ENZYME SYSTEMS IN RELATION TO THE ELECTRICAL PROPERTIES OF CELL PARTICLES

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The cell is a mysterious and dynamic unit, bestowed with the power of coordination both at structural and functional levels of its organization. Hence the cell, its intracellular regulation and coordination of catabolic and anabolic phases of the metabolism, has been a very fascinating subject of the present-day scientific field. The metabolic machinery of the cell is being specifically driven by the biological catalytic agents, the enzymes. The activities of these enzymes have to be systematically coordinated by the cell environment to arrive at a coherent resting metabolism. As these enzymes are for established to be the proteins and as the proteins in a suspension possess charges on their molecular pattern, the charge density on these molecules might have been playing a dynamic role in the expression of the enzyme activities. Since the active centres of the enzyme are known to be specifically governed by the charge density prevailing on its protein component and since the proteins in a heterogeneous suspension are known to interact to change their charges and achieve the electrostatic equilibrium, the author had been very much fascinated to find out the role of these charges present on the particulate fractions of the medium over the expression of the enzyme activities. The author aims at exploring a possible relationship between the charges in the medium and regulation of the enzyme activities, at least to a considerable extent though not to the perfect levels.
The author could not proceed to the interior and perfect levels of this problem because of unavoidable restrictions posed by the modern equipment and its lack of availability. The study of this problem at the perfect levels requires the availability of high speed centrifugation apparatus like ultracentrifuge in order to isolate a pure enzyme and to study its kinetic properties as proposed in the present investigation. Even for the estimations of cytochrome C content and mitochondrial protein content in the present investigation, the author could get the permission to use the semi-ultracentrifuge from the other source with a Herculean task. The research facilities like gel-electrophoresis, microtitration techniques and such other important modern equipments are not within the reach and hence the author had been restricted by several barriers from the further exploration of the riddles in the expression of the enzyme activities. Most of the instruments and chemicals for histochemical studies are not available.

Hence the author tried his level best to extract as much information as possible, pertaining to the problem of enzymes and their dependence on the protein charges, with the available equipment. Inspite of all these, the author is highly indebted to the University authorities for having provided with the sufficient equipment and facilities atleast to proceed into the considerable depth of the problem.
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1. Charge on the subcellular components and their electro-migration in a field of direct electric current

The cell structure does not simply consist of a random array of molecules, but a hierarchically ordered molecules, molecular groupings and supramolecular entities and the life activity depends on the order of their interactions. Once the cell is acknowledged to be systematically organised community of molecular population with dynamic interactions, the understanding of the dynamic organization of the system will be the immediate problem posing the scientific world of the present day (Siekevitz, 1962).

One of the examples of such delicate organization is that of the electrical activity of the cell which manifests as a delicately organized potential difference (Grundfest, 1955; Hodgkin, 1951 and 1958). Any alteration of this potential difference affects the enzymatic pattern of the cells since significant changes are induced in the colloidal system of the protoplasm (Heilbrunn, 1934). The enzymatic reactions inside the cell are delicately balanced and governed by the colloidal properties of the protoplasm (Heilbrunn, 1956; Bayliss, 1959). The protoplasmic response to an applied external voltage gradient to a large extent depends on the strength of the electric field (Heilbrunn, 1956). Higher strength of voltage is known to cause a permanent injury to the dynamic organization of the protoplasm
(Heilbrunn, 1952 and 1956). The information known about the effects of electric field on the nature of the molecular structure of subcellular particles in an intact living cell and the physicochemical processes that accompany with them is very meagre (Liberman and Chailakhian, 1959). Both visible and structural changes which accompany the electrical stimulation in a variety of cells have been studied by various investigators (Schmitt and Schmitt, 1940; Flaig, 1947; Hill and Keynes, 1949; Tobias and Solomon, 1950; Heilbrunn, 1952).

The protein molecules are widely accepted to be the amphoteric electrolytes, as they can get ionized both positively and negatively (Neurath et al., 1954). As the surface of different subcellular components is proteinaceous with differing characterizations, they get ionized and carry different signs of the charges at the intracellular pH. Thus the subcellular components are supposed to carry charges due to the ionization of their protein components.

The information available about the nature of the subcellular charges of living protoplasm is very fragmentary. Kuhne (1864) and Hardy (1913) exposed the slime moulds and the onion root tip cells respectively to the direct electric field and observed the movement of the cytoplasmic granules towards the cathode. Similar studies conducted by Churney and Klein (1937) and Dahlgren (1915) revealed that the
nucleus as a whole migrated towards the cathode whereas the chromatin inside moved towards the anode. Investigations conducted on similar trends (Heilbrunn, 1956; Swami, 1960) have revealed the presence of a net negative and net positive sign of the charges on the surface of a cell and its internal contents respectively. Hence the electrophoretic studies are known to explore the nature of the ionizable protein groups present on the subcellular components (Ponder et al., 1960; Bangham, 1961; Heard et al., 1961). Investigation on the Elodea leaf revealed the presence of a net positive sign of the charge (Heilbrunn and Daugherty, 1939) and a net negative sign of the charge (Tobias and Solomon, 1950) on the chloroplasts. These studies suggested that the charge on the subcellular components depend on the state of the metabolic activities of the cell. Hence for the investigations of the charge properties of the subcellular components, the metabolic status of the cell, should be considered. Thus the further studies on the electrophoretic behaviour of the cell components (Heilbrunn and Daugherty, 1939; Heilbrunn, 1956; Swami, 1960 and 1961) have indicated the possession of the charges over them. In some cases the charge was found to be positive and in other cases it was negative.

Similar type of electromigration studies were extended, in this laboratory, on vertebrate tissues using the gastrocnemius and sartorius muscles of frog. The suitability of frog muscle for the investigations on the charge properties
of subcellular organization of a cell was studied by Swami et al., (1962). The rate of lactic acid production and the glycogen breakdown was estimated in muscles exposed to both direct and alternate currents (Swami et al., 1962) and it was found that a field of direct current was more suitable for these investigations since the rate of lactic acid production was very low. From the studies of Swami and Krishnamoorthy (1964), a correlation had been suggested between the charges over the subcellular components and their influence on the enzyme activities. It was also suggested that the positive charges on the whole accelerate the enzyme activities (Swami and Krishnamoorthy, 1964). Thus these studies revealed the gross pattern of the sarcoplasm of the muscle. In this connection, it is essential to make an attempt to investigate the charge pattern on the subcellular components and their electromigration in detail, in order to explore the riddles of the organization of the sarcoplasm and its role in the phenomenon of muscular contraction.
2. Subcellular distribution of enzyme systems and their electronegativity

The cell, as a unit, retains its unity by virtue of the power of subordination which it exerts upon its constituent enzyme systems in such a manner that their individual activities instead of being free and unrelated, will be restrained and directed towards a combined unitary resultant (Siekevitz, 1962). In order to achieve this unitary resultant metabolism, the enzyme systems are specifically lodged in different subcellular components (De Duve et al., 1962). The activities of these enzyme systems will be governed by the charge on the subcellular particulate fractions in which they are located. Hence there seems to be a sort of "division of labour" amongst the subcellular components as far as their enzyme systems are concerned and each system is dependent on the other in order to evolve the continuous and coherent metabolic interconnections in the cell.

In a living cell, the structure and organization of the subcellular particles are undissociably associated with the function. Hence in order to understand the functional significance of the enzyme systems, the particulate fractions should be kept intact to preserve their organization.

The recent and elegant method of studying the particulate fractions and the activities of the contained enzymes is
by using differential and gradient centrifugation technique. The work of differential centrifugation lead to the recognition that the mitochondria contained all the enzymes necessary for the complete oxidation of pyruvate to carbon-dioxide and water and also for coupling this oxidation with the esterification of inorganic phosphate. Regaud (1911) concluded on the basis of staining reactions that the sarcosomes of muscle were identical with the mitochondria. Slater (1950 and 1960) isolated the sarcosomes by differential centrifugation from the extracts of heart and showed that they brought about oxidative phosphorylation. Cleland and Slater (1953) separated cat heart muscle into various fractions which were identified microscopically and determined the distribution of the cytochrome oxidase activity. The sarcosome fraction represented 6 to 7 times the specific respiratory activity, as exemplified by cytochrome oxidase, of the whole muscle. Studies by Chappel and Perry (1953) of the distribution of succinic oxidase and pyruvic oxidase in pigeon breast muscle gave the concluding proof of the presence of these enzymes in the muscle sarcosomes.

Through the epoch making studies based on differential centrifugation, the present scientific world became aware of the intracellular particulate localization of the enzyme systems. The presence of various oxidative enzyme systems like succinate oxidase (Schneider and Hogeboom, 1950),
choline oxidase (Kessler and Langeman, 1951), oxalacetate oxidase (Schneider and Potter, 1949), cytochrome C reductase (Hogeboom, 1949; Hogeboom and Schneider, 1950) and cytochrome oxidase (Schneider, 1946) in mitochondria has been proved beyond doubt. The isolation of cytochrome C from liver fractions has been reported and a large percentage of the pigment recovered appeared to be associated with the mitochondrial fraction (Schneider and Hogeboom, 1950).

The ability to oxidise succinate with any given electron acceptor has always been found to be associated with the cytoplasmic particles, and these have in a number of cases been identified conclusively with the mitochondria. The same situation exists with the cytochrome oxidase. Hence the activities of succinic and cytochrome oxidases have been extensively used as convenient markers to assess the proportion of mitochondria present in a given subcellular fraction (De Duve et al., 1962).

The density of mitochondria and respiratory activity have drawn a distinction between different types of muscle fibres. Paul and Sperling (1952) have sorted out the myofibrils into two categories depending on the density of the mitochondria in their composition. White muscle fibres have less mitochondrial density and considerably low respiratory activity when compared to the red muscle fibres which have high density of mitochondria with greater respiration.
The various investigations reviewed above have firmly established that the granules which have been described by the cytologists under the names, sarcoosomes or mitochondria, are the respiratory granules of the cell lodging the enzyme systems concerning the oxidative phosphorylation.

Most of the enzymes concerned with the glycolysis have been reported to be present in the cytoplasm (soluble protein fraction) of an intact cell (De Duve et al., 1962). As per the suggestion of Johnson (1960), it is inferred that most of the glycolytic activity had been recorded from the supernatant fraction. But Lang and Siebert (1951) and Roodyn (1956) claim that the glycolytic activity is associated with the nucleus due to the presence of aldolase, an important glycolytic enzyme, in it. One can endorse the opinion of Johnson, who feels that trapping of soluble enzymes by fibrous material and incompletely disrupted cell debris could easily account for particulate glycolytic activity (De Duve et al., 1962).

The investigations of Zamecnik (1960 and 1962) inferred that the ribonucleoprotein particles or ribosomes, associated with the endoplasmic reticulum, are the sites of protein synthesis. Subsequent experiments with cell free systems demonstrated the requirement of ribosomes as subcellular particles and adenosine triphosphate or ATP-generating system for the synthesis of proteins (Ochea, 1962). The
ribonucleic acid was shown to be localized in the ribosomes (Rich, 1962; Zamecnik, 1960 and 1962). The enzyme L-amino acid oxidase had been shown to be constantly in association with the ribosomes (De Duve et al., 1962).

Thus enzyme systems are specifically localized inside the subcellular components. As the surface of these subcellular components carry charges, they will tend to migrate in a field of direct current to the opposite pole of their respective charge. Along with the migration of these subcellular components the enzyme systems, lodged in them also will be migrating towards their respective poles. Hence the intracellular electromigration of the subcellular particles may be looked upon as resulting in a net movement of the enzyme systems. As enzymes are proteins, they also contribute to the electrophoretic phenomenon with the rest of the protein constituency. Hence it is essential to explore the nature and organization of enzyme systems in intact cell. In the light of these observations, an attempt must be made to investigate the relationship between the electrophoretic characteristics of the enzyme systems and the pattern of metabolism prevailing in the inner depths of a living intact cell.

3. Charges on the protein molecules and their electromigration

Proteins are amphoteric electrolytes, as they have both acidic and basic properties (Alberty, 1963) and get ionized
both in the acid and alkaline media. The principal contribution to the behaviour of a protein as an electrolyte will come from the ionizable groups in the amino acid side chains (Fruton and Simmonds, 1960). The ionization characteristics of the proteins are highly dependent on the hydrogen ion concentration of the medium (Stern, 1956). The ionization of the proteins manifest itself as charge on the molecules. The protein molecules get positively charged towards the acidic side and negatively charged towards the alkaline side of their respective isoelectric points (Fruton and Simmonds, 1960). Owing to their dipolar behaviour, the protein molecules play a dynamic role in the buffering processes of the biological systems. In addition to this role of biological buffering, the protein molecules due to their amphoteric electrolytic nature plays significant role in their stability, chemical interactions and the enzyme activities. In fact almost all the functions of the biological systems are dependent on the electric charge on the protein molecules, governed by the pH of the medium.

If a solution, containing ions bearing electric charges, is placed between the two electrodes of direct electric field, the ions migrate to the opposite pole of their respective charge (Fruton and Simmonds, 1960). In the light of this finding, the protein molecules should exhibit electromigration as they are electrolytes. The protein molecules in the acid media tend to migrate towards the cathode as they
will be positively charged and towards the anode in the alkaline media since they will be negatively charged.

Isoelectric pH of the protein is defined as that hydrogen ion concentration at which the protein molecule carries a net zero charge over it and migrates to neither of the poles. The first systematic electrophoretic study of the protein was carried on by Hardy in 1899, who showed that the denatured egg albumin migrated towards the cathode in acid media and towards the anode in the alkaline media. Since then, the electromigration studies of proteins have been extensively conducted by various investigators. Depending on the electrophoretic behaviour of the proteins, different types of proteins have been isolated and characterized through the process known as electrophoresis. The electrophoretic mobility of the protein molecule depends on the density of charges on its surface (Stern, 1956). The electrophoretic mobility of colloidal ions is not only affected by the hydrogen ion concentration but also by the ionic strength and the chemical composition of the solvent in which they are suspended (Stern, 1956). Since the enzymes are proteins, their chemical activity is largely dependent on the ionization characteristics of protein component (London et al., 1953) which in turn manifests in terms of electrophoretic characteristics. Hence study of the electrophoretic pattern may reveal the pattern of activation associated with the enzyme activity.
4. Enzyme activity - optimum pH; isoenzymes

The hydrogen ion concentration of the medium has a profound and remarkable role to play in the expression of enzyme activities. At a particular pH value of the medium, the given enzyme exhibits its highest activity, which was termed as "optimum pH" of that enzyme and was first pointed out by Sørensen in 1909. Some enzymes exhibit relatively sharp pH optima. Towards the either side of this optimum pH value, there will be a decrement of the enzyme activity. The enzyme activities depend on the ionization pattern of their protein moiety (London et al., 1958), which on the other hand depends on the hydrogen ion concentration of the medium. Hence it is essential to maintain the pH value of the medium constant to study the enzyme activity (Fruton and Simmonds, 1960). The reasons for the drop in the enzyme activity towards the either side of the optimal pH may be the inactivation of the enzymes due to denaturation of its protein part or change in the ionization pattern of the molecule affecting the catalytic centres or due to the alteration in the dissociation of the substrate. Thus the hydrogen ion concentration can either activate or inactivate the enzyme molecule affecting the catalytic activity.

In recent years an increasing amount of evidence has shown that many enzymes exist in multiple forms with
relatively variable pH optima, called as isoenzymes or isozymes. The investigations have concluded that there are multiple forms of the same enzyme within the cells of a single organism even when the organism is homozygous (Weiland and Pfleiderer, 1957; Vessel and Seara, 1957; Markert and Müller, 1959). The several forms of an enzyme may differ significantly in primary protein structure and thereby be under the control of multiple genes as had been clearly demonstrated with respect to lactic dehydrogenase isozymes. These isozymes characteristically differ in different tissues of an organism (Markert and Müller, 1959), having the same molecular weight but differing in electrophoretic mobility. The two extreme forms of lactic dehydrogenase, LDH - 1 and LDH - 5 are drastically differing in their amino acid composition, immunological specificity, heat stability and in such other characters (Cohn et al., 1962; Markert, 1963b; Kaplan, 1964). Lactic dehydrogenase was found to consist of four subunits of two types A and B (Appella and Markert, 1961; Markert, 1963a). Each type of the sub unit was found to have a separate genetic control (Shaw and Barto, 1963; Boyer et al., 1963; Blanco et al., 1964; Kraus and Weely, 1964).

Aldolase was also shown to exist in isozymic forms of A and B (Blostein and Rutter, 1963) and these two forms were shown to differ specifically in tissue localization,
immunological specificity and the reaction rates with different substrates.

Malate dehydrogenase was shown to exist at least in two isozymic forms, both differing significantly in their electrophoretic mobility, amino acid content and kinetic properties. Amongst the two isozymic forms of malate dehydrogenase, one was shown to be localized in the mitochondrial fraction and the other in the supernatant fraction of the tissue homogenates (Delbrück, et al., 1959a & b; Siegel and England, 1960; Grimm and Soherty, 1961; Thorne and Cooper, 1964).

Isocitrate dehydrogenase is another enzyme in which three different isozymes are identified. Amongst the three isozymes, one specific to NAD and the rest of the two specific to NADP have been clearly demonstrated (Kornberg and Pricer, 1951; Lowenstein and Smith, 1962). The three isozymes were shown to function similarly in catalyzing the oxidative decarboxylation of isocitrate and requires the presence of a divalent metal ion (Henderson, 1964). The NAD and NADP specific isozymes were shown to differ significantly in K\textsubscript{m} value of substrate enzyme complex, optimum pH, stability and the cofactor requirement (Plaut, 1963). These isozymes were shown to differ in their intracellular location and distribution in various tissues and these could be separated by electrophoresis of tissue homogenates.
(Lowenstein and Smith, 1962; Plaut, 1963; Bell and Baron, 1964).

The existence of the enzymes in multiple forms differing in tissue specificity and intracellular location had been suggested to play an efficient role in different intracellular environments. As the cell being a complex system of several enzyme systems, their enzymic interaction should be coupled with each other in order to keep up the unitary resultant which exemplifies in the form of cell metabolism. A detailed study can be made to investigate the reasons for the existence of the same enzyme in different isozymic forms, to visualise the riddles of the metabolic interactions inside an intact living cell.

5. **Enzyme activity and its dependence on the protein ionization**

Enzymes are the biological catalysts, through whose catalytic activities, the different physiological phenomena are possible at the conditions prevailing in the cell interior. The investigations of Northrop et al. (1948) established the protein nature of the enzymes beyond doubt. Enzymes known upto the present time are proved to be proteinous in character. The enzyme activity was supposed to be dependant on the characteristics of its protein moiety to a greater extent (London et al., 1962). Hence
the understanding of the mechanism through which enzymes exert their catalytic action depends, in large measure, on the understanding of the details of the protein structure and characteristics (Fruton and Simmonds, 1960).

The conversion of an enzyme-precursor to the active form may be regarded as a model system for the study of the relation between the chemical nature and biological activity of the enzymes (Neurath et al., 1954). During the enzymatic activation of chymotrypsinogen and trypsinogen, one or more peptide bonds are presumably opened and hence it can be expected that on activation there is an alteration in the exposure of the new end groups or side groups on the enzyme molecule (Neurath et al., 1954). As the end groups are involved in the expression of the enzyme activity, the activation phenomenon of the zymogen molecules can be looked upon as a change in the ionization pattern of the protein component and thereby their alteration in the charge density of the enzyme molecule. Hence it may be inferred that the activation and inactivation of the enzymes is governed by the charge pattern of the molecular species. The specificity of the dehydrogenases was shown to depend largely on the protein moiety of the enzyme with which the coenzyme is associated (Cantarow et al., 1954). Thus the investigations on activation of these enzymes revealed the dependence of biological activity of the enzyme molecules on the protein constituency.
The enzyme molecule is not catalytically active entirely but carries certain centres, which selectively bind the substrate, called active centres (Fruton and Simmonds, 1960). The active centre is defined as that part of the enzyme molecule which cannot be modified without the total loss of the biological activity (Sriram et al., 1962). It was also demonstrated (Sriram et al., 1962) that there was indeed a dispensable part which can be removed without loss of biological activity. But it should be noted that the activity of the active centres is dependent on the dispensable part and its ionization invivo (Fruton and Simmonds, 1960). The configuration of the active centres is dependent on the ionization pattern of the other part of the enzyme molecule. From the elegant investigations, a theory was postulated (London et al., 1958) suggesting that apart from the site of the enzyme substrate combination, secondary factors concerning charged sites and tensions within the enzyme molecular structure conferred specificity on the enzyme. These factors were referred to as secondary specificity site whereas the active centres were referred as the primary specificity site of the enzyme molecule (London et al., 1958). It had been further demonstrated that a positively charged group and a hydrogen bonding type group are a likely pair which made the active substrate combining site of the enzyme molecule (London et al., 1958).
High degree of specificity also is attributed on the secondary specificity site as the order of distribution of charges and their density in this region are known to govern the activity of the enzyme molecule (London et al., 1953). It is postulated that the entire enzyme molecule will be under the thermal vibrations and when the proper array of charges are available on the protein molecule, the active centre will be in harmonic movement with the substrate and thereby the union between the enzyme and substrate becomes possible (London et al., 1953). Thus in the light of the information available it is essential to visualize the key role of the protein part in the activation and inactivation of the enzyme molecules. From the pH-activity curve of invertase, it was shown (Myrbäck, 1960) that the isoelectric point of this enzyme lies within the range of its optimum pH value. As the isoelectric point of the enzyme happened to be within the range of the pH in which it was maximally active, it was generally assumed that the specific configuration requires the prevalence of a net zero charge on the enzyme molecule for its effective expression of catalytic activity (Myrbäck, 1960). With shift of pH to either side will affect the specific configuration, which results in the appearance of an excess of positive or negative charge (Chase et al., 1962) and thereby may be responsible for the decrease in the activity of the enzyme. It had been suggested that the like charges existing in the neighbouring medium might affect the stability of a protein
by causing repulsion between the groups of the molecules (Kauzmann, 1954 and 1956). Thus in the presence of either net positive or negative sign of the charges over the surface, the protein molecules may be constantly under motion and thereby fold or unfold different groups regularly. In the case of an enzyme, this folding and unfolding of its molecular configuration may result in a structural alteration of the enzyme molecule which might either mask the catalytic sites or in some other way prevent the normal binding between the enzyme and its substrate (Chase et al., 1962). Thus the enzyme studies on invertase at different pH values indicated valuable information and it is likely that the study of other enzyme systems in the same way may contribute to the understanding of the riddle of the mechanism of enzyme action.

The activation of pepsinogen is found to be associated with the removal of a polypeptide from the enzyme protein and thereby suggesting that the polypeptide masks the active centres of the enzyme (Beyer et al., 1960). It is also possible that the removal of polypeptide from the enzyme molecule might have resulted in a proper configuration of the protein moiety of the enzyme and thereby might be responsible for the activity expressed by the active centres.

Recent studies in this laboratory contributed much of the information with regards to the activation and inactivation of the enzyme systems in relation to the charges on
the protein molecules. It was shown that the density of charges on the protein molecule directly governs the enzyme activities. The activities of succinic dehydrogenase, cathepsin, catalase and carbonic anhydrase are shown to increase with the increase in the density of positive charges. (Swami and Krishnamoorthy, 1964). Decrease in the density of negative charges is known to release the inhibitory effect on the expression of the activity of certain enzymes (Swami and Govindappa, 1964; Govindappa and Swami, 1965; 1966). Increase in the density of negative charges accelerated the protease activity (Indira and Swami, 1965). The inactivation and reactivation of the enzyme systems were correlated to the change in the charge pattern of the protein constituency (Indira and Swami, 1965; Swami and Govindappa, 1965). Infact ionisation and deionisation of the carboxyl groups are shown to play a vital role in the activation and inactivation of the enzyme systems such as dehydrogenases (Indira and Swami, 1965; Indira, 1966).

These studies pertaining to the activation and inactivation of the enzyme systems have been extended to the fatigue phenomenon of the muscle. It was shown that during the fatigue, the protein ionisation varies due to the denaturation (Kumudavalli, 1965). Consequently the enzyme systems were inactivated during the fatigue and this factor was correlated to the changes in the ionisation pattern of the proteins (Kumudavalli, 1965).
These investigations can be extended in this connection to find out the dependence of the enzyme activity on the charge pattern of the protein constituency in order to understand some aspects of the mechanism of activation and inactivation of the enzyme systems in living cells.

6. Effect of inorganic ions on the enzyme activation and inactivation

As the inorganic ions are known to be bound by the proteins and result in a change (Klotz, 1953) in the ionization pattern, which has a direct bearing on the enzyme activation, it will be interesting to study the role of the ions in the enzyme activities. Proteins are shown to combine with inorganic cations like Ca$^{++}$ and K$^{+}$ with the basic groups in the same manner as done by the hydrogen ion (Klotz, 1953). In general, it is assumed that the inorganic cations are bound by the carboxylate ions of proteins (Klotz, 1953) and thereby become responsible for the change in the charge on the protein molecule. A variety of biological responses are sensitive to the concentration of specific molecules, particularly ions (McLean and Hastings, 1934). If ions are bound by a protein, their electrostatic fields alone are known to affect the acid base character of the titratable side chains of the protein molecule (Klots, 1953). The titration curves carried over on wool proteins
were shown to be sensitive to the presence of cations leading to shifts along the pH axis (Steinhard and Zaiser, 1950). If an ion is bound to a protein, there will be additional effect on the pH of the medium because the bound ion changes the ionization pattern of the protein molecule. Thus binding of anions by a protein was shown to produce a shift in the isoionic point towards a higher pH (Klotz, 1953). Interactions of protein molecules with small ions lead to the formation of protein dimers or aggregates (Hughes, 1947).

When a metal combines with a protein molecule, it was shown that the net charge of the complex differs from that of the original charge (Klotz, 1954). This pattern of the charge may induce a change in the affinity of the enzyme for the substrate on purely electrostatic grounds. As a result of combination, groups involved in the catalytic activity may have their dissociation curves shifted markedly along the pH axis (Klotz, 1954). In fact, the pH-activity curve of the enzyme may be shifted so that the measurements of activity in a restricted pH range would be markedly influenced by the presence of metallic ions (Klotz, 1954).

Many hydrolytic enzymes appear to associate with a variety of salts which have an important function in the catalysis of these enzymes (Giese, 1960). On dialysis, it
was demonstrated that some of the hydrolytic enzymes became inactive or greatly reduced in their catalytic activity (Cantarow and Scheprutz, 1954), since it loses the sodium chloride. Hence it was shown that the chloride ion was very essential for the activation of these enzymes. Activation of pepsinogen to a pepsin form by the hydrochloric acid of gastric mucosa involves the participation of proton or a charge (Neurath et al., 1954). This activation of the enzyme was associated with the removal of a polypeptide. It was suggested that the polypeptide in the zymogen might be masking the active centres (Boyer et al., 1960), and thereby the combination between the enzyme and substrate was prevented. Thus hydrochloric acid eliminates a polypeptide from the enzyme molecules and thereby providing a proper immobilization pattern for the expression of catalytic activity. Enzymes which require free sulphydryl groups for their catalytic activity, must be reduced after purification for the expression of their activity (Fruton and Simmonds, 1960). For example, the protease papain, when extracted was inactive until it was reduced by the reducing agents such as glutathione, or hydrogen sulphide (Fruton and Simmonds, 1960), which convert the disulphide bonds of the enzyme into thiol groups.

The investigations of London et al. (1962) have revealed that simple anions like chloride, bromide or thiocyanate can
inhibit an enzyme, prostatic acid phosphatase, in a solution both competitively with regards to substrate and non-competitively. The non-competitive inhibition had been suggested to occur by an alteration of the charges on the protein molecule (London et al., 1962). It was clearly shown that the secondary specificity site of the enzyme molecule, which bears an array of charges over its surface was under the regulation of anions and protons (London et al., 1962). As the secondary specificity sites play a dynamic role in the configuration of the active centres, which are involved in the expression of the catalytic activity, the protons and anions can alter the enzyme activity through a change over the molecule (London et al., 1962). It was suggested (London et al., 1962) that a positively charged group and a hydrogen bonding type group were the likely pair which forms the substrate combining site on the enzyme molecule. The anions which are capable of reacting with the positive site would behave as competitive inhibitors of the enzyme (London et al., 1962). It was suggested by the same authors that there were many positive sites other than substrate combining sites, and these sites too were capable of reacting with the anions and the binding of anion in the case alter the charge prevailing on the enzyme molecule. It was also pointed out that the anions studied were also effective accelerators of the thermal denaturation process (London
et al., 1962). The ions are actually known to react with all enzyme species regardless of net charge but only molecules at the extreme left and right denature. Sodium ion, as it happens to be less reactive, produces slight denaturation (London et al., 1962). The doubly charged anions like sulphate exhibited maximum denaturation enhancement. But citrate produces maximum effect even at molar concentrations lower than the sulphate (London et al., 1958).

It had been pointed out that multiple charges on an anion may introduce a stabilising factor. Thus the inorganic ions seem to play vital role in the activation and inactivation of the enzymes. Hence this type of study can be extended in relation to the enzyme systems in order to understand the intricate mechanisms in the metabolic reactions.

7. Present study

In the present study, it is proposed to investigate the charge properties of different subcellular components and to correlate their charge pattern with metabolism as exemplified by the enzyme activities. There are certain enzymes which specifically lodge in subcellular components and thereby identify different specific subcellular components. These enzyme activities can safely be taken as the marker enzymes for the identification of different subcellular components in the tissue fractions. Hence in the present
study an attempt is made to study the distribution of these subcellular components using marker enzyme activities. The charge on the subcellular components will be traced in terms of marker enzymes.

As the enzyme activities are dependent on the charge properties of the subcellular components, an electrophoretic separation of different subcellular components had been achieved in order to study the effect of charges on enzyme activities.

There was shown to be change in the optimum pH of the same enzyme as exemplified by the isozyme study. Hence an attempt is made to study the behaviour of the enzymes as exemplified by their catalytic activity in different set of conditions pertaining to the charges on the protein molecules. In order to understand the interaction between the surrounding proteins and the expression of the enzyme activity, the enzyme activity is proposed to be estimated on the addition of different charged protein components to the medium.

Thus in the present study an attempt is made to draw the correlations between the enzyme activities and the charge density on the surrounding protein molecules.
MATERIAL AND METHODS
Procurement of the experimental material

The gastrocnemius muscles of frogs, *Rana hexadactyla*, were isolated from both the legs with least injury, after pithing the animal. The muscles were washed three to four times in amphibian Ringer (Savannah, 1956) and allowed to stay for 15 minutes in fresh Ringer to recover from shock effects. The muscles were taken out and gently pressed against the folds of a filter paper in order to remove the droplets of Ringer solution. One of the two muscles was subjected to a long axis voltage gradient of 10 volts D.C. per cm. for 20 minutes in nitrogen environment to provide anaerobic environment. The electric field was applied to the muscle with platinum electrodes, each one placed one centimeter apart from the centre of the muscle. Immediately after the exposure to the electric field, the muscle was sliced transversely into two halves and chilled to 0°C. The muscle halves which were in contact with anode and cathode poles of the electric field were referred as anode half (AH) and cathode half (CH) respectively.

Another muscle was kept in nitrogen environment for 20 minutes and referred as control. This muscle was also sliced into two halves at the centre transversely and the two muscle halves were referred as control\(_1\) (C\(_1\)) and Control\(_2\) (C\(_2\)). The control muscle halves were also chilled to 0°C immediately after the muscle was sliced.
In order to perform the experiments on the reversal of polarity of the applied voltage gradient at the middle of the experimental period, the muscle was arranged in the nitrogen medium with two electrodes placed at one cm. apart from the centre of the muscle. The voltage gradient of 10 volts D.C. per cm. was applied for 10 min. and after this period, the polarity of the electric field was reversed and the current was allowed to pass for another 10 min. Immediately after the disconnection of the power supply, the muscle was sliced transversely into two halves and they were called as Experimental$_1$ ($E_1$) and Experimental$_2$ ($E_2$) respectively. These muscle halves were chilled to $0^\circ$C. The control muscle was prepared as mentioned previously.

**Assay of dehydrogenases (Triphenyl tetrazolium chloride method)**

Control, AH and KH muscle halves were homogenized at $0^\circ$C with mortar and pestle using acid washed sand in 0.1M potassium phosphate buffer at pH 7.0 and 10% (W/V) homogenates were obtained. These homogenates were centrifuged at 2,500 rpm for 15 min. and the supernatants were separated. With these supernatants the activities of succinic, malic and glutamic dehydrogenases; xanthine and l-amino acid oxidases were estimated by triphenyl tetrazolium chloride method (Srikantan et al., 1955). The incubation mixture had 0.5 ml of 0.1M substrate (sodium succinate, sodium malate, sodium glutamate, xanthine and l-leucine for the estimations of succinic, malic
and glutamic dehydrogenases; xanthine and L-amino acid oxidases respectively), 1 ml 0.1M potassium phosphate buffer at pH 7.0, 0.5 ml triphenyl tetrazolium chloride (0.5% solution in distilled water) and 1 ml supernatants.

The optimum time required for the complete expression of the activity was determined for succinic and glutamic dehydrogenases by incubating the above mentioned incubation mixture for different periods of time at 37°C and arresting the reaction with the addition of 6 ml glacial acetic acid. The formazan formed as a reduction product of triphenyl tetrazolium chloride was extracted into 6 ml of toluene layer overnight at 0°C and optical density values at 495 mp were obtained from UVI Spectrophotometer (Hilger and Watts, England) using silica cuvettes of 10 mm path length. The same period of incubation had been taken as standard incubation period for the estimation of all the enzyme activities studied and the formazan formed in the reaction was extracted as stated in the previous case.

**Estimation of structural (buffer-insoluble) and soluble (TCA insoluble) proteins (BIURET METHOD).**

10% (W/V) homogenates of control, AH and KH muscle halves were prepared by grinding the tissues at 0°C with mortar and pestle using acid washed sand in potassium phosphate buffer of 0.1M at pH 7.0. The homogenates were centrifuged at 2,500 rpm
for 15 min and the supernatants were separated. To the supernatants equal quantities of 10% TCA was added to precipitate the proteins. The suspensions were allowed to stay for 15 min at cold to settle the proteins from the medium. The suspensions were centrifuged at 2,500 rpm for 15 min and the residues were separated. The proteins obtained in the first residue (buffer insoluble) and second residue (TCA insoluble) were estimated by Biuret method.

The residues were suspended in 1 ml of phosphate buffer of 0.1M strength at pH 7.0 separately and 4 ml Biuret reagent was added and stirred well. The suspensions were centrifuged and the supernatants were collected. The optical density of the colour developed was read at 545 nm in JVI Spectrophotometer (Hilger and Watts, England). The protein contents were represented as mg per gm wet weight of the muscle tissue.

Estimation of cytochrome C in the control and experimental muscles

The cytochrome C content was estimated by the method elaborated by Skinner, 1963.

The muscle tissue was accurately weighed and homogenised at 0°C in 10 ml of 0.7% sodium dithionate in a mortar using acid washed sand. The suspension was kept at room temperature for five hours and after which it was frozen to ensure the cell lysis and complete extraction of cytochrome C. After
thawing, the extract was centrifuged in a Semi Ultra refrigerated centrifuge (MSE, England) at 15,000 g for 30 min. The cytochrome C extracted will be in the reduced state in the presence of dithionate. The optical density of the reduced cytochrome C was determined at 550 nm in UNI spectrophotometer (Hilger and Watts, England). The reduced cytochrome C was oxidised by adding potassium ferricyanide and the optical density was determined at the same wave length. The cytochrome C content was calculated employing the extinction coefficient of horse heart cytochrome C at this wave length as given by Skinner.

**Estimation of mitochondrial protein content in the control and experimental muscles—Differential centrifugation technique**

Control, AH and XI muscles were collected from a dozen frogs and 50% (w/v) homogenates of them were prepared in 0.25 M sucrose solution at 0°C. The homogenates were centrifuged preliminarily at 2,500 rpm for 30 min in order to eliminate the cell debris and the nuclei. The supernatants were separated and they were centrifuged at 15,000 rpm for 20 min in Beckmann Ultracentrifuge (USA). The supernatants were discarded and they were resuspended in 10 ml of 0.25 M sucrose solution and they were centrifuged again at the same speed and for the same period. The supernatants were discarded.

The mitochondrial of pellets obtained on the differential centrifugation, were resuspended in 2 ml of 0.25 M sucrose
solution. To these mitochondrial suspensions, equal quantities of 10% TCA was added to precipitate the proteins. The suspensions were shaken well and allowed to stand for 10 min at room temperature and centrifuged at 2,500 rpm for 15 min. The residue in each case was suspended in 1 ml of 0.25% sucrose solution and to it 4 ml Biuret reagent was added, stirred well and allowed to stay at room temperature for the development of the colour. The suspensions were again centrifuged and the absorbancy of the clear supernatants were measured at 545 μm using Beckmann Junior Spectrophotometer. The protein content was calculated in each case from their absorbance units.

Study of the ionization pattern of bovine albumen and hemoglobin Spectrophotometric study

The ionization characteristics of a protein can be studied spectrophotometrically. The proteins get ionized depending on the H⁺ concentration of the medium. At the isoelectric point, they will be having equal charges and they tend to precipitate from the medium and consequently get eliminated from the solution on centrifugation. The supernatant will be devoid of the ionization characteristics of these proteins. Towards either side of isoelectric pH, proteins will be ionized either as cations or anions depending on the H⁺ concentration. The optical density values will be in correspondence with the ionisation of the protein. Hence this method can be employed to study the ionization pattern of the proteins in the solutions at different pH values.
In the present method, 0.2% (w/v) solutions of bovine albumen and hemoglobin were prepared in 0.25M sucrose solution. 0.1M acetate buffers at pH 5.0 and 5.4 and 0.1M phosphate buffers at pH 5.7, 6.0, 6.5, 7.0, 7.5 and 8.0 were prepared and their pH values were tested by Beckmann pH meter using KCl and glass electrodes.

To 2 ml of buffer solution, 2 ml of 0.2% bovine albumen was added and allowed to stay at room temperature for 10 min. In the same proportions the bovine albumen was added to all the buffer solutions indicated above and allowed to stay for 10 min. The solutions were centrifuged at 2,500 rpm for 30 min and the supernatants were separated. The optical density of the supernatants was determined at 260 μm and 280 μm in UVI Spectrophotometer (Hilger and Watts, England) employing silica cuvettes of 10 mm path length.

Isoionic solutions of CaCl₂, sodium citrate and EDTA with 0.03 ionic strength were prepared in 0.25M sucrose solution. To 2 ml of buffer 2 ml of 0.2% bovine albumen was added followed by 0.5 ml of CaCl₂, sodium citrate and EDTA separately. In the same proportions the mixtures were prepared with the buffer solutions of different pH values cited above. The solutions were allowed to stay for 15 min at room temperature and centrifuged at 2,500 rpm for 30 min. The optical density values of supernatants were determined at 260 μm and 280 μm respectively in UVI Spectrophotometer.
In the same way the optical density values were taken with normal solution of 0.2% hemoglobin and on the addition of CaCl₂, sodium citrate and EDTA at different pH values. These ionic solutions were added to the reference solutions also while taking the optical density readings of the samples.

**Study of the ionization pattern of the proteins in control, AH and KH muscles**

The control, AH and KH muscle halves were homogenized at 0°C using acid washed sand and 10% (w/v) homogenates were prepared in 0.25% sucrose solution. The homogenates were centrifuged at 2,500 rpm for 30 min and the supernatants were isolated.

Potassium acetate buffers of 0.1M with pH 5.0 and 5.4 and potassium phosphate buffers of 0.1M with pH 5.7, 6.0, 6.5, 7.0, 7.5 and 8.0 were prepared (Solowick and Kaplan, 1957). The pH values of the buffers were tested with Beckmann pH meter.

1 ml of the supernatants were added separately to 2 ml of different buffer media and allowed to stand for 10 min at room temperature. The suspensions were centrifuged at 2,500 rpm for 30 min to eliminate the precipitated protein. The supernatants were separated and their optical density values were determined at 260 and 280 μm respectively in UVI Spectrophotometer (Hilger and Watts, England) using silica cuvettes of 10 mm path length.
Solutions of CaCl₂, sodium citrate and EDTA of isotonic strength of 0.03 were prepared in 0.25M sucrose solutions. To 2 ml of buffer media taken separately as listed above, 1 ml of the homogenate supernatants of the control and experimental muscles were added separately followed by 0.5 ml of CaCl₂, sodium citrate and EDTA separately. The solutions were shaken well and centrifuged for 15 min at 2,500 rpm. The optical density values of the clear supernatants were determined as in the previous case.

Electrophoretic analysis of the sucrose soluble proteins in control, AH and KH muscles

10% (w/v) homogenates of control, AH and KH muscle tissues were prepared by grinding the tissue with mortar and pestle using acid washed sand in 0.25M sucrose solution at 0°C. The homogenates were centrifuged at 2,500 rpm for 30 min and the supernatants were separated.

Whatman NOI filter paper strips of 1" x 13" dimensions were trimmed and their central positions were marked transversely with pencil lines and the polarity was also indicated on the strips. The strips were soaked in the suitable buffer of 0.1M and mounted on the stage of the electrophoretic cell. The pockets of the electrophoretic apparatus were filled with the appropriate buffer and the apparatus was set right. A potential gradient of 100 volts D.C. was applied to the electrophoretic cell for 30 min to ensure equilibration so
that a thin and uniform film of buffer is maintained on the filter paper strips. The power supply was disconnected and 0.025 ml of the supernatants were spotted separately over the pencil lines of the strips. Then the potential gradient of same strength was applied up to 24 hours at cold. After 24 hours, the power supply was disconnected and the filter paper strips were removed from the stage of the electrophoretic cell. The paper strips were dried in air and stained in 0.1% (W/V) light green in 1.5 acetic acid (Rideout & Prichard, 1955). The excess of the stain was washed with 1% acetic acid solution and the strips were dried in hot-air-oven at 110°C. These strips were analysed by Dr. Lange's photovolt densitometer, fitted with tungsten lamp. The absorbance units were plotted against the length of the band on the strip and the peaks resulted thereby were supposed to indicate the individual soluble proteins, present in the tissue homogenate.

The electrophoretic mobility of each soluble protein was calculated as

\[
\text{distance of the band in cm from the origin} \times \frac{1}{100} = \text{cm/volt/hr}
\]

Titration curves of the proteins in the control, AH and KH muscle homogenates

10% (W/V) homogenates of control, AH and KH muscles were prepared at 0°C in 0.25 M sucrose solution as indicated
previously. 10 ml of the control homogenate was taken in a beaker and its pH value was determined by Beckmann pH meter using KCl and glass electrodes. To the homogenate 0.05 ml of 0.1N HCl was added with "agla micrometer syringe" (England) and the drop in the pH was determined. The addition of the acid in 0.05 ml instalments and recording down of the drop in the pH was continued till a constant pH value was obtained. Another 10 ml of the control homogenate was taken in a separate beaker, its pH value was determined and 0.05 ml of 0.1N NaOH was added with the microsyringe and increase in the pH was noted. The addition of alkali in 0.05 ml instalments and the recording of the raise in pH was continued till a fairly constant pH value was obtained in this side also.

The same titrations with acid and alkali were conducted with AH and KH muscle homogenates also. In a similar way the titrations of sucrose solution (0.25%) were conducted both in the acid and alkali sides of the homogenate pH.

Graphs were plotted with ml of acid or alkali added against the change in pH value. The buffering capacities of different ionizable groups in the proteins were determined by area weight method.

**Area-weight method**

The method facilitates an idea about the intensity of different groups ionized on the titrations with acid and
alkali in terms of relative weight of the paper.

The titration curves of the homogenates and the sucrose solution were plotted on an uniformly thin paper, both towards the acidic and alkaline sides.

The different ionizable groups depending on their pKa values were sorted out in accordance with their range of pH as follows:

<table>
<thead>
<tr>
<th>S.N.</th>
<th>pH range</th>
<th>Name of the group</th>
<th>Groups ionized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.7 to 5.6</td>
<td>a</td>
<td>α Carboxyl and phosphate groups.</td>
</tr>
<tr>
<td>2</td>
<td>5.6 to 6.0</td>
<td>a¹</td>
<td>Distal carboxyl groups.</td>
</tr>
<tr>
<td>3</td>
<td>6.0 to 7.0</td>
<td>b</td>
<td>Phosphate and imidazolyl groups.</td>
</tr>
<tr>
<td>4</td>
<td>7.0 to 10.0</td>
<td>c</td>
<td>-SH, amino and phenolic groups.</td>
</tr>
<tr>
<td>5</td>
<td>10.0 above</td>
<td>d</td>
<td>Omega amino, phenolic, amino and guanidine groups.</td>
</tr>
</tbody>
</table>

The titration curves drawn on the uniformly thin paper were divided into different ranges as listed above. The paper was cut into the bits with suitable range of pH and weighed. The buffering capacity of different groups was represented in terms of mg. paper.
Effect of isionic CaCl₂, sodium citrate and EDTA on the
titration curves of control, AH, and EH muscle proteins

Isionic solutions of CaCl₂ (0.01M) sodium citrate
(0.00125M) and EDTA (0.0033M) were prepared with the ionic
strength of 0.03. To 10 ml of the homogenates of control
and experimental muscle halves, 5 ml of CaCl₂, sodium
citrate and EDTA were added separately and titrations
towards acidic and alkaline sides were conducted as de-
scribed above. The sucrose solution (0.25M), was also
titrated against acid and alkali as a control on the addi-
tion of CaCl₂, sodium citrate and EDTA separately. Graphs
were plotted as shown previously and the buffering capacities
of different ionizable groups were determined by area- weight
method as described previously.

Isolation of positive and negative types of proteins from
the muscle tissue

Gastrocnemius muscles were isolated from a freshly
pithed frog, Rana hexadactyla. 20% (W/V) homogenate of the
muscles was prepared in 0.25M sucrose solution at 0°C. The
homogenate was centrifuged at 2,500 rpm for 30 min and the
supernatant was separated.

0.1% potassium acetate buffer at pH 5.0 and potassium
phosphate buffer at pH 8.0 were prepared (Colowick and
Kaplan, 1957). The pH values were tested by Beckmann pH meter.
5 ml of the supernatant was added to 1 ml of acetate buffer at pH 5.0 and 1 ml of phosphate buffer at pH 8.0 separately. The contents were shaken well and allowed to stay for 1 hour at cold. The proteins precipitated were separated on centrifugation at 2,500 rpm for 30 min. The protein fractions precipitated were suspended in 5 ml of 0.25% sucrose solution at pH 6.8. The protein fraction precipitated from buffer at pH 5.0 will be negatively charged on suspension in the sucrose medium at pH 6.8 and hence provides a negative type of protein as far as the net charge is concerned. The protein fraction separated from the buffer at pH 8.0, when suspended in sucrose medium at pH 6.8, gets positively charged and thereby provides a positive type of protein, as far as the net charge is concerned.

1 ml of positive and negative proteins were taken separately and the amount of protein present in 1 ml of the suspension was determined by the Biuret method (elaborately dealt previously). The protein concentration in both the cases was made same with proper dilutions.

**Succinic dehydrogenase activity at different pH values**

(a) Triphenyl tetrasolium chloride method (Srikantan et al., 1955).

Control, AH and KH muscle tissues were homogenised at 0°C with a mortar and pestle using acid washed sand and
prepared 10% (w/v) homogenates in 0.25% sucrose. The homogenates were centrifuged at 2,500 rpm for 30 min and the supernatants were separated.

0.1M potassium acetate buffers at pH 5.0, 5.4 and potassium phosphate buffers at pH 5.7, 6.0, 6.5, 7.0, 7.5 and 8.0 were prepared (Colowick and Kaplan, 1957) and the pH values of the buffers were tested with Beckman pH meter.

The incubation mixture for the enzyme activity consisted of 0.5 ml of 0.1M sodium succinate, 0.5 ml triphenyl tetrazolium chloride (0.5% solution in distilled water), 1 ml of the suitable buffer solution and 1 ml of the supernatant. The mixture was incubated up to 18 hours as it was previously found to be an optimum period of incubation and the reaction was arrested by the addition of 6 ml glacial acetic acid. The formazan formed as a consequence of the reduction of the dye, triphenyl tetrazolium chloride, was extracted into a layer of 6 ml toluene overnight in cold. The optical density values were taken at 495 μm UVI Spectrophotometer (Hilger and Watts, England) using silica cuvettes of 10 mm path length.

(b) Ferricyanide reduction method—Spectrophotometric assay

The assay depends on the reduction of ferricyanide to ferrocyanide which in turn will be denoted by a drop in the optical density.
Control, AH and KH muscle tissues were homogenized at 0°C and 10% (W/V) homogenates were prepared in 0.25M sucrose solution. The homogenates were centrifuged at 2,500 rpm for 30 min and the supernatants were separated.

Acetate and phosphate buffers were prepared as described previously.

The incubation mixture consisted of 0.3 ml of 1M sodium succinate, 0.3 ml 0.1M potassium ferricyanide, 0.3 ml of 0.1M KCN, 1.1 ml suitable buffer and 1 ml of the supernatant of the tissue homogenates. KCN was employed here to arrest the activity of cytochrome system in the tissue extract. The reagents were added in such a way to give 0.1M sodium succinate, 0.01M KCN and potassium ferricyanide in a total of 3 ml incubation mixture.

The time required for the attainment of the equilibrium of the enzyme activity was determined for enzyme extracts of control, AH and KH muscle halves separately.

The incubation mixture was kept in the light path of the Spectrophotometer and the drop in the optical density as a consequence of the reduction of ferricyanide was determined at the intervals like 20 seconds, 40 seconds, and 60 seconds, at 400 mp.

The study of the reduction of ferricyanide was conducted at different pH values by the enzyme extracts of control, AH and KH muscles up to 60 seconds.
(c) SDH activity in the dialysed extracts of control, AH and KH muscle tissues

Control, AH and KH muscle halves were homogenized separately at 0°C and 10% (w/v) homogenates were obtained in 0.25M sucrose solution. The homogenates were centrifuged at 2,500 rpm for 30 min and the supernatants were separated. The supernatants were dialyzed in cold against phosphate buffer at pH 6.3 for 3 hours by changing the buffer at the end of each hour. The dialysis was conducted with a cellophane bag, in which the supernatant was taken.

SDH activity was studied by ferricyanide reduction method and triphenyl tetrazolium chloride method as described earlier using the dialyzed enzyme extracts. In this case also the activity was studied at different pH media.

(d) Effect of isionic CaCl₂, sodium citrate and EDTA on SDH activity at different pH media

Isoionic solutions of CaCl₂ (0.01M) sodium citrate (0.00125M) and EDTA (0.0038M) were prepared with ionic strength of 0.03.

10% (w/v) homogenates of control, AH and KH muscle halves were prepared at 0°C in 0.25M sucrose solution. The supernatants were obtained on centrifugation as described above. The enzyme activity was studied by triphenyl
tetrazolium chloride method (Srikantan et al., 1955) as described previously with the addition of 0.5 ml of CaCl₂, sodium citrate and EDTA separately to the incubation mixture. The pH of the buffers on the addition of the inorganic ions was adjusted to their respective values before the addition of the homogenate. The effect of these ions on SDH activity at different pH media was studied.

(e) Effect of positive and negative proteins of the muscle tissue on the SDH activity

Positive and negative types of proteins were isolated as described earlier from the muscle tissue.

The SDH activity was studied at different pH values by Triphenyl tetrazolium chloride method (Srikantan et al., 1955) by the addition of 0.5 ml of positive and negative proteins separately to the incubation mixture. The effect of proteins on the SDH activity was studied in the normal enzyme extract of the gastrocnemius muscle of frog. The enzyme extract was prepared as given previously.