/R). Conductivity measurements are generally performed with AC supply. The conductivity is a linear function of the ion concentration; therefore, it can be used for sensor applications. The measurement of conductance is extremely fast and sensitive under sophisticated modern analytical techniques, making conductometric microbial biosensors very attractive. There are two general types of devices for measuring conductance. The first and most widely used employs a pair of contacting electrodes, frequently platinum, immersed in the liquid. The second type instrumentation is non contacting or "electrodeless" and depends on the inductive or capacitive effects to measure conductance. Development of these technologies is mainly driven by the need for in vivo applications for medical diagnosis and may not find immediate use in the agricultural and food industries. In most cases conductometric devices have been strongly associated with enzymes, where the ionic strength, and thus the conductivity, of a solution between two electrodes changes as a result of an enzymatic reaction. Thus, conductometric devices could be used to study enzymatic reactions that produce changes in the concentration of charged species in a solution (D'Orazio, 2003). The variable ionic background of clinical samples and the requirement to measure small conductivity changes in media of high ionic strength limits the applicability of such enzyme-based

conductometric devices for biosensing (Thevenot *et al.*, 2001). Another approach is to directly monitor the changes in conductance of an electrode as a result of the immobilization of e.g. enzymes, complementary antibody-antigen pairs, etc. onto the electrode surface.

2.6.2.2.4.2 Potentiometric biosensors

In this type of biosensor the measured parameter is oxidation or reduction potential of an electrochemical reaction. In other words, in potentiometric biosensors, the potential difference between the reference electrode and the indicator electrode is measured without polarizing the electrochemical cell, that is, very small current is allowed. The reference electrode is required to provide a constant half-cell potential. The indicator electrode develops a variable potential depending on the activity or concentration of a specific analyte in solution. The change in potential is related to concentration in a logarithmic manner. The Nernst equation relates the potential difference at the interface to the activities of species in sample phases (s) and in the electrode phase (β):

$$E = E_0 + \frac{RT}{Z_i F} \ln \frac{a_i^s}{a_i^\beta}$$

where E_0 is the standard electrode potential of the sensor electrode; α_i is the activity of the ion, R is the universal gas constant; T is the absolute temperature; F is the Faraday constant; Z_i is the valency of the ion. The lowest detection limits for potentiometric devices are currently often achieved with ion-selective electrodes (ISE) (Battilatti *et al.*, 1989; Soldatkin *et al.*, 2000a,b). The ion-selective electrode (ISE) for the measurement of electrolytes is a potentiometric technique routinely used in clinical chemistry. In many cases, the potentiometric biosensor comprises a membrane with a unique composition, noting that the membrane can be either a solid (i.e., glass, inorganic crystal) or a plasticized polymer e.g. potentiometric biosensors based on biologically active membrane (Soldatkin *et al.*, 2002b), and the ISE composition is chosen in order to impart a potential that is primarily associated with the ion of interest via a selective binding process at the membrane-electrolyte interface. These are semiconductor Field Effect Transistor (FETs) having an ion-sensitive surface. The surface electrical potential changes when the ions and the semiconductor interact. This change in the potential could be subsequently measured. The ion sensitive field effect transistor (ISFET) is constructed by covering the sensor electrode with a polymer layer. This polymer layer is selectively permeable to analyte ions. The ions diffuse through the polymer layer and in turn cause a change in the

FET surface potential. This type of biosensor is also called an enzyme field effect transistor (ENFET) and used primarily for pH detection e.g. a biosensor based on nonactine/polyphenyl acetylene (Battilotti *et al.,* 1989). The variety of ions, for which low detection limits are possible, is currently quite limited and missing such important analytes as: nickel, manganese, mercury and arsenate ions. The sensitivity and selectivity of potentiometric biosensor are outstanding due to the species-selective working electrode used in the system. However, a highly stable and accurate reference electrode is always required and challenging to maintain, which may potentially limit the application of potentiometry in microbial biosensors.

2.6.2.2.4.3 Amperometric biosensors

Amperometry is the electrochemical technique usually applied in commercially available biosensors for clinical analysis. Amperometric biosensor is a high sensitivity biosensor that could detect electroactive species present in biological test samples. Amperometry is a method of electrochemical analysis in which the signal of interest is current that is linearly dependent upon the concentration of the analyte. In contrast to the logarithmic relationship in potentiometric systems, amperometric systems possess a linear concentration dependence and measure change in the current on the working electrode. Amperometry is a specific electrochemical technique taking advantage of the fact that certain electroactive species are oxidized or reduced (redox reactions) at inert metal electrodes, driven at a constant applied potential. As certain electroactive species are oxidized or reduced (redox reactions) at inert metal electrodes, electrons are transferred from the analyte to the working electrode or to the analyte from the electrode. The direction of flow of electrons depends upon the properties of the analyte and could be controlled by the electric potential applied to the working electrode. Typically, the current is measured at a constant potential and this is referred to as amperometry. These measure the movements of electrons produced in a redox reaction and response measured in amperes e.g. oxalate biosensor (Yadav *et al.,* 2011a), xanthine biosensor (Devi *et al.,* 2011a,b). The electrochemical cell, where the amperometric experiment is carried out consists of working electrode (WE), a reference electrode (RE) and usually a counter (auxillary) electrode (CE). The WE is the one, where the reaction or transfer of interest taking place and usually constructed from a metal such as platinum or gold. The RE provides a known and stable potential, against which the potential of WE is compared. The most common RE systems used in aqueous medium are the silver-silver chloride

(Ag/AgCl) and saturated calomel (Hg/Hg_2Cl_2) which have electrode potential independent of the composition of electrolyte. The CE is a current carrying electrode, via which the current is measured. Inert conducting materials such as a platinum wire or a graphite rod are often used as CE. If the working electrode is driven to a positive potential, relative to the reference electrode, an oxidation reaction is supported at the working electrode, and it is referred to as the anode of the electrochemical cell. When applied to biosensors, the anode monitors formation of a product or depletion of a reactant resulting from a bioreaction (e.g., an enzyme-catalyzed reaction) involving an analyte of interest, near the surface of the electrode. The species to be monitored diffuses to the surface of the electrode, where it undergoes a redox reaction. The measured cell current (diffusion current) is a quantitative measure of the analyte of interest. Due to the different electron transfer process, there are three so-called "generations" of biosensors: first generation biosensors where the normal product of the reaction diffuses to the transducer and causes the electrical response, second generation biosensors which involve specific "mediators" between the reaction and the transducer in order to generate improved response, and third generation biosensors where the reaction itself causes the response and no product or mediator diffusion is directly involved **(Fig. 7).**

Amperometry is an electrochemical technique that measure current as function of potential, when the current recorded at a fixed potential as a function of time, the technique is called chronoamperometry and when potential vary with time in a predetermined manner and the current is measured as a function of potential is called voltammetry or voltamperometry. At voltammetry, an increasing (decreasing) potential is applied to cell until the oxidation (reduction) of the substance to be analyzed occurs and there is sharp rise (fall) in the current to give a peak current. The height of the peak current is directly proportional to the concentration of electroactive material. the appropriate oxidation (reduction) potential is known, one may step the potential directly to the value and observe the current. It involves probing a small region of a solution containing, for example, metal ions, by performing small-scale electrolysis between an indicator microelectrode and a reference electrode. A reference electrode, such as the saturated calomel electrode (SCE), is by definition nonpolarizable i.e. its potential remains the same regardless of the potential difference imposed between it and the indicator electrode. The latter is described as polarizable, because it faithfully adopts any potential imposed on it relative to the reference. If the potential difference between indicator and reference electrode could be controlled accurately and varied uniformly,

criteria which modern potentiostatic devices ensure, the corresponding current that flow reflects the nature and concentration of oxidizable or reducible solutes in solution. Current flow because of the exchange of electrons between the indicator electrode and electroactive solutes. The term voltammetry encompasses a broad area of electroanalytical chemistry that includes polarography, linear scan voltammetry, cyclic voltammetry, pulsed voltammetry and stripping voltammetry. Overall voltammetry is very versatile and could be used for the analysis of many redox active species. Amperometric biosensors also have the advantages of being more highly sensitive, rapid, inexpensive and disposable as compared to conductometric and potentiometric biosensors.

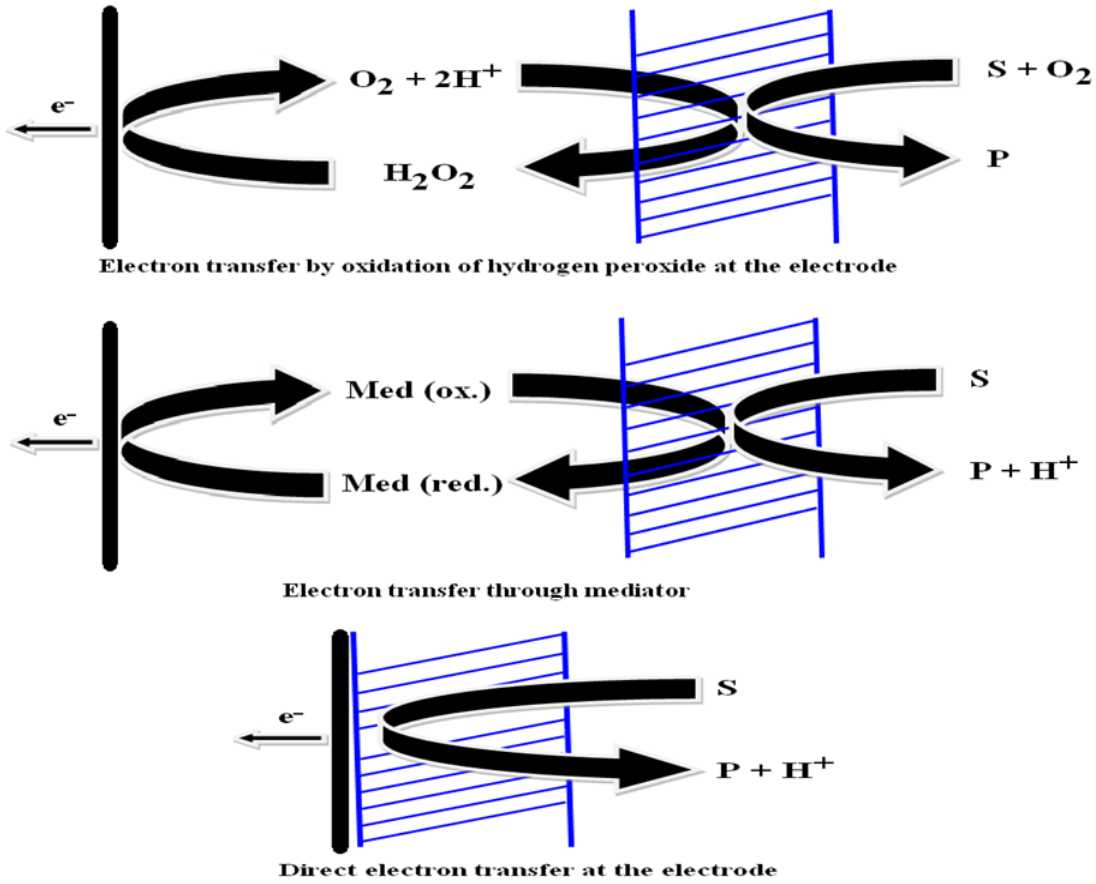


Fig. 7. Mechanism of electron transfer in amperometric biosensing system

2.7 CREATININE BIOSENSORS

The first biosensors for creatinine detection based on ammonia-sensing electrode was developed in 1976 (Meyerhoff and Rechnitz, 1976). In the past three decades, various biosensors have been developed for creatinine determination, some of which have been used as conventional devices for routine analyses. Although detection of creatinine can be

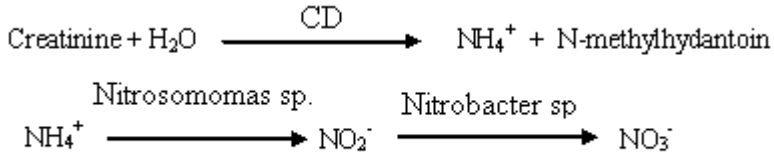
made in the presence of some interferents but the selectivity was found to be poor and continuous efforts have been made for such improvements. A number of biosensor systems aimed at the specific measurement of creatinine are discussed below:

2.7.1 Electrochemical creatinine biosenosrs

2.7.1.1 Dissolved oxygen (DO) meter based creatinine biosensors

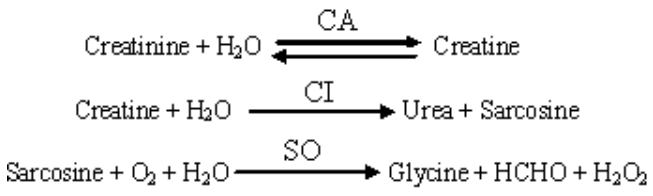
Dissolved oxygen (DO) meter based creatinine biosensor have been made based on immobilized CA, CI, SO, CD, GLDH, glutamate oxidase (GLOD, EC 1.4.3.7) and nitrifying bacteria onto membrane, sensing part of combine electrode of DO meter. Three types of electrode preparation were used in DO metric creatinine biosensors for amperometric determination of creatinine.

Principle: The DO metric biosensors are based on the consumption of oxygen detected by an oxygen electrode. A selective creatinine biosensor was developed consisting of immobilized CD and immobilized nitrifying bacteria for determination of creatinine (Kubo *et al.,* 1983). This sensor is based on amalgamation of an enzyme reaction and bacterial metabolism. CIH hydrolyzes creatinine to N-methylhydantoin and ammonium ion, and the ammonia produced is successively oxidized to nitrite and nitrate by nitrifying bacteria. The bacteria have not been completely characterized, but are known to be a mixed culture of *Nitrosomonas sp.* and *Nitrobacter sp.* The sequence of reactions is



The reacting bacteria consume oxygen, so that the oxygen decrease may be detected by an oxygen electrode.

Further DO metric creatinine biosensor were developed consisting CA, CI and SO enzymes co-immobilized to the surface of membrane of a Clark-type electrode responsive to oxygen (Nguyen *et al.,* 1991; Suzuki *et al.,* 2001). The coupling of these three enzymes allows the transformation of creatinine without coenzymes. The reaction sequence is as follows:



The reaction of creatinine in reaction 1 leads to oxygen consumption in reaction 3.

A flow-injection biosensor system was developed for creatinine, with a single injection and one detector. The amperometric detection of urea or creatinine was based on coupled reactions of three sequentially alligned enzyme reactors, CD, GLDH and GLOD (Rui *et al.*, 1992, 1993a,b). The reaction sequence is as follows:

$$\text{Creatinine} + H_2O \xrightarrow{CD} NH_4^+ + \text{N-methylhydantoin}$$

$$\text{2-Oxoglutarate} + NH_4^+ + \text{NAD(P)H} \xrightarrow{GLDH} \text{L-glutamate} + \text{NAD(P)+} + H_2O_2$$

$$\text{L-glutamate} + O_2 + H_2O \xrightarrow{GLOD} \text{2-oxoglutarate} + NH_4^+ + H_2O_2$$

Ammonia produced by the enzymatic hydrolysis of creatinine was converted to glutamate, and the oxygen consumption due to the oxidation of glutamate by GLOD was detected with an oxygen electrode. **Table 2** provides a comparison of analytical properties of DO metric creatinine biosensors

Merits: DO metric creatinine biosensors are easy to use, can be operated at the bedside of patient/outside, can easily and conventionally be employed without special expertise and training

Demerits: Interference due to atmospheric O_2 which make these biosensors less sensitive.

2.7.1.2 Amperometric creatinine biosensors

The majority of amperometric creatinine biosensors rely on the three-enzyme method first described by Tsuchida and Yoda (1983). This involves the three-stage conversion of creatinine to creatine, creatine to sarcosine and sarcosine to glycine. In this final stage, consumption of electrochemically detectable oxygen and liberation of hydrogen peroxide (H_2O_2) occurs. The enzyme catalyzed reaction of creatinine is shown as

$$\text{Creatinine} + H_2O \xrightarrow{CA} \text{Creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{CI} \text{Urea} + \text{Sarcosine}$$

$$\text{Sarcosine} + O_2 + H_2O \xrightarrow{SO} \text{Glycine} + HCHO + H_2O_2$$

The last step of the sequence involves the flavin-containing enzyme SO, which reacts as follows:

$$\text{Sarcosine} + \text{SO}(FAD^+) \longrightarrow \text{Glycine} + HCHO + \text{SO}(FADH_2)$$

$$\text{SO}(FADH_2) + O_2 \longrightarrow \text{SO}(FAD^+) + H_2O_2$$

$$H_2O_2 \longrightarrow 2H^+ + O_2 + 2e^-$$

Detection of H_2O_2 liberation is the preferred technique in amperometric systems, although oxygen electrodes have also been used (Kubo *et al.,* 1983, Nguyen *et al.,* 1991, Suzuki *et al.,* 2001). This preference is due to the classic problems associated with interference at oxygen electrodes (i.e. the high potentials required to bring about its reduction). Amperometry usually requires a system consisting of a reference, a counter (auxiliary), and working electrodes. The most common reference electrodes are the Ag/AgCl or the saturated Hg_2Cl_2; the counter electrode is usually an inert metal such as platinum or stainless steel. The fundamental process in electrochemical reactions is the transfer of electrons between the working electrode surface (area of interest) and the species at the interfacial area (in solution or those immobilized at the electrode surface). The surface topography and the nature of the functional groups on the surface significantly affect the kinetics of the reaction. Working-electrode materials for creatinine biosensors include platinum as a bare electrode or as a disk, platinized gold, platinized shapable electroconductive (SEC) films, cellulose acetate, PbO_2 oxidizing layer, polyvinyl alcohol, poly(carbomoyl sulfonates)-hydrogel and carbon-paste electrodes alone or mixed with platinum powder. It is obvious that platinum has provided a highly catalytic surface where H_2O_2 oxidation can proceed at an accelerated rate. Although composite materials are a cheaper alternative, they do not reach the operational standards that precious metal surfaces are capable of. Thus, the preferred electrode material is platinum because of its stability in aqueous solutions, high catalytic activity and good conductivity. Platinum, silver, carbon and Ag/AgCl ink also can be used in screen-printing methods to create thick- and thin-film sensors for the fabrication of miniaturized, planar, solid-state electrodes (Killard and Smyth, 2000; Lad *et al.,* 2008). **Table 3** provides a comparison of analytical properties of amperometric creatinine biosensors.

Merits: These biosensors achieve excellent operational stability, long storage lifetimes, relatively short response time and high sensitivity. In addition, other interferences (e.g. ammonia) are not a problem and nearly all biosensors achieve the required analytical range.

Demerits: The added complexity of a three-enzyme system has slowed down the development of these biosensors and the presence of three enzymes means a loss in system sensitivity. Creatine potentially causes interference and dual sensors that measure and subtract creatine from creatinine are required, thus adding to system complexity, variability and error.

Table 2. Comparison of analytical properties of DO metric creatinine biosensors

Sr No.	Support for immobilization	Enzymes	Method of immobilization	Optimum pH	Detection limit (μM)	Linear range (μM)	Response time (s)	Interfering compounds	Storage stability	Reference
1	Triamine and acetylcellulose membrane	CD, Nitrifying bacteria	Covalent	8.5	50	44-8840	180	No interference	300 assays over 3 weeks	Kubo *et al.*, 1983
2	Poly(γ-methyl-L-glutamate)	CD, Nitrifying bacteria	Entrapment	8.5	44	88-8800	60	No interference	-	Kubo and Karube, 1986
3	Polypropylene	CA, CI, SO	Entrapment	8.0	3	3-1000	<60	Creatine and Sarcosine	100 assays over 3 months	Nguyen *et al.*, 1991
4	Propylamine and scuccinate CPG	CD, GLDH, GLOD	Crosslinking	8.0	100	100-5000	180	-	-	Rui *et al.*, 1992
5	Propylamine and scuccinate CPG	CD, GLDH, GLOD	Crosslinking/Covalent	8.0	20/100	100-2000	120	Ascorbic acid, ATP, GTP	90% with in one month	Rui *et al.*, 1993a
6	Propylamine and scuccinate CPG	CD, GLDH, GLOD	Crosslinking/Covalent	8.0	200	200-5000	-	-	-	Rui *et al.*, 1993b
7	Silicone gas-permeable membrane	CA, CI, SO	Crosslinking	9.0	20	20-500	360-540	-	-	Suzuki *et al.*, 2001

Table 3. Comparison of analytical properties of amperometric creatinine biosensors

Sr No	Support for immobilization	Enzymes	Working electrode	Method of immobilization	Optimum pH	Detection limit (µM)	Linear range (µM)	Response time (s)	Sensitivity µA/µM/cm^2	Potential applied (V)	Interfering compounds	Storage stability (Days)	Reference
1	Cellulose acetate	CA, CI, SO	Pt	Crosslinking	6-10	8.8	Upto 880	20	-	0.65	-	270	Tsuchida and Yoda, 1983
2	Controlled pore glass	CA, CI, SO	Pt	Entrapment	7.7	25	25-750	-	0.0000208	0.65	-	180	Sakslund and Hammerich, 1992
3	Polypyrrole doped with sulfonated phenoxy resin	CA, CI, SO	-	Adsorbtion	-	200	200-5000	100	-	0.4	-	-	Yomato *et al.*, 1995
4	Gas Permeable Membrane	CD	-	Adsorption	8.5-9.5	-	20-1000	-	0.000001	0.525	-	-	Osborne and Girault, 1995
5	Poly-2-hydroxyethyl methyacrylate	CA, CI, SO	Pt	Crosslinking	7.3-7.4	30	Upto 2000	300	0.0000139	0.6	Creatine, sarcosine	90	Madaras *et al.*, 1996

6	Poly (1,3-diaminobenzene)	CA, CI, SO	Platinized Au	Crosslinking	-	20	900-1200	60	-	0.65	Creatine, sarcosine	30	Madaras and Buck, 1996
7	Poly (carbamoyl) sulfonate-hydrogel matrix	CA, CI, SO	Pt	Entrapment	7.0-8.0	0.3	1-150	20	0.034	0.6	Low selectivity	180	Schneider *et al.*, 1996
8	Polypyrrole	CIH	Pt	Crosslinking	-	88	Upto 2000	-	-	0.3	-	-	Trojanowicz *et al.*, 1996
9	Platinized-SEC (shapable electroconductive) film	CA, CI, SO	-	Crosslinking	-	1-2	10-5000	-	0.023	0.4	Creatine, sarcosine	30	Khan and Wernet, 1997
10	Ferrocene embedded carbon paste	CA, CI, SO, Peroxidase	-	-	8.0	0.01	Upto 15	120	-	0.1	No interference	-	Kinoshita *et al.*, 1997
11	Carbon paste electrode containing 10% Pt powder	CA, CI, SO	-	Adsorption	7.5	200	200-2000	90	-	0.5	-	-	Kim *et al.*, 1999
12	Polyaniline-nafion	CD	Glassy carbon	Crosslinking	7.5	0.5	0.5-500	60	-	-0.2	NH^+, other cations	60	Shih and Huang, 1999

13	PbO_2 oxidizing layer over HPU	CA, CI, SO	Pt	Entrapment	-	0.8	1-1000	98	-	0.8	-	35	Shin *et al.*, 2001
14	Poly (carbamoyl) sulfonate-hydrogel with nafion	CA, CI, SO	Pt	Entrapment	7.5	5	5-150	-	0.005	0.6	-	-	Tombach *et al.*, 2001
15	Polished Pt electrode with alumina and diamond suspension	CA, CI, SO	Pt	Crosslinking	7.4	4.5	4.5-500	60	-	0.7	ND	-	Walsh and Dempsey, 2002
16	Polyvinyl alcohol	CA, CI, SO	Pt	-	7.6	10	10-1000	104	0.0001256	0.8	-	-	Choi *et al.*, 2002
17	Nafion/poly (1,2-diaminobenzene)	CA, CI, SO	Pt disc	-	7.5	-	1-100	-	-	0.5	No interference	90	Yao and Kotegawa, 2002
18	Poly (carbamoyl) sulfonate-hydrogel matrix	CA, CI, SO	Pt	Entrapment	7.4	-	5-1000	25-80	0.00024-0.00046	0.6	-	75	Erlenkotter *et al.*, 2002
19	Carbon paste electrode	CA, CI, SO	Ag wire	-	7.6	0.002	0.004-0.1	30	-	0.65	-	-	Stefan *et al.*, 2003
20	Polypropylene	CA, CI, SO	Pt	-	7.0	-	3.2-320	-	-	-	-	-	Hsiue *et al.*, 2004
21	Carbon paste electrode	CA, CI, SO	Ag wire	-	7.6	0.004-0.006	0.004-0.4	-	-	0.42-0.65	-	-	Stefan-van Staden *et al.*, 2006

2.7.1.3 Nanomaterials based amperometric creatinine biosensors

Compared with traditional biosensors, nanomaterial based electrochemical amperometic creatinine biosensors have marked advantages such as enhanced detection sensitivity and specificity, and possess great potential in applications in clinical samples (Yadav *et al.*, 2011, 2012). In this section, we discussed the recent development of enzymatic creatinine biosensors based on various nanomaterials such as CNTs, ZnO and Fe_3O_4 nanoparticles. In **Table 4** we tabulate the naoparticles based different creatinine biosensors reported so far and give brief descriptions in terms of the sensitivity, detection limit, response time and applied potential.

2.7.1.3.1 Zinc oxide nanoparticles and carboxylated multiwalled carbon nanotubes based creatinine nanobiosensor

The advantages of CNTs, such as high surface area, favorable electronic properties and electrocatalytic effect attracted very recently considerable attention for the construction of electrochemical enzyme biosensors. This configuration assures an enlarged area for immobilization of biomolecules. An amperometric creatinine biosensor was developed by covalently co-immobilizing the CA, CI and SO onto electrochemically synthesized zinc oxide nanoparticles/chitosan/carboxylated multiwall carbonnanotube/polyaniline (ZnO-NPs/CHIT/c-MWCNT/PANI) composite film on Pt electrode (Yadav *et al.*, 2011). CNTs in a suspension individually could be cytotoxic but cytotoxicity is avoided by immobilizing CNTs on surface or within composite. The addition of nanoparticles (NPs) to the CNTs films could generate new nanostructures with excellent behavior in the fields of optics, electronics, and electrocatalysis. Metal nanoparticle (NPs) modified electrodes present unusual advantages in electroanalysis such as improved catalysis, enhancement of electron transport, high effective surface area and control over electrode microenvironment. The enzyme electrode detects creatinine level as low as 0.5 µM (S/N=3) within 10s at pH 7.5 and 30°C when polarized at 0.5 V vs Ag/AgCl. The fabricated creatinine biosensor showed linear working range of 10–650 µM, creatinine with a sensitivity of 0.030 µA/µM/cm^2. The biosensor shows only 15% loss of its initial response over a period of 120 days when stored at 4 °C. The app K_m was calculated to 0.35 mM.

2.7.1.3.2 Iron oxide nanoparticles based creatinine nanobiosensor

Recently an amperometric creatinine biosensor was fabricated by covalently co-immobilizing CA, CI and SO onto iron oxide nanoparticles/chitosangraft-polyaniline (Fe_3O_4-NPs/CHIT-g-PANI) composite film (Yadav *et al.,* 2012). Fe_3O_4-NPs have been considered as interesting for the immobilization of desired biomolecules due to biocompatibility, strong superparamagnetic behavior which provide better contact and low toxicity. Immobilization of bioactive molecules onto a surface charged with superparamagnetic nanoparticles is of special interest, since the magnetic behavior of these bioconjugates may result in improved delivery and recovery of biomolecules for desired biosensing applications. The biosensor exhibited an optimum response within 2s at pH 7.5 and 30°C, when polarized at 0.4 V vs Ag/AgCl. The electrocatalytic response showed a linear dependence on creatinine concentration ranging from 1-800µM. The sensitivity of the biosensor was 3.9 µA/µM/cm^2, with a detection limit of 1µM (S/N = 3). Apparent K_m value for creatinine was 0.17 mM. The biosensor showed only 10% loss in its initial response after 120 uses over 200 days, when stored at 4°C.

2.7.1.4 Potentiometric creatinine biosensors

The first potentiometric creatinine biosenor was developed by Meyerhoff and Rechnitz (1976) based on the ammonia-sensing electrode. Mostly potentiometric creatinine biosensors are based predominantly on the hydrolysis of creatinine by CIH or CD, which generates ammonia that can be detected by any pH, ammonia gas, or ammonium ion-selective electrode (ISE) (Killard and Smyth, 2000; Lad *et al.,* 2008). The enzyme catalyzed reaction of creatinine is shown as

$$\text{Creatinine} \xrightarrow{\text{CIH/CD}} \text{N-methylhydantoin} + NH_3$$

Three enzyme [CA, CI, urease (EC 3.5.1.5)] system also used for construction of potentiometric creatinine biosensors (Jurkiewicz *et al.,* 1998a; Premanode and Toumazoub, 2007). The three enzyme catalyzed reaction of creatinine for potentionmeric biosensor is shown as

$$\text{Creatinine} + H_2O \xrightarrow{\text{CA}} \text{Creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{CI}} \text{Urea} + \text{Sarcosine}$$

$$\text{Urea} + 2H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

Potentiometric techniques involve a nonfaradaic electrode process and measurement of the potential difference between a working electrode and a reference electrode. The potential is proportional to the logarithm of the analyte concentration in the sample and is measured relative to an inert reference electrode. The electrodes have been adapted to varying biosensor designs, including macroelectrodes, wire type and thick and thin films, which have been used to measure ammonia, urea, and oxygen. If the potentiometric sensor is based on a field-effect transistor (FET) chip, then it can be specifically configured to be an ion selective FET (ISFET). A biosensor of this type is from the metal-oxide-semiconductor FET family, in which the usual metal-gate electrode is replaced by a suitably sensitive membrane and a reference electrode. If a thin layer of enzyme is immobilized on the ion-selective membrane of an ISFET, the result is an enzyme-sensitive FET (EnFET). EnFETs usually are based on pH sensitive ISFETs but also can be based on an ammonium-gas sensitive FET. **Table 5** provides a comparison of analytical properties of potentiometric creatinine biosensors.

Merits: These systems have the advantage of relative simplicity because they require only a single enzyme and are based on well established gas-sensing electrode technologies; they also avoid interference from creatine.

Demerits: However, there are significant problems caused by interference from endogenous NH_4^+ in blood and more significantly, in urine specimens, low detection limits in biofluids and poor stability of the enzyme.

2.7.1.5 Conductimetric creatinine biosensor

Conductometric sensors for biosensing devices have first been introduced by Watson *et al.* (1987-1988). The device consisted of a planar glass support with interdigitated gold electrode pairs on one surface in a planar configuration. The operation of the biosensor device was based on measurement of the bulk conductance of the sensitive membrane due to biochemical reaction in solution. A novel, highly sensitive and stable conductometric biosensor was developed for creatinine determination (Isildak *et al.*, 2012). The biosensor was based on solid-state contact ammonium-sensitive sensor. Creatininase was chemically immobilized on the surface of the solid-state contact ammonium-sensitive membrane via glutaraldehyde covalent attachment method. The detection limit of the biosensor was about 2×10^{-6}M and the response time was shorter than 10 s in phophate buffer solution at pH 7.20. The linear dynamic range of the biosensor was between 1×10^{-1} and 9×10^{-6}M creatinine concentration in phosphate buffer solution at pH 7.20.

The biosensor exhibited good operational and storage stability for at least 4 weeks kept in dry at 4–6 °C. It had a reproducible and stable response during continuous work at least for 10 h with the relative standard deviation of 0.5% (n = 48) for creatinine of 1×10^{-3}M in phosphate buffer solution. A schematic diagram of conductometric creatinine biosensor based on solid-state contact ammonium-sensitive sensor is shown in **Fig. 8.** Conductometric transducers are considerably beneficial since construction in a single way, high compatibility, rugged and relatively cheap, no need of any reference electrode.

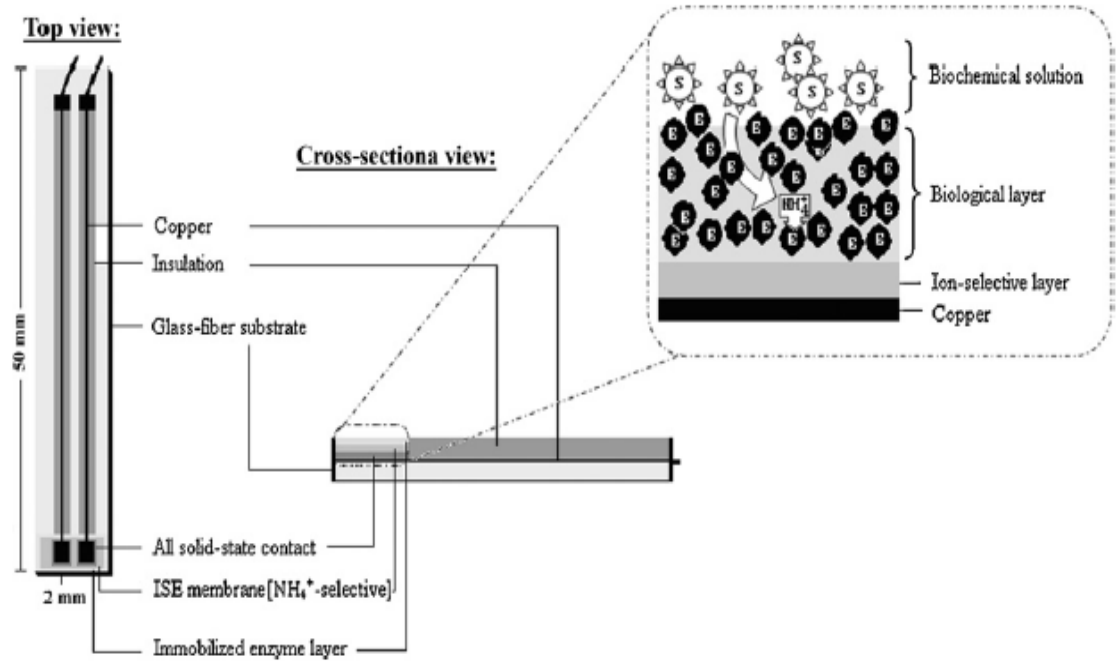


Fig. 8. Schematic diagram of the conductometric creatinine biosensor (Source: Isiladak *et al.*, 2012)

2.7.1.6 Enzymeless creatinine biosensors

Enzymeless creatinine biosensors based on different principles were developed. They are as:

2.7.1.6.1 Capacitive creatinine sensor based on a photografted molecularly imprinted polymer (MIP)

A capacitive chemosensor based on the photopolymerization of the monomer acrylamidomethylpropanesulfonic acid and the cross-linker methylenediacrylamide to create an artificial receptor layer was developed for detection of creatinine (Panasyuk-Delaney *et al.*, 2002). A gold electrode surface was modified with a self-assembled monolayer of alkane thiol followed by adsorption of a photoinitiator, the monomer, the

cross-linker, and the template (creatinine). Treatment with UV radiation formed an ultrathin polymer layer. Removal of the template yielded an electrode surface that is sensitive to creatinine. Creatinine binding was detected by a decrease in the electrode capacitance. The sensor is reversible and highly selective: no response to addition of creatine, urea and glucose were observed.

2.7.1.6.2 Voltammetric behaviour study of creatinine at phosphomolybdic-polypyrrole film modified electrode

The electrochemical behaviour of creatinine was studied by using Keggin-type phosphomolybdate (PMo_{12})-doped polypyrrole (PPy) Film Modified glassy carbon electrode (PMo_{12}-PPy/GCE) (Guo and Guo, 2005). The redox behaviour of the modified electrodes was described by cyclic voltammetry. The electrochemical behaviour of creatinine at this modified electrode was studied by 0.5 order differential voltammetry. In this method creatinine has high inhibitory activity towards the reduction of modified electrode in 0.5 M H_2SO_4. 0.5 order differential voltammetry technology offers great advantages because of rapid, simple, high sensitivity and low-cost, which is employed to monitor the inhibitory activity towards the PMo_{12}-PPy/GC electrode processes and characterize their electrochemical behaviour.

2.7.1.6.3 Novel biomedical sensors for flow injection potentiometric determination of creatinine in human serum

Coated-wire (CW) and tubular (Tu) type membrane sensors were developed consistisitng of creatinine tungstophosphate (CTP), creatinine molybdophosphate (CMP) and creatinine picrolonate (CPC) ion-pair complexes as electroactive materials dispersed in plasticized poly(vinyl chloride) matrix membranes for creatinine determination (Hassan *et al.,* 2005). Tubular and coated wire CTP membrane sensors were incorporated in flow-through cells and used as detectors for flow injection analysis (FIA) of creatinine. The intrinsic characteristics of the detectors under hydrodynamic mode of operation in a low dispersion manifold were determined and compared with data obtained under static mode of operation.

2.7.1.6.4 Enzymeless electrochemical sensor for the selective determination of creatinine

An enzymeless electrochemical approach was developed for the selective and quantitative recognition of creatinine in human urine by using a preanodized screen-printed carbon

electrode (SPE) (Chen *et al.*, 2006). This enzymeless approach, adopted from the Jaffe´ reaction, uses a preanodized, screen-printed carbon electrode. The electrode and the active methylene group in creatinine form a stable and selective carbon-carbon bond in the presence of chloride ions. Creatinine was measured by square-wave voltammetry in phosphate buffer saline at pH 6.7. A simple medium exchange procedure can then lead to the selective recognition of creatinine without any interference from co-existing urinary chemicals like AA, UA, urea, ammonia and creatine **(Fig. 9)**.

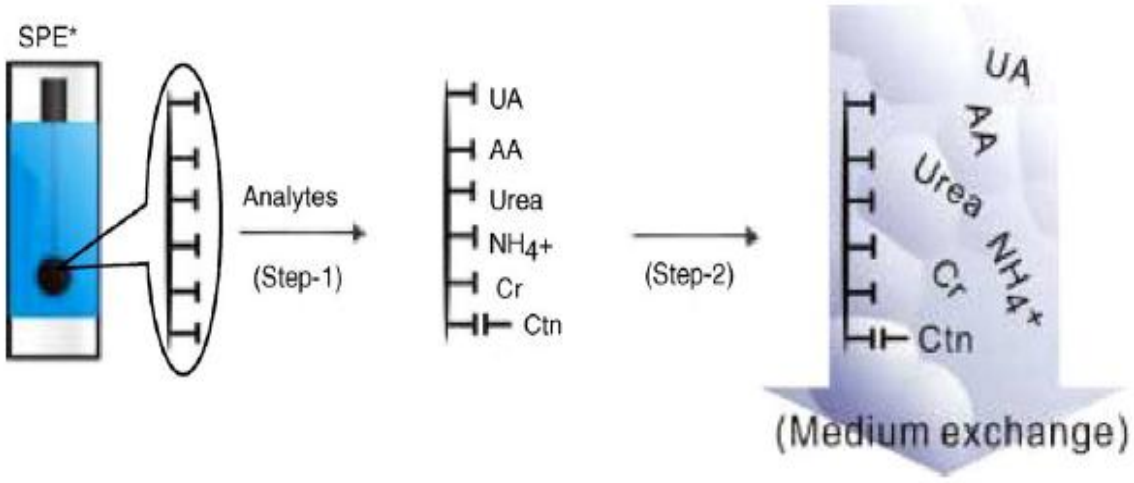


Fig. 9. Conceptional representation for the selective recognition of creatinine (Ctn) in presence of uric acid (UA), ascorbic acid (AA), urea, ammonium chloride (NH_4^+) and creatine (Cr) at the SPE. Step-1: preconcentration of SPE at 1.8V vs. Ag/AgCl. Step-2: medium exchange of the SPE where the preconcentrated electrode is submerged in water. (Source: Chen *et al.*, 2006)

2.7.1.6.5 Potentiometric sensors based on dibenzo-30-crown-10 (DB30C10) with potassium tetrakis(p-chlorophenyl)borate

Three types of enzymeless creatinine potentiometric membrane sensors were developed based on the use of dibenzo-30-crown-10 (DB30C10) with potassium tetrakis(*p*-chlorophenyl)borate type (I), dibenzo-30-crown-10 alone type (II), and potassium tetrakis(*p*-chlorophenyl)borate alone type (III), incorporating in poly(vinyl chloride) matrix membrane plasticized with either o-nitrophenyl octyl ether or dioctylphthalate (Elmosallamy, 2006). The sensors were used for monitoring creatinine after soaking the membranes in 0.1 $moll^{-1}$ creatinine solution for at least 2 days. These sensors were based on the neutral carrier alone or neutral carrier with anionic additives or anionic additives alone have almost the same potentiometric response characteristics.

2.7.1.6.6 Potentiometric electronic tongue for clinical analysis of human urine

A electronic tongue (ET) composed of miniaturized metallic sensors (metal of high purity and alloys) and ion-selective electrodes with PVC solvent polymeric membranes was developed for the detection of urinary system dysfunctions and creatinine levels (Lvova *et al.,* 2009). The ET showed a good predictive power for the total content of alkalimetal ions, total phosphorous and chloride anions content and urinary creatinine.

2.7.1.6.7 Chronoamperometric determination of urea and creatinine based on the organic nickel (II) complexes catalytic systems

Creatinine and urea were determined with the help of catalytic activity of organic nickel (II) complexes in the electrochemical oxidation of creatinine and urea (Kozitsina *et al.,* 2009). The signals of electrocatalytic oxidation of the studied carbonyl containing amines in model solutions were obtained. The oxidation catalytic activity of some nickel (II) complexes based on di- and triketones, tetrazine derivatives, and macrocyclic derivatives of the porphyrin and tetraaza-porphyrin series have been studied.

2.7.1.6.8 Electrochemical sensor for detection of urea and creatinine based on molecularly imprinted polymers (MIP)

Enzymeless electrochemical biosensor was fabricated based on molecularly imprinted polymers (MIP) for creatinine and urea selective determination using solvent evaporation processing of poly(ethylene-co-vinyl alcohol), (EVAL) to form MIP (Khadro *et al.,* 2010). The ratio of ethylene and vinyl-alcohol of EVAL was chosen in order to obtain the higher sensitivity of detection.The carbonyl functions assigned on the spectra confirmed capture and removal (after rinsing with 20 ml of ethanol) of template molecule.

2.7.1.6.9 Enzymeless creatinine estimation using poly(3,4-ethylenedioxythiophene)-β-cyclodextrin

A novel enzymeless creatinine biosensor was constructed for the quantitative estimation of creatinine using β-cyclodextrin (β-CD) incorporated poly-3,4-ethylenedioxythiophene (PEDOT) modified glassy carbon electrode (GCE) (Kumar *et al.,* 2011). The molecular recognition interactions between β-CD and creatinine occurs via weak non-covalent interactions between amide hydrogen of creatinine and glucopyranose oxygen atom in the β-CD. Complex formation between β-CD and creatinine was inferred from regular shifts in the electrode potential versus creatinine concentration. Potentiometric evaluation of the present modifier exhibits Nernstian response per decade concentration change of

creatinine in a range of 10^{-4}–10^{-1} M. The selective interactions of the β-CD incorporated PEDOT film with creatinine in neutral Tris buffer solutions were elucidated using electrochemical impedance analysis.

2.7.1.6.10 A novel structural specific creatinine sensing scheme for the determination of the urine creatinine

Another highly structural dependent amperometric scheme was developed for the determination of creatinine without enzymatic assistance (Chen and Lin, 2012). The principle of this novel method was based upon the formation of a soluble copper-creatinine complex on the copper electrode surface. Subsequently, an oxidative current from the regeneration of the surface oxide layer is monitored and it is proportional to the concentration of the creatinine. The selectivity and sensitivity of this novel method is based upon the chelating ability between creatinine and the copper layers rather than the redox behavior of creatinine itself. **Table 6** provides a comparison of analytical properties of enzymeless electrochemical creatinine biosensors.

Merits: An application of enzymeless chemical sensors and sensors arranged in an array may appear a possible alternative method, which can be used for routine urine analysis even in a small and simply equipped laboratory. Moreover, it permits either to identify various urine samples or to detect at the same time several parameters of the same sample.

Demerits: Complicated preparation process, less recognition sites inside matrix particles obtained via crushing and grinding the imprinted monolith and greater diffusion barrier for template molecule.

2.7.2 Creatinine immunosenors

Biosensors combining immunology and chip-based electrochemistry are called immunosensors. Similar to conventional immunoassays, these devices are based on the principles of solid-phase immunoassay with an antibody or antigen immobilized on the sensor surface. Immunosensors have two modes-an indirect (heterogeneous) immunosensor uses a separate labeled species that is detected after binding by fluorescence or luminescence and a direct (homogeneous) immunosensor detects binding by a change in potential or current. The homogeneous format is a more sensitive approach with fewer problems.

An electrochemical creatinine sensor was developed by using an indirect competitive assay method (Benkert *et al.,* 2000a). The sensing electrode was made up of a platinum surface covered with a creatinine-modified electrode incorporated into an electrochemical cell. The sample, a mixture of anti-creatinine antibody and anti-IgG (mouse)-GOx conjugate, was added to the cell. The creatinine to be measured competes with the membrane-immobilized creatinine for the antigen-binding sites of the conjugated anticreatinine antibodies. Following a washing step, glucose was added, and the H_2O_2 produced was measured amperometrically. It was suggested that the membrane reduces unspecific binding of antibodies or redox-active proteins, preventing any unwanted reactions at the electrode. The measuring range of the sensor was 0.09-90 μM with a lower detection limit of 40 nM, the lowest detection limit for a creatinine sensor. This high sensitivity is advantageous not only for very low levels of creatinine but also for a highly diluted or limited-volume sample, for example, from newborns or blood taken by a capillary. However, one measurement cycle takes 30 min. Calibration curve data for higher concentrations were not mentioned.

The group later reported on a homogeneous immunosensor based on a size exclusion redox-labeled immunoassay that demonstrated a better analytical range of 0.09-900 μM (Benkert *et al.,* 2000b). In this method, creatinine from the sample and synthetic redox-labeled creatinine compete for the antigen-binding sites of the anticreatinine antibodies. Redox-labeled creatinine not adsorbed by the antibodies passes through the cellulose membrane to the glassy carbon electrode. The redox label was a quinone derivative that was electrochemically indicated at an interference-free, working potential similar to artificial Meds used with enzymes **(Fig. 10).** If the sample creatinine concentration is high, then the signal response caused by unbound redox-labeled creatinine would also be high.

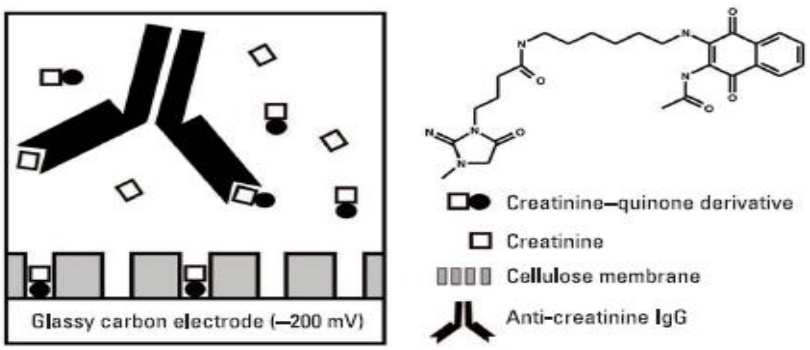


Fig. 10. Principle of size exclusion redox-labeled immunoassay (Source: Benkert *et al.,* 2000b)

Merits: The sensor required no regeneration and displayed an adequate analytical range and sensitivity.

Demerits: The sensor consumed a large amount of expensive anticreatinine antibodies.

2.7.3 Optical creatinine biosensor

Creatinine is also be monitored by using an optical sensor. The detection of analytes by optical sensors usually requires the development of fluorescent transducers which are specific for different analytes. Optical transducers was coupled to the detection of creatinine via the creatinine deiminase driven hydrolysis of creatinine, with the optical transducer modulated by ammonium or ammonia. Detection of ammonium requires an ammonium specific ionophore coupled to a chromophore that changes its absorption spectrum upon protonation, and a lipophilic anionic site. Detection of ammonia requires a protonated pH sensitive indicator which changes its absorption or fluorescence spectrum upon deprotonation. As such, sensors based on the detection of ammonium can be expensive and complex.

A creatinine sensor material was invented (Munkhomm, 1999) comprising the three layer system: first layer comprising a pH sensitive fluorophore immobilized in a first, hydrophobic polymer, wherein the fluorophore can react quantitatively with ammonia and the transducing moiety of the fluorophore is neutrally charged when deprotonated; a second layer comprising creatinine deiminase and a polymer; and a third layer comprising a polymer. The present invention provided a method for measuring creatinine comprising the fluorescence of the creatinine sensor material; exposing the sensor material to a solution comprising creatinine; measuring the fluorescence change; and determining the concentration of the creatinine. The optical biosensor shows the detection limit of 200 μM.

Merits: An advantage of optical sensors is their ability to resolve information from different analytes via their discrete wave-bands. In this way one could couple an ammonia sensor together with a sensor for a different analyte in the same membrane, but collect the readout information at separate wavelengths.

Demerits: It suffers from laborious labeling processes that may also interfere with the function of a biomolecule. Quantitative analysis is challenging due to the fluorescence signal bias, as the number of fluorophores on each molecule cannot be precisely controlled.

Table 4. Comparison of analytical properties of nanomaterials based amperometric creatinine biosensors

Sr No	Support for immobilization	Enzymes	Working electrode	Method of immobilization	Optimum pH	Detection limit (µM)	Linear range (µM)	Response time (s)	Sensitivity µA/µM/cm^2	Potential applied (V)	Interfering compounds	Storage stability (Days)	Reference
1	ZnO-NPs/CHIT/c-MWCNT/PANI	CA, CI, SO	Pt	Covalent	7.5	0.5	10-650	10	0.030	0.5	Creatine	120	Yadav *et al.*, 2011
2	Fe_3O_4-NPs/CHIT-g-PANI	CA, CI, SO	Pt	Covalent	7.5	1	1-800	2	3.9	0.4	Creatine, Sarcosine	200	Yadav *et al.*, 2012

Table 5. Comparison of analytical properties of potentiometric creatinine biosensors

Sr No	Support for immobilization	Enzymes	Working electrode	Method of immobilization	Optimum pH	Detection limit (µM)	Linear range (µM)	Response time (s)	Sensitivity (mV/conc.)	Interfering compounds	Storage stability (Days)	Reference
1	Cellophane dialyzing and gas permeable membrane	CIH	NH_4 gas sensing	Entrapment	8.5	520	70-8900	120-600	44.4-49.4	NH_4^+	8	Mayerhoff and Rechnitz, 1976
2	-	-	-	-	-	44	44-8800	300-360	-	-	-	Guilbault *et al.*, 1980
3	Nylon coil	CIH	NH_4^+ sensitive	-	-	2	-	90	-	-	-	Mascini and Palleschi, 1982
4	Collegen and pig intestine membrane	CD	NH_4 gas sensing	-	8.7	53	88-8800	120-600	-	NH_4^+	21	Guilbault and Coulet, 1983
5	Nylon tubes	CIH	NH_4^+ sensitive	-	8.5	10	-	-	-	-	-	Mascini *et al.*, 1985
6	Poly (vinyl chloride)	CD	NH_4^+ sensitive	Adsoption	8.5-9.5	44-88	88-4400	180	-	-	180	Kihara and Yasukawa, 1986
7	Controlled pore glass and eupergit	GLDH, CIH	NH_4^+ sensitive metal oxide semiconductor	-	7.5-7.9	0.2	Upto 30	-	-	-	-	Winquist and Lundstorm, 1986

8	-	CIH	pH sensitive	-	9.5	-	50-1000	-	-	-	-	Collison and Mayerhoff, 1987
9	Nonactine/polyphenyl acetylene	CD	EnFET/ISE	-	-	0.1/100	-	-	-	-	-	Battilotti *et al.*, 1989
10	Triacetate cellulose membrane	CD	NH_3 gas sensing	Entrapment	8.0	200	200-6000	<60	-	-	7-10	Campanella *et al.*, 1990a
	Cellulose acetate/ Polyazetidine		ISE		-	-	100-30000	180	35.8-37.2	Urea, ethanolamine, diethanolamine, triethanolamine, alanine	4-9	
11	Cellulose acetate/ Polyazetidine	CD	Gas diffusion electrode	Entrapment	-	-	200-60000	60	-		7-10	Campanella *et al.*, 1990b
12	Cubic liquid crystalline phase	CD	pH sensitive	Entrapment	-	50	500-2000	600-900	-	-	20	Razumas *et al.*, 1994
13	Controlled pore glass beads/Nylon membrane	CA, CI, Urease	NH_4^+ sensitive	Covalent	7.5	-	-	108-126	50.8-57.9	-	90	Jurkiewicz *et al.*, 1998a
14	Graphite epoxy resin	CD	NH_4^+ sensitive	Covalent	7.5	10	50-1500	420	52.3	NH_4^+	30-60	Jurkiewicz *et al.*, 1998b
15	Polyinon complexes/ polypyrrole film	CIH	pH sensitive	-	-	1	-	-	29.28	-	-	Osaka *et al.*, 1998

16	Poly(methyl vinyl ether)/maleic anhydride	CD	AC impedance detection	Adsorption	-	-	-	-	-	-	-	Ho *et al.*, 1999
17	Polyinon complexes/ polypyrrole film	CIH	pH sensitive	Entrapment	6.86	1	-	-	-	ND	-	Osaka *et al.*, 2000
18	Poly(vinyl alcohol) containing stryl pyridinium	CD	ISFET	-	7.4	20	20-1000	90-120	-	-	180	Soldatkin *et al.*, 2002a
19	Biologically active membrane	CD	ISFET	Crosslinking	7.4	10	0-5000	-	-	-	180	Soldatkin *et al.*, 2002b
20	Chitosan	CIH	NH_4^+ sensitive	Adsoption	7.0	100	100-1000	30-60	-	-	44	Magalhaes and Machado, 2002
21	Organically modified sol gel glass and p-toulenesulfonate doped polyaniline	CA, CI, urease	pH sensitive	Adsorption/Entrapment	7.0	100	-	900	-	-	30	Pandey and Mishra, 2004
22	Carboxylated-polyvinyl chloride	CD	NH_4^+ sensitive	Covalent	8.1	15	20-20000	-	-	NH^+, other cations	180	Radomska *et al.*, 2004a
23	Carboxylated-polyvinyl chloride	CD, urease	NH_4^+ sensitive	Covalent	-	-	-	-	-	-	180	Radomska *et al.*, 2004b
24	PVA-SbQ	CIH	NH_3 gas sensing	-	8.5-9.5	20	-	60	-	-	-	Suzuki and Matsugi, 2004
25	Polyvinyl alcohol	CD	ChemFET	Entrapment	-	-	10-1000	300	30	-	7	Sant *et al.*, 2004

26	PVA-SbQ	CD	pH sensitive	-	10	20	-	10	-	-	-	Suzuki and Matsugi, 2005
27	Directly onto gate	CA, CI, urease	ISFET	-	6-8	-	0-20000	-	-	-	-	Premanode and Taumazou, 2007
28	Nylon membrane	CD	-	-	7.4	0.3	-	30	58.78	-	100	Rasmussen *et al.*, 2007
29	Polyvinyl chloride	CD	NH_4^+ sensitive	Covalent	-	-	-	-	-	-	-	Grabowska *et al.*, 2007
30	Carboxylated-polyvinyl chloride	CD, urease	NH_4^+ sensitive	Covalent	7.5	0.1	-	60-120	50	-	-	Gutierrez *et al.*, 2008
31	CHIT-SiO_2-MWCNTs	CIH	-	Covalent	7.0	-	-	-	-	-	240	Tiwari and Dhakate, 2009
32	CHIT-g-PANI	CIH	-	Covalent	7.0	-	-	-	-	-	300	Tiwari and Shukla, 2009
33	Conductive layer	CD	NH_4^+ sensitive	-	7.5	3	5-255	25	-	-	-	Hsiung *et al.*, 2010
34	Chemical current conveyors	CD	ISFET	-	7.4	-	44-106	-	-	-	-	Pookaiyaudom *et al.*, 2011

Table 6. Comparison of analytical properties of enzymeless electrochemical creatinine biosensors

Sr No.	Electrode		Method of detection	Detection limit (µM)	Linear range (µM)	Response time (s)	Interfering compounds	Storage stability (Days)	Reference
1	MIP modified Au electrode		Impedometric	10	50-600	120	No interference	180	Panasuk-Delaney *et al.*, 2001
2	Phosphomolybdic-polypyrrole film modified glassy carbon		Cyclic voltammetry	0.005	1-100	-	-	7	Guo and Guo, 2005
3	Screan printed carbon		Squarewave voltammetry	8.6	370-3600	-	-	-	Chen *et al.*, 2006
4	Creatinine tungstophosphate	Tubular	Potentiometry	3.5	5-10000	<20	No interference	70	Hassan *et al.*, 2005
		Wire coated		5.6	10-10000	<10			
	Creatinine molybdophosphate	Tubular		50	75-10000	<20			
		Wire coated		5.6	8-10000	<10			
	Creatinine picrolonate	Tubular		20	31-10000	<20			
		Wire coated		15	31-10000	<20			
5	Dibenzo-30-crown-10 with potassium tetrakis (p-chrolrophenyl) borate	o-NPOE	Potentiometry	11	50-10000	6	NR	180	Elmonsallamy, 2006
		DOP		8		8	NR	180	
	Dibenzo-30-crown-10 alone	o-NPOE		15	60-10000	5	NR	180	
		DOP		14		4	NR	180	
	Potassium tetrakis (p-chlorophenyl) borate alone	o-NPOE		27	70-10000	4	NR	150	
		DOP		20		10	NR	150	
6	Mettalic electrode/Ion selective		Potentiometry	-	-	-	-	-	Lvova *et al.*,

	electrode							2009
7	Organic nickel (II) coplexes modified GCE	Chronoamperometry	27	50-1000	70	-	-	Kozitsina *et al.,* 2009
8	Poly (ethylene-co-vinyl alcohol) modified Au electrode	Electrochemical impedance spectroscopy	-	0.44-17.6	20	-	-	Khadro *et al.,* 2010
9	β-cyclodextrin incorporated poly-3,4-ethylene dioxythiophene modified glassy carbon electrode	Potentiometry	50	100-100000	60	Ascorbic acid, Uric acid	-	Kumar *et al.,* 2011
10	Copper-platinum electrode	Cyclic voltammetry	0.5	1.8-108	-	Uric acid	-	Chen and Lin, 2012