PHOTOSYNTHETIC REGENERATION OF ATP USING NATIVE AND IMMOBILIZED BACTERIAL CHROMATOPHORES

by

Ho Seung Yang

B.S., Seoul National University (Korea)
(1969)

M.S., University of Minnesota
(1971)

SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF
PHILOSOPHY
at the
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June, 1976

Signature of Author ..... Signature redacted ....
Department of Nutrition and Food Science
May 7, 1976

Certified by ..... Signature redacted .....
Thesis Supervisor

Accepted by ..... Signature redacted ....
Chairman, Departmental Committee on Graduate Studies

PHOTOSYNTHETIC REGENERATION OF ATP USING NATIVE AND IMMOBILIZED BACTERIAL CHROMATOPHORES

by

Ho Seung Yang

Submitted to the Department of Nutrition and Food Science
on May 7, 1976
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy.

A B S T R A C T

Development of cell-free enzyme-catalyzed systems for synthesis of useful products must include a method for regeneration of ATP. One approach to ATP regeneration is to utilize light as an energy source with chromatophores (photosynthetic apparatus from bacteria) to catalyze the photophosphorylation.

Continuous photophosphorylation by Rhodospirillum rubrum chromatophores was carried out in an ultrafiltration cell reactor and the stability of chromatophores during use was studied. Increased inactivation of the chromatophores was shown to occur in the early phase of continuous operation, in particular at high ADP concentrations. Inhibition by some factor(s) produced during the photophosphorylation process, but not by the product (ATP) itself, was hypothesized to be responsible for the inactivation. The stability of chromatophores was also affected by light intensity, temperature, and other substrates in the reactant solution.

A coupled system of yeast adenylate kinase and the chromatophores regenerated ATP from AMP using light energy. The photophosphorylation activity of chromatophores, both specific activity and stability, was the limiting factor in the performance of the coupled reaction.

The immobilization of R. rubrum chromatophores was successfully accomplished by entrapping them in polyacrylamide gel. Their photophosphorylating activity was increased to 40 % of native chromatophores by optimizing the conditions in the preparation. The temperature and pH optima for immobilized chromatophores were similar to the native photo-

synthetic apparatus. Kinetic parameters showed that the rate of photophosphorylation in polyacrylamide gel particles was diffusion controlled. Light penetration of the gel particles was not a limiting parameter under the conditions employed. Immobilization considerably increased the stability of the chromatophores toward denaturation.

The immobilized chromatophores were utilized for the photosynthetic regeneration of ATP in the continuous reactors- packed column reactor and continuous-flow stirred tank reactor. The inactivation of chromatophores due to the inhibition during continuous photophosphorylation was overcome by maintaining a high rate of flow of the reactant solution in the continuous reactor with immobilized chromatophores. The productivity of the chromatophore reactor was comparable to the catalytic activity of immobilized chromatophores.

Co-immobilization of adenylate kinase and chromatophores was demonstrated for a regeneration of ATP from AMP.

Thesis Supervisor : Michael C. Archer

Title : Associate Professor of
Biological and Food Chemistry

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Professor Michael Archer, whose good-natured guidance and encouragement allowed me to successfully carry out my Ph.D. research. He made himself always available for helpful discussion. I appreciate the advice he gave me, not only concerning academic problems but also in more personal matters such as how to prepare for the arrival of our first baby. My gratitude is extended to my Ph.D. thesis committee, Professors C.L. Cooney (chairman), N. Catsimpoolas, A.J. Sinskey, and S.R. Tannenbaum, for their valuable criticism and guidance.

Many thanks also to the faculty and students in the Biochemical Engineering Seminar and in my research groups. My association with these groups at M.I.T. has been very enjoyable even under the heavy academic pressure. Special thanks are extended to my labmates, Gary Pace, Lynn Reed, Ron Dagani, and Jim Okun for their companionship and help.

I would like to convey my affection to my parents back home who made me what I am today. Lastly, but not least, my sincere gratitude goes to my loving wife, Sook, for her admirable endurance and continuous encouragement which has been the basis of my drive in everyday work.

This work was supported by a grant from the National Science Foundation (G.I. 34284).

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Many biosynthetic reactions require ATP as a driving force. Cell-free enzyme syntheses of useful products such as antibiotics, Gramicidin S and Bacitracin (Hamilton, 1974) are examples of reactions requiring a continuous supply of ATP. Energy is supplied by the hydrolysis of ATP to ADP or AMP, depending on the particular biosynthetic reaction.

ATP is a very expensive chemical ($1,000/lb; Langer, 1974); hence its regeneration from ADP and AMP is desirable. There are several alternative methods of ATP regeneration: a) Chemical addition of phosphate to ADP or AMP, b) Mitochondrial oxidative phosphorylation, c) Microbial conversion in vivo, d) Photosynthetic phosphorylation using whole cells, e) In vitro enzyme catalysis, and f) Photosynthetic phosphorylation using isolated bacterial chromatophores. The last two approaches are suitable for providing a continuous supply of ATP during biosynthetic reactions. Both approaches utilize a favorable aqueous enviroment, are specific, do not produce by-products, and produce very good yield of ATP.

Enzymatic regeneration using two kinases, in which AMP is converted to ADP by adenylate kinase followed by conversion to ATP with a high energy phosphate donor by acetate kinase, has been studied (Langer, 1974). The success of the method in practical use depends on the cost of phosphate donor, the ease of enzyme preparation, and the stability of enzyme during use.

The photosynthetic regeneration of ATP from ADP and inorganic phosphate using the bacterial chromatophores (particulate membrane fragments from photosynthetic bacteria)

has some potential advantages over the single enzyme catalyzed method. First, the chromatophores are readily isolated in relatively homogeneous form, and second, inexpensive inorganic phosphate (Pi) is utilized as phosphate donor concomitant with a light energy source ($h\nu$) as the driving force for regeneration instead of using a high energy phosphate donor. The feasibility of using the photosynthetic apparatus from Rhodospirillum rubrum in ATP regeneration has been demonstrated and the conditions for the photophosphorylation have been optimized (Pace, 1975).

Since the catalysts are in continuous use during the ATP regeneration, their stability is an important consideration. The performance of the chromatophores is dependent on their stability during reaction. Immobilization of chromatophores may increase their stability, as is often observed for many immobilized enzymes. Immobilization also allows various modes of continuous operation.

An enzymatic reaction can be coupled to photosynthesis for the regeneration of ATP from AMP. Adenylate kinase converts AMP to ADP, which is then photophosphorylated by chromatophores to ATP;

$$\text{AMP} + \text{ATP} \underset{\text{adenylate kinase}}{\rightleftharpoons} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{Pi} \xrightarrow[h\nu]{\text{chromatophores}} 2\ \text{ATP}$$

$$\text{net,}\quad \text{AMP} + 2\ \text{Pi} \longrightarrow \text{ATP}\ .$$

There is no available literature data on the stability of chromatophores. The factors affecting the stability of R. rubrum chromatophores in the ultrafiltration reactor during continuous photophosphorylation are described in the

first part of this thesis. The feasibility of the coupled system of adenylate kinase and chromatophores for photosynthetic regeneration of ATP from AMP is explored. Finally the immobilization of chromatophores with high photophosphorylating activity and their use in ATP Regeneration are demonstrated.

2. PERTINENT LITERATURE SURVEY

2.1. Photosynthetic Phosphorylation by Bacterial Chromatophores.

2.1.1. General.

Photosynthesis is the process in which light energy is captured and converted into chemical energy by cells. The capacity to carry out photosynthesis is found in a wide variety of organisms. In eucaryotic cells the photosynthetic system is localized in membranous organelles called chloroplasts. The molecular components of the photosystems of photosynthetic bacteria are located in the cytoplasmic membrane. Mild disruption of the cells yields small fragments of membrane which spontaneously reseal to form small particles called chromatophores. Illumination of either isolated chloroplasts or chromatophores in the presence of ADP and inorganic phosphate causes formation of ATP in a process involving electron transport (Lehninger, 1971). Unlike mitochondrial oxidative phosphorylation, there is no consumption of either oxygen or substrate in this process.

A cell free fraction isolated from the photosynthetic bacterium Rhodospirillum rubrum has been found to be photosynthetically active and capable of photophosphorylation (Frenkel, 1956).Basic aspects of photophosphorylation by this system have been extensively studied as reviewed by Baltscheffsky and Baltscheffsky (1971) and Gest(1972). Pace(1975) studied factors affecting the initial rate of photophosphorylation by the R. rubrum chromatophores and

demonstrated the feasibility of photosynthetic regeneration of ATP using the chromatophores.

The organism, R. rubrum, is a gram negative, spiral shaped, dark red colored bacterium. Young cultures are motile (Reed et al., 1957). R. rubrum is a facultative phototroph. Under lighted anaerobic conditions it generates energy from light and uses organic compounds as electron donors. The photosynthetic apparatus is not formed when cells grow in the absence of light and under aerobic conditions (Oeleze and Drews, 1972).

R. rubrum chromatophores are spherical particles with diameters ranging from about 50 to 100 nm (Lascelles, 1962). They are arranged in the cytoplasmic membrane. The main components of chromatophores are protein (46 %) and lipid (32 %). The Bacteriochlorophyll content of chromatophores is variable but the protein content is relatively constant.

2.1.2. Mechanism of photosynthetic energy conversion by R. rubrum.

A summary of current views of photosynthetic energy conversion in the purple bacteria is shown in Figure 1.

Pigments in chromatophores are responsible for light absorption. Variuos pigments allow a rather broad range of wave lengths to be absorbed. R. rubrum contains bacteriochlorophyll a and carotenoid pigments. The absorbance maximum of bacteriochlorophylls is in the range of 700-900 nm (Cusanovich and Kamen, 1968). The carotenoid pigments harvest light at shorter wave lenghts, 400-600 nm (Kondrat'-eva, 1965),and the harvest light is chanelled to the bacteriochlorophyll reaction center, whose absorption

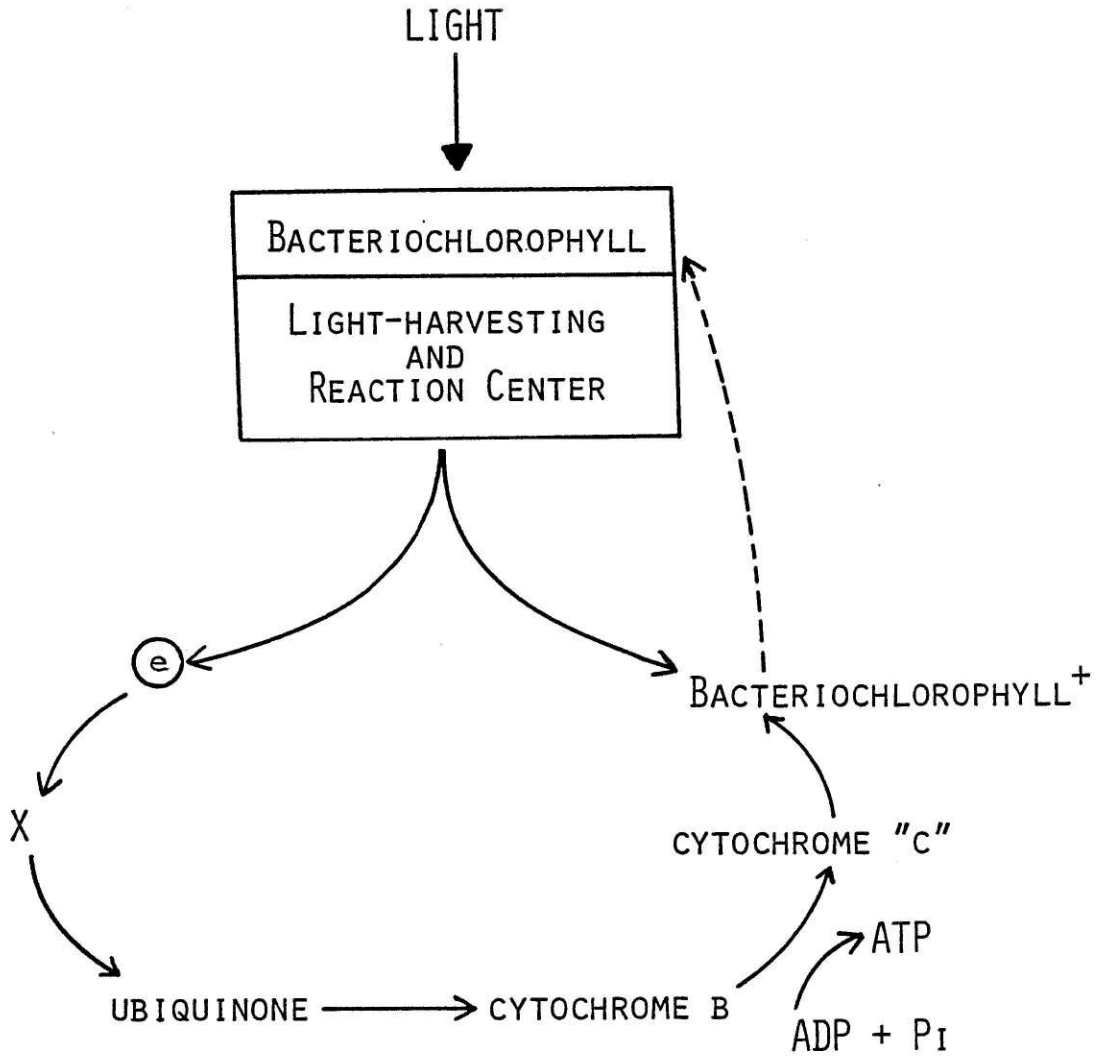


FIGURE 1.

General outline of a scheme for light-dependent cyclic electron transfer and phosphorylation in purple bacteria. X is the unknown acceptor of an electron(e) from bacteriochlorophylls. Adapted from Gest(1972).

maximum is at 890 nm.

The photoexcited reaction center (P890) reacts initially with an unknown acceptor (X) to produce an oxidized P890. A series of cyclic oxidation-reduction reactions then ensues with the oxidized P890 finally being reduced back to its ground state form by cytochrome c.

Phosphorylation coupled to the cyclic flow of electrons occurs to convert the absorbed light energy into chemical energy. As a consequence ADP is phosphorylated to ATP in the presence of inorganic phosphate.

2.1.3. Photophosphorylation kinetics and stability of chromatophores.

Typical values for the initial rate of photophosphorylation by bacterial chromatophores reported in the literature e.g. Baltscheffsky (1961) are in the range of 500 umoles of ATP produced/hour/mg of bacteriochlorophylls. Rates of photophosphorylation, however, are dependent on the reaction conditions as follows:

Temperature

Most studies on photophosphorylation were performed at room temperature (25°). Pace(1975) observed that the optimum temperature for chromatophore activity is 45°. At 25°, chromatophores had about 30 % of their maximum activity, However, the stability of chromatophores at the high temperature was low so that reactions in Pace's study were routinely performed at 30°.

pH

A pH profile with maximum activity at 8.0 has been reported (Yamashita et al., 1967). Pace(1975) observed a pH optimum at 7.8 but the value was affected by light intensity and substrate concentrations.

Light

A plot of the reciprocal of the initial rate of photophosphorylation versus the reciprocal of the light intensity was linear (Baltscheffsky and Baltscheffsky, 1971). Km for light was 1.5×10^3 ergs $cm^{-2}sec^{-1}$ (Pace, 1975). The reaction rate stayed constant when the light intensity was above a certain level i. e. light was saturating (Keister and Yike, 1967).

Red-ox potential

Horio and Kamen (1962) found that the optimum activity of R. rubrum chromatophores occured at a redox potential of 0.0 ± 0.10 volts corresponding to an ascorbic acid concentration of 0.013 M under anaerobic condition. Succinate, ascorbate, lactate, NADH, and phanazinemethosulfate (PMS) were effective reductants.

The ascorbic acid concentration for the maximal rate of photophosphorylation was found to be 0.065 M under aerobic conditions and 0.017 M under anaerobic conditions. These values were equivalent to redox potentials of 0.061 and 0.089 volts, respectively, determined by the direct measurement of redox potentials (Pace, 1975).

The role of PMS in the stimulation of photophosphorylation is thought to be first that it acts as a modifier

of redox potential (Horio and Kamen, 1962) and secondly that it acts as an redox dye and functions as an component of the cyclic electron transport system (Geller, 1969).

Substrate and product concentrations

Reported Km values for ADP are 0.9×10^{-5} M (Horio et al., 1966) and 1.5×10^{-5} M (Nishimura, 1962), and for inorganic phosphate 1.1×10^{-4} M (Nishimura, 1962). Pace (1975) reported that high ADP concentrations, greater than 0.02 M, resulted in inhibition and that high Pi concentrations in the range of 0.02 - 0.2 M did not give any inhibition.

Magnesium ion concentration

Metal ion plays a role in photophosphorylation as in the other transphosphorylation reactions. A magnesium requirement for photophosphorylation by chromatophores has been reported (Frenkel, 1954 and 1956). Over the range of concentration of 0.001-0.1 M, the effect of magnesium was not sensitive to changes in substrate concentrations and chromatophore concentration (Pace, 1975). A magnesium concentration of 0.01 M is sufficient for the maximal rate of photophosphorylation by chromatophores.

Buffer composition

The composition of pH buffers has been found to affect the ATP production in photophosphorylation by Rhodopseudomonas spheroides chromatophores (Culbert-Runquist et al., 1973). Pace (1975) observed that Tris buffer gave a higher initial rate than other buffer systems tested, HEPES, glycylglycine, and phosphate only. Good and Izawa (1972) reviewed the

hydrogen buffers used in the biological reactions. Several buffers were discussed in relation to photosynthetic reaction of chloroplasts. As an example, Tris was a weak uncoupler of photophosphorylation and not recommended in chloroplast study.

Inhibition on equilibrium conversion

The study of kinetics of ATP regeneration via photophosphorylation by bacterial chromatophores concerns the entire time course of the reaction as well as initial rates. Pace(1975) observed a low equilibrium conversion of ADP to ATP, a yield of about 50 %, when a closed reaction system (sealed tubes) was employed. The low conversion phenomenon was eliminated by bubling nitrogen through reaction mixture. Yet there was no difference in initial rates of the reactions of both closed and nitrogen bubbled systems. The inhibition was also relieved by addition of a low level of fumarate. Addition of 0.1 or 1 mM fumarate resulted in a 89 % yield of ATP at 1.8 mM ADP. However, the fumarate addition was effective at a low level of substrate and a short reaction time. The rection containing fumarate stopped when ATP production reached 2 mM or after about 2 hours of reaction in the closed system.

The nature of the inhibition in the closed system was explored (Pace, 1975). The mechanisms of effects of nitrogen bubbling and fumarate addition on photophosphorylation by chromatophores seem to be different. The presence of volatile inhibitors hypothesized could not be demonstrated. A slight change in pressure of the reaction system resulted in low conversions. The pressure effect was reversed by fumarate

addition. The mechanism of effect of fumarate was postulated to be due to its action on succinate dehydrogenase of chromatophores. Small amounts of hydrogen which produced during photophosphorylation feedback inhibited ATP formation. Gas phase contact was hypothesized to stimulate photophosphorylation by sweeping hydrogen out of the system, and fumarate effectively removed hydrogen by reacting with it (this reaction is known to be catalyzed by succinate dehydrogenase).

STABILITY of chromatophores has received little attention. A chromatophore preparation which contained 5 % DMSO showed no loss of activity at -196° (liquid nitrogen) for a long period of time (Pace, 1975). By extrapolation of the deactivation rate curve of chromatophores, the half-life at 30° was about 8 days when they were suspended in water (Pace, 1975).

No information is available on stability of chromatophores under reaction conditions.

2.2. Properties of Adenylate Kinase.

2.2.1. General properties.

Adenylate kinase (E.C. 2.7.4.3) catalyzes the transfer of one phosphate group from ATP to AMP,

$$AMP + ATP \underset{<}{\xrightarrow{Mg^{++}}} 2ADP$$

Adenylate kinases are found in the various tissues where energy turnover is high. Adenylate kinases have been isolated from various sources(see Appendix IV) and their properties have been studied. The role of adenylate kinase in the cell is believed to be to facilate the storage and the use of energy of adenine nucleotides (Noda, 1973).

A fair amount of data is available in the literature on myokinase -adenylate kinase extracted from muscle particularly rabbit muscle. Myokinase is available commercially (Sigma, St.Louis, MO) but it is expensive. Adenylate kinase was isolated in large quantity from Baker's yeast (Saccharomyces cerevisiae) by the Chemical Engineering group at M.I.T. (Quintero-ramirez, 1974) for their use in the enzymatic regeneration of ATP. Eighty units of adenylate kinase was isolated from a gram of dry yeast and the enzyme was purified to a specific activity of 600 units per mg protein.

The properties of the yeast adenylate kinase have been studied only by a group in the University of Kansas in addition to the Chemical Engineering group at M.I.T. The present review will be concentrated mainly on the properties of yeast enzyme based on the studies of those two groups.

2.2.2. Mechanism of adenylate kinase reaction.

Adenylate kinase is a phosphoryl transfer enzyme in which one substrate is bound to a divalent metal ion. There are two independent sites for binding substrates - one for MgATP or MgADP and the other site for AMP or ADP (Noda, 1973).

Kinetic studies employing Baker's yeast adenylate kinase (Su and Russell, 1968 and Khoo and Russell, 1970) indicate that the mechanism for phosphate transfer is, according to the nomenclature of Cleland, random bi bi. This means that a ternary comples involving the enzyme, ATP, and AMP is formed in one direction and a ternary complex involving the enzyme and two molecules is formed in the other direction. However, there is no required order in which the substrates bind to the enzyme or products dissociate from the enzyme. A hypothesis for the mechanism of the enzyme is that the two substrate in either direction of the adenylate kinase reaction are a chelated and unchelated nucleotide and that the chelated and unchelated nucleotides have their own binding sites. This last observation has also been noted from muscle adenylate kinase (Noda, 1962).

Su and Russell (1968) have also, through the use of isotopic exchange studies, found that the rate limiting step for the reaction is the interconversion of the central ternary complex and not the binding of substrates or the dissociation of products.

Recently Nemet(1976) has proposed a complete kinetic model of yeast adenylate kinase reaction and is currently studying the kinetics of the enzyme reaction.

2.2.3. Factors affecting the reaction kinetics and stability.

pH

Chiu et al. (1967) found that in phosphate buffer the pH value at which the rate of the reverse reaction (2ADP -> ATP + AMP) is maximum is 7.7. For the forward reaction the optimum pH was 7.2 and, in contrast to myokinase (Noda, 1958), the pH-activity curve exhibited a sharp maximum. Su(1966) examined the effect of pH on the activity of the yeast enzyme in Tris Buffer and found an identical pH optimum in the forward direction and a difference of 0.2 units for the reverse reaction. Equilibrium calculations for the distribution of nucleotides among possible species in solution (Langer, 1974) showed that the concentrations of different nucleotides are identical in both Tris and phosphate buffers for the forward reaction, but differ for the reverse reaction. These calculations also suggest that ADPH(protonated ADP) might act as an inhibitor for the reverse reaction, since in phosphate buffer the level of ADPH calculated from equilibrium considerations was higher than that in Tris buffer at all pH's investigated.

At pH's below the observed optima the lower initial rates are due to the inhibitory effectd of the partially deprotonated nucleotides coupled with changes in the ionization state of the enzyme, while at pH's above the optima solely by changes on the enzyme (Alberty and Bock, 1953).

Magnesium ion

Magnesium ions (or other divalent cations) are needed

for activity of kinases in general (Morrison and Hyde, 1972). For yeast adenylate kinase the order of reactivity of ions was Mg> Ca > Mn > Ba (Su and Russell, 1967). The maximum enzyme activity was reached when the molar ratio of Mg to total nucleotides was about two.

The magnesium ions form Mg-Nucleotide complex for adenylate kinase reactions. Langer(1974) gave a complete list of dissociation sonstant for important species of the adenine nucleotide-metal complexes. Thus if the initial concentrations of adenine nucleotides, pH, and Mg are specified, the concentration of each species can be calculated.

Buffers

Slight depression in the initial rate with 33 mM phosphate buffer was observed as compared to the initial rate with the same concentration of Tris buffer (Su, 1966). Phosphate binds Mg whereas Mg binding by Tris is not significant.

Substrate concentrations: Michaelis constants

The substrate specificity of yeast adenylate kinase has been reported by Su and Russell (1967). The Michaelis constants determined assuming a random bi bi model are as follows; MgATP, 5.4×10^{-5} M; AMP, 5.8×10^{-5} M; MgADP, 2.7×10^{-4} M; (conditions, pH 7.0, 33 mM phosphate buffer, 3.3 mM $MgCl_2$ and 25°).

The Michaelis constants determined by Khoo and Russell (1970) without substrate inhibition are; MgATP, 9.4×10^{-5}M, and AMP, 9.6×10^{-5}M (conditions, pH 8.0, 25°, and 0.0165 M Tris).

Substrate and product inhibition.

Su(1966) reported substrate inhibition in the forward direction both by ATP and AMP at concentrations higher than about 0.5 mM. AMP appears to be a noncompetitive inhibitor of ATP, while ATP a competitive inhibitor of AMP at higher concentrations of the second substrate.

Khoo(1971) observed no inhibition by $MgATP^{2-}$ at concentration up to 3.3 mM. At high concentrations, AMP^{2-} was a competitive inhibitor for $MgATP^{2-}$ at pH's lower than 7, but it did not inhibit the reaction at higher pH's. A possible explanation for this behavior is that at pH's lower than 7, a considerable fraction of the total AMP exist as $AMPH^{-}$, a species which binds to the enzyme but it does not form a reactive enzyme-substrate complex.

Stability of yeast adenylate kinase

The absence of free sulfuhydryl group in the yeast adenylate kinase (Chiu et al., 1967) suggests that the addition of reducing agents would not affect the stability of the yeast enzyme. Myokinase required the presence of reducing agents for its stability (Noda, 1967).

The stability of yeast adenylate kinase is currently under study by the Chemical Engineering group at M.I.T. Quintero-ramirez(1974) reported the half-life of the yeast enzyme at 25° as more than 10 days. Nemet(1976)(personal communication) showed that the stability of the yeast enzyme is dependent on the protein concentration in the enzyme solution. Addition of bovine serum albumin enhanced the stability of yeast adenylate kinase during storage.

No published data is available for stability of the yeast adenylate kinase during reaction.

2.3. IMMOBILIZATION OF ENZYMES

2.3.1. General

During last several years an ever-increasing amount of work has been reported on different aspects of the immobilization of enzymes and other macromolecules (Melrose, 1973 and Zaborsky, 1973). Most of the reports are concerned with enzymes. Immobilization techniques employed for enzymes, however, has been applied to other biological materials such as whole cells (Franks, 1972), antigens and antibodies (Silman and Katchalski, 1966), protease inhibitors (Feinstein, 1970), hormons (Selinger and Civen, 1971), nucleic acids (Wagner et al., 1971), mitochondria (Arkles and Bringar, 1975), and even small molecules such as amino acids (Saxinger et al., 1971) and vitamins(Oleson et al., 1971). Thus, "immobilization of enzymes" is not limited to enzymes but also implies other biological materials in the following review.

Immobilized enzymes can be defined as enzymes bound to or within a synthetic matrix. There are two broad classes of immobilization (zaborsky, 1973), - chemical and physical immobilizations. Chemical methods include the formation of a covalent bond between enzyme and a functionalized, water-insoluble polymer. Physical methods include any that involve non-covalent interactions or entrapment within the matrix of a polymer.

Immobilized enzymes may exhibit selectively altered properties. This is probably due to alteration of functional groups and of being changed to different structure and reactivity of the enzymes during immobilization. Changes in pH-activity behavior, Km and specificity for substrate, and stability were reported for immobilized systems compared

to native ones.Chemical methods of immobilization are more likely to produce these effects than physical methods. The effects of changes in enzymes as well as in the micro-enviroments on the reaction will be discussed in detail related to gel entrapment later.

Immobilization gives considerable operational advantages in the application of enzymes (or other biological materials) for industrial purposes (Lilly and Dunnill, 1971 and Hamilton, 1974). It allows a reuse of enzymes and thus various modes of operation, either batch or continuous, a great variey of engineering design for continuous process, and efficiency in multistep reactions. It also often increased the stability of enzymes, which is one of the most important factors for continuous use of enzymes.

Now our scope is narrowed to the gel entrapment, a physical method, which is employed for immobilization of chromatophores in this thesis. Enzymes (or other biological materials) are entrapped within the interstitial space of cross-linked water insoluble polymers. Entrapped enzymes cannot permeat out of gel matrix but small substrate and product molecules can transfer across and within the polymer net work. Since the first successful entrapment of the enzymes, trypsin and α-chymotrypsin, by Bernfeld and Wan (1963), numerous enzymes (Zaborsky, 1973), antigens and antibodies (Goodfriend et al., 1969 and Carrel and barandun, 1971). DNA(Cavalieri and Carroll, 1970) and whole cells (Mosbach and Larsson, 1970 and Franks, 1972) have been immobilized by the entrapment in gels.

Polyacylamide gel is most commonly used but silica gel, silicon rubber, and starch were also employed

Polyacylamide produces a chemically stable and physically uniform gel. The gel has a uniform porosity and is charged neutrally and hydrophilic. It is resistant to hydrolysis in the pH range between 1 and 10 (Zaborsky, 1973). Thus, these factors provide suitable enviroments for high activity of enzymes in general. Exposure to mild conditions during immobilization provides high retention of activity of the native enzymes. In addition, immobilization by polyacrylamide gel entrapment is inexpensive. Polyacrylamide gel can be prepared in a highly transparent form which is a primary requirement for immobilizing materials of the photosynthetic apparatus such as chromatophores.

2.3.2 Methods of polyacrylamide gel entrapment.

The entrapment methods involve formation of a highly cross-linked network of polyacrylamide in the presence of the system to be immobilized (enzymes, organelles, cells, etc.). The polyacrylamide gel system is produced by the reaction of acrylamide and N,N'-methylenebisacrylamide. The preparation procedures are similar to those for polyacrylamide gel for chromatography (Hjerten and Mosbach, 1962 and Hjerten, 1968) and for electrophoresis (Chrambach and Rodbard, 1971).

The effectiveness of polymerization is enhanced by adding a catalyst. Hicks and Updike (1966) examined the nature of polymerization catalysis. Catalytic systems employed for polymerization during immobilization of enzymes are persulfate and tetramethyleneethylenediamine (TEMED) (Mosbach and Mattiasson, 1970, Nilsson et al., 1972, and Franks, 1972), riboflavin and light (Hicks and Updike, 1966),

β-dimethylaminopropionitrile (DMAPN) and persulfate (Mosbach, 1970, Brown et al., 1968, and Mori et al., 1972), and γ-ray radiation (Maeda et al., 1973).

Convertional gel immobilization consists of the preparation of a gel block containing entrapped biological materials followed by mechanical fragmentation (Mosbach, 1970, Franks, 1972, and Maeda et al., 1973). This procedure results in a wide range of size and shape of particles.

Bead polymerization of acrylamide for immobilization of enzymes has been described (Nilsson et al., 1972). The procedure involves dispersion of an aqueous solution of enzyme and acrylamide in a hydrophobic phase (toluene or chloroform) with the aid of surface active agents, and then initiation of polymerization. The spherical beads obtained containing entrapped enzyme show excellent mechanical stability and could overcome the diffusional limitation shown by large particles. The average bead diameters were able to be controlled by changing polymerization conditions. Well-defined spherical beads of specific size allow high flow rates in a packed column.

2.3.3. Properties of gel entrapped enzymes.

Activity

Activity retention of entrapped enzymes depends on preparations. The activity retentions are in the range of 2 to 60 % by polyacrylamide gel entrapment (Zaborsky, 1973). The afactors affecting the retention of activity were gel concentration, percent cross-linking (Degani and Miron, 1970, Franks, 1972, and Mori et al., 1973), and polymer substances employed (Zaborsky, 1973). Relatively high

concentrations of monomer and cross-linking agents showed higher activity and a more rigid gel. Above a certain concentration, however, the denaturing effect of acrylamide (Degani and Miron, 1970) and limitations in diffusion of substrate become problems.

pH-activity

No shift in the pH-activity curves was reported for several immobilized enzymes in polyacrylamide gel (Bernfeld and Wan, 1963 and Bernfeld and Bieber, 1969). This behavior is expected due to the electrically neutral character of the polyacrylamide gel. An unexplained shift toward the acid side was also observed (Bernfeld and Wan, 1963 and Mori et al., 1973).

Diffusinal effect : Km for substrates

The Michaelis constant (Km) of enzymes can be altered as a result of immobilization. Changes in the enzyme conformation and the microenviroments affect the Km for the enzymes (Kobayashi and Laidler, 1973).

For enzymes entrapped in polyacrylamide gel, which is neutral in charge, the main cause of changes in Km can be explained in terms of substrate diffusion (Hinberg et al., 1974 and Kobayashi and Laidler, 1973). A theoretical expression of the diffusional effect on the kinetics of immobilized enzymes has been developed by Lasch (1973), Hornby et al. (1968), and Moo-young and Kobayashi (1972). In homogeneous solution even the fastest of enzyme-catalyzed reactions appears to be little influenced by diffusional effects, but with immobilized enzymes, especially when the enzyme is fairly active and the membrane thickness or

particle diameter is not too small, diffusion effects are quite significant.

The kinetic characteristics of immobilized enzymes were analyzed in terms of an effectiveness factor (Moo-young and Kobayashi, 1972, Kobayashi and Laidler, 1972, and Hamilton et al., 1974). The effectiveness factor is defined as the ratio of the total rate when there are diffusional effects within the support to the rate when diffusion within the support is infinitely rapid. This concept has been found to be useful for the description of the importance of diffusional effects in immobilized enzymes and for an analysis of the experimental results in terms of kinetic parameters and diffusivity of the substrate within a support such as polyacrylamide gel.

Various results have been reported for Km values as compared to native enzymes. Higher Km, lower Km, or unchanged Km have been observed (Zaborsky, 1973). No good explanations could be found for the observations.

Stability

Storage stability is one of the main advantages of immobilization. Many studies of storage stability of enzymes immobilized in the polyacrylamide gel have been reported. Enhanced thermal stability by gel immobilization was observed for several enzymes (Zaborsky, 1973). Dimished stability also has been reported (Guilbault and Hrabankova, 1970). It is, however, not clear whether the diminished stability was solely a thermal effect.

The amount of data on stability under operational conditions, which is important for continuous use of the

immobilized enzymes, is still limited. When polyacrylamide gel entrapped lactate dehydrogenases in a column was perfused continuously with lactate at 37°, no loss of activity was noticed for at least 10 hours and after about 20 hours activity decreased to 50 % (Hicks and Updike, 1966). The native enzyme used in the preparation was, however, very unstable in solution when maintained at 37° losing 90 % of its activity in only 2 hours.

2.3.4. Immobilized enzyme reactors.

One of the advantages of the immobilization of enzymes is that it allows easy retention or recovery of the enzymes and hence various modes of continuous enzyme reactors are possible. Lilly and Dunnill (1971 and 1972) and their colleagues ahve made major contributions in this area.

Various types of reactors have been used with either native or immobilized enzymes. Most frequently employed for immobilized enzymes, or whole cells (Franks, 1972 and Yamamoto et al., 1974),are the packed-bed column reactors.

The performance of an immobilized-enzyme column reactor is expressed as follows: $PS_o = Km\ln(1-P) + kE\beta/Q$ --(Eq.1), where P is the fractional conversion of substrate to product, S_o is the inlet substrate concentra ion, Km is the apparent Michaelis constant for substrate, k is a rate constant for the enzymic reaction, E is the total amount of enzyme in column, β is a void coefficient of the column, and Q is the flow rate. $kE\beta$ (C) is a measure of reaction capacity of a column reactor. At a constant C, conversion of substrate to product depends on the flow rate.

The relationship between fractional conversion and flow rate of the column reactor was studied theoretically and experimentally by Lilly et al. (1966) with ficin covalently bound to CM-cellulose. The apparent Michaelis constants and the reaction capacity were determined for any flow rate of substrate through the column by plotting P against ln(1-P).

Continuous-flow stirred tank reactors(CSTR) have been often employed as enzyme reactors either in batch or continuous operation mode. Kinetic analysis of the CSTR with immobilized enzymes has been given by Lilly and Sharp (1968). The following equation (Eq. 2), relating the fraction of substrate converted to product in such a reactor with Km, C, and Q, was derived in a fashon analagous to that for Eq.1, $PS_o = -(P/1-P)Km + kE\beta/Q$ --- (Eq. 2). The validity of this equation was examined by O'Neill et al., (1971) with amyloglucosidase bound to DEAE-cellulose and it was found to be a suitable model for the reactor system.

Theoretical and experimental comparisons of the packed -bed column reactor and the CSTR have been made by O'Neill et al. (1971) and Lilly and Sharp (1968). Under most conditions the column reactor is more efficient on the basis of product output per unit of enzyme than the CSTR. The CSTR is, however, more efficient at low levels of substrate conversion and at high substrate concentrations. The CSTR becomes more favorable when substrate inhibition is present. On the other hand, when product inhibition occurs, the CSTR is less efficient than the column reactor.

Other factors affecting the choice of enzyme reactors

(beside reaction kinetics mentioned above) have been discussed by Lilly and Dunnill (1972). The CSTR is normally the cheapest because of its relatively simplicity and it is extreamly flexible in terms of operation. One of the most important factors in the choice of reactor is the operational stability of immobilized enzymes in the reactor. Reuse of immobilized enzymes, which is one of the main reasons for immobilization, will depend on the stability. There are many reports of increased stability of enzymes on immobilization. Unfortunately the amount of data on stability under operational conditions is, however, very limited.

2.4. Coupled Enzymatic Reactions

2.4.1. General : Examples of coupled enzyme system producing ATP.

The conversion of AMP to ATP in the presence of phosphocreatine by a partially purified rabbit muscle preparation was reported (Morrison and Doherty, 1961). This reaction (AMP + 2 Phosphocreatine $\longrightarrow$ ATP + 2 Creatine) also occured by combined action of highly purified adenylate kinase and creatine kinase (Doherty and Morrison, 1963). Hence the orginal observation was due to contamination of creatine kinase with adenylate kinase and a small amount of ATP in the preparation. The overall reactions were,

$$\text{AMP} + \text{ATP} \xrightarrow{\text{Adenylate kinase}} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{Phosphocreatine} \xrightarrow{\text{Creatine kinase}} 2\text{ATP} + 2\ \text{Creatine}$$

$$\text{AMP} + 2\ \text{Phosphocreatine} \longrightarrow 2\ \text{ATP} + 2\ \text{Creatine}$$

In the time curve for the release of creatine from phospho creatine, a lag period which was obtained by a extrapolation of the linear part of the curve with the base line, was observed. This suggests that ADP is formed during the reaction and that the velocity increases until such time as creatine kinase is saturated with substrate; the reaction rate then becomes linear and is function of the concentration of creatine kinase.

Regeneration of ATP from AMP using a coupled enzymatic reaction was studied (Langer, 1974 and Hamilton, 1974). Thermodynamics of the coupled enzyme system of adenylate kinase and acetate kinase has been investigated by Langer (1974).

$$\text{AMP} + \text{ATP} \xrightarrow{\text{Adenylate kinase}} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{Acetylphosphate} \xrightarrow{\text{Acetate kinase}} 2\text{ATP} + 2\text{Creatine}$$

$$\text{AMP} + 2\ \text{Acetylphosphate} \longrightarrow \text{ATP} + 2\ \text{Acetate}$$

At equilibrium ATP yields are not strongly dependent on the value of the adenylate kinase equilibrium constant when the stoichiometric amount of AMP and acetate phosphate(BP), BP/AMP = 2, are initially added. He demonstrated the complete conversion of AMP to ATP. Other phosphotransferase systems related to the ATP regeneration were also reviewed.

The conversion of AMP to ATP by a crude extract of R. rubrum with light in the presence of inorganic phosphate was observed (Frenkel, 1956). The conversion from AMP to ATP, however, did not occur when a partially purified phosphorylating fraction was isolated from the crude extract. The observation is explained by the coupled reactions of adenylate kinase and chromatophores in the cell extract (see Figure 2).

2.4.2. Kinetics of coupled enzymatic reaction.

In the coupled enzymatic reactions, one enzyme uses the products of the other enzyme reactions as its substrate. Metabolic pathways of intermediary metabolism in living systems, where a series of enzymes (multienzyme) catalyze a set of consecutive chemical reactions, is an example of a series of coupled reactions. (Westley, 1969). When more than one equation is used to represent the observed changes, more than one kinetic expression is needed to follow the changing composition of

of all the reaction components. Descriptions of the kinetics of the consecutive chemical reactions (non-enzymatic) can be found in Levenspiel (1972).

The study of coupled enzyme systems must be dealt with both the transient and steady-state regions of the reaction. The analytical solutions for the pre-steady-state phase of a coupled enzymatic reaction were presented by Kuchel and Roberts (1974). A solution of differential equation describing the coupled reactions was fitted to the experimental data. Rate equations for consecutive irreversible reactions such as one encountered in coupled enzyme assays have been solved (McClure, 1969). The rate equations were derived assuming that the rate of the first reaction is zero-order and the second one is first-order. The steady-state product concentration was determined by the ratio of rate constants of the two enzyme reactions. The time required to reach the steady-state was inversly propotional to the rate constant of the second reaction.

Esterby(1973) presented general expressions to describe the transient state in a multienzyme sequence in which the initial enzyme is rate limiting. The initial enzyme did not contribute to the transient time but determined the steady -state velocity. Each enzyme has a transient time given by the ratio of its Michaelis constant to its maximum velocity. The transient time (or lag phase) for a complete sequence is a simple sum of the individual transient time.

One of the commonest kinetic complexities to be encountered in coupled enzyme systems is the cyclic form (Westley, 1969). Reactions of adenylate kinase coupled to acetate kinase or chromatophores (Figure 2) for regeneration of ATP

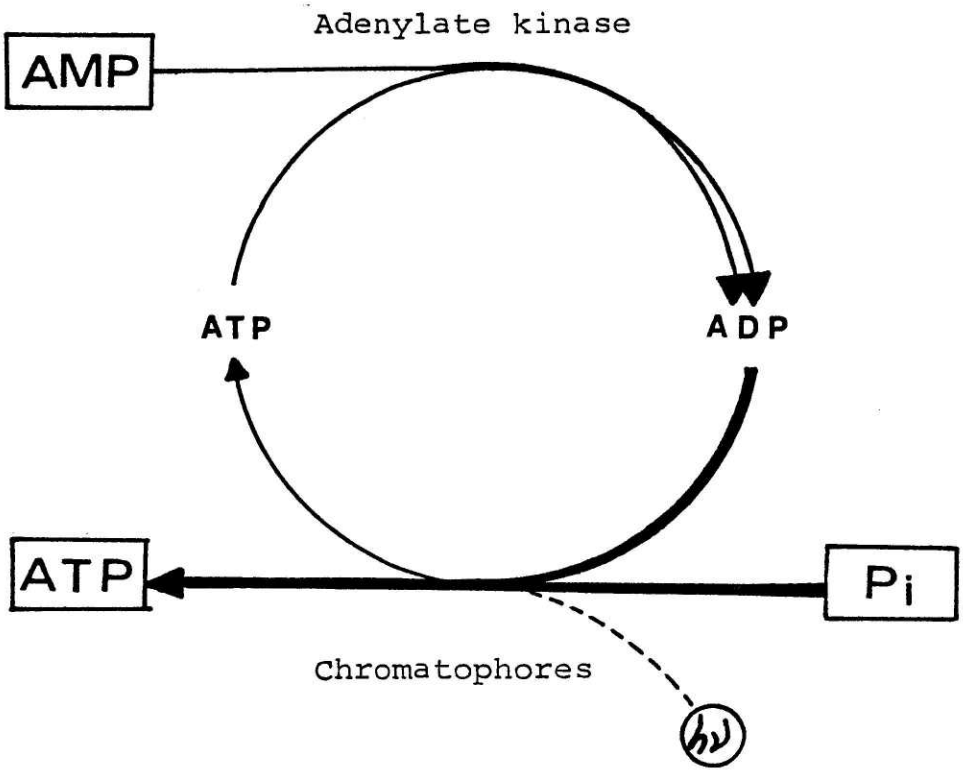


FIGURE 2.
Coupled reactions of adenylate kinase and chromatophores in enzymatic and photosynthetic regeneration of ATP from AMP.

from AMP are such examples. The product of second reaction is a substrate of the first reaction. Kopp and Miech (1972) derived mathematical models for the simplest type of non -linear enzymatic cycling system.

Immobilization of coupled enzymes in a single matrix allow the enzyme system to mimic the living state, where the multienzyme system is bound to a solid phase such as membrane. The proximity of enzymes in an immobilized system increases efficiency over the enzymes free in solution (Mosbach, 1972) since the product of the first reaction can be transformed by the second reaction before it has time to diffuse away from the reaction center. Goldman and Katchalski (1971) presented a theoretical analysis of the kinetics of two-enzyme membrane carrying out two consecutive reactions. The absolute values of rate constants of the two reactions and their ratios determined the behavior of the two-enzyme system. Mattiasson and Mosbach (1971) observed a decrease in the lag phase during the formation of NADPH by a matrix-bound three-enzyme system. The decrease was assumed to be a result of the build-up of an increased concentration of the intermediates in the microenviroment of the enzyme complex.

3. MATERIALS AND METHODS

3.1. Preparation of Chromatophores and Adenylate Kinase.

3.1.1. Chromatophores from Rhodospirillum rubrum.

Cell growth

R. rubrum (ATCC 11170) was obtained from the American Type Culture Collection. The seed culture was maintained by storing at 3° in agar slants. The medium of Woody and Lindstrom (1955) was used for growing cells. Cells were grown at 30° in light under anaerobic condition in a 14 l stirred fermentor for 4-5 days. Typical yields of cells were 1 gram dry weight per liter.

Isolation of chromatophores

Several disruption methods- growing with sand, French press, sonication, Manton-Gaulin homogenizer, blade homogenizer, Potter homogenizer, and Rotary cell (Braun) homogenizer, were attempted to find a satisfactory cell disruption method. The Braun homogenizer, 3 min at 5-10°, gave good yields and specific activity of chromatophores and, hence, was routinely employed for cell disruption. The procedures used in the preparation are outlined in Figure 3.

Storage

Chromatophores suspended in 0.1 M Tricine buffer, pH 7.8, with 5 % dimethylsulfoxide (DMSO), frozen, and stored in liquid nitrogen (-196°) maintained full activity for at least 3 months. Samples of the chromatophore suspension were frozen and kept in 2 ml plastic vials.

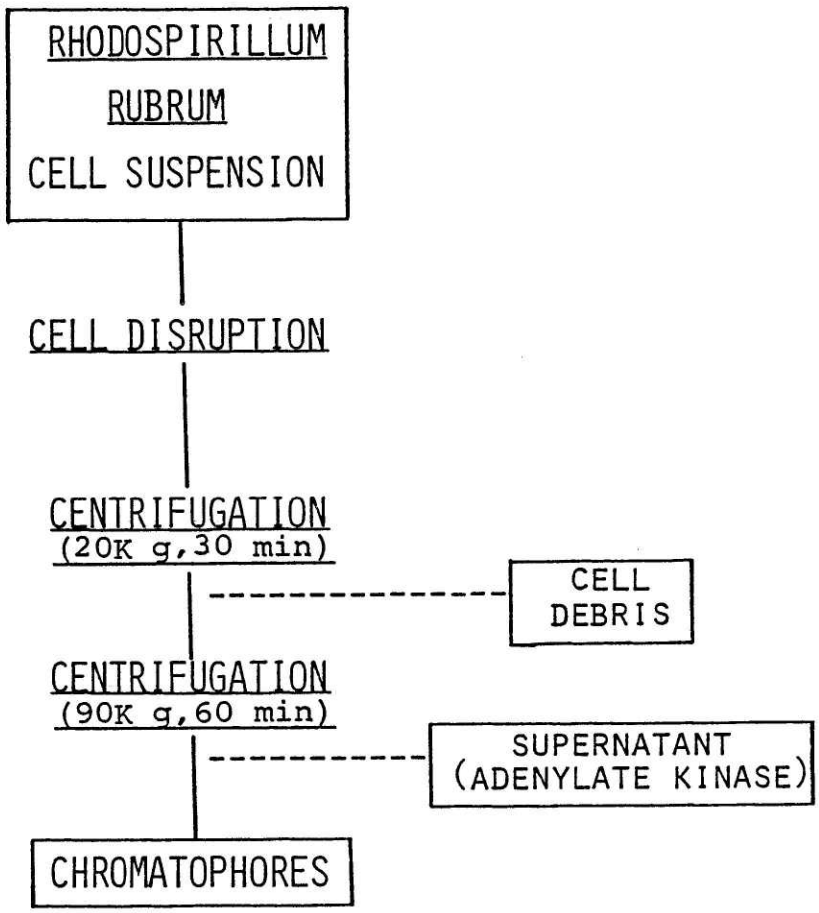


FIGURE 3.

Generalized outline for the preparation of the chromatophores (and adenylate kinase) from Rhodospirillum rubrum.

3.1.2. Adenylate kinase from Baker's yeast.

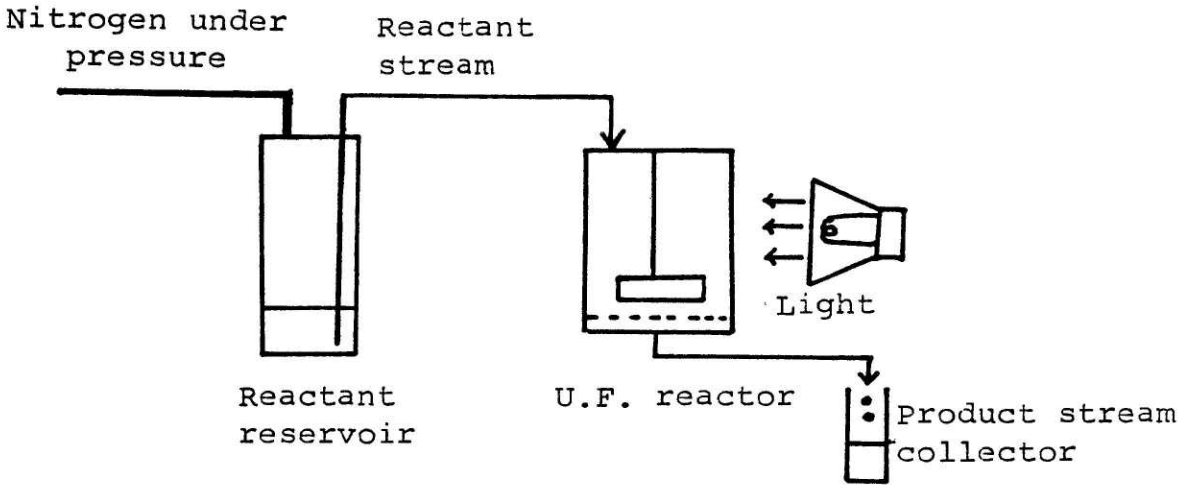
Adenylate kinase from Baker's yeast was prepared by the Chemical Engineering group at M.I.T. (Quintero-Ramirez, 1974). The steps included for the partial purification of the enzyme were acetone fractionation, heat treatment, ammonium sulfate fractionation, and gel filtration. The prepared enzyme was stored at 3° in 0.2 % bovine serum albumin (BSA) buffer at pH 7.4. The specific activity was claimed to be approximately 300 units/mg protein before adding BSA. The enzyme preparation was free of protease and ATPase activities. Dilution of adenylate kinase was made with buffered BSA solution to retard denaturation.

3.2. Ultrafiltration Cell Reactor for Continuous ATP Regenration.

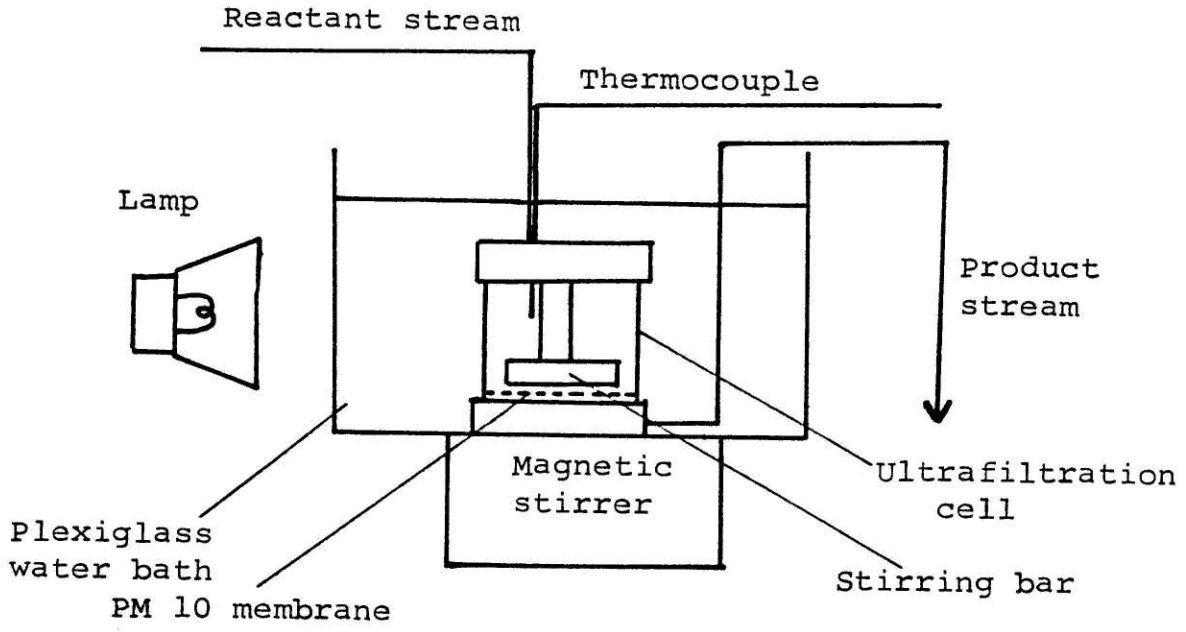
An ultrafiltration cell with a PM 10 membrane (Amicon Model 12, Lexington, MA) was employed as the reactor for continuous ATP regeneration with native chromatophores. The set-up of the reactor is shown in Figure 4. The diameter of the unit was 25 mm. The volume of the reactant solution in the ultrafiltration cell (U.F.) reactor was kept at 12.5 ml unless specified.

The u.F. cell was immersed in a water bath (transparent Plexiglass) with a constant temperature control. The temperature inside the U.F. cell was monitored with a thermocouple. Illumination was via photoflood lamps located outside the water bath.

The reactant solution was stored in a reservoir pressurized with nitrogen gas. This also maintained the reactants in an anaerobic condition. The solution in the



Scheme of Continuous U.F. Reactor



Details of U.F. Reactor

FIGURE 4.

Continuous ultrafiltration reactor for ATP regeneration.

U.F. reactor was stirred at a constant speed (approximately 500 r.p.m.) using a magnetic stirrer.

The U.F. reactor system was operated in a cold room so that the reactant (inlet) and product (outlet) streams were matained at 3 to 5°. The flow rate of the product stream was a function of the pressure of the reactant reservior, the concentration of chromatophores in the reactor, temperature, the age of the membrane. By controlling these variables, the desired flow rate was obtained. The average residence time (Tr) was calculated as Tr = V/Q where V is the reactor volume and Q is the flow rate.

Continuous operation was begun by adding the chromatophore solution to the reactor containing reactant solution previously drawn from the reservior and then finally opening the inlet valve. No loss of chromatophores, measured by the concentration of bacteriochlorophyll and protein inside the U.F. reactor, was observed throughout the operation.

The product stream was collected using an automatic fraction collector. The product solutions were assayed for ATP concentration.

3.3. Immobilization of Chromatophores.

Chromatophores were immobilized by entrapment in polyacrylamide gel. Acrylamide, NN'-methylene-Bis-acrylamide (BIS), and ammonium persulfate was purchased from Bio-Rad Laboratories (Richmond, CA). Tetramethyleneethylenediamine (TEMED)was obtained from Eastman Kodak(Rochester, NY).

3.3.1. Procedures for immobilization of chromatophores.

The following procedure was employed in general, although various conditions were altered in subsequent

experiments. To 2 ml of 0.2 M Tricine buffer solution (pH 7.8) containing 14.2 % acrylamide monomer and 0.83 % BIS, 0.2 ml of 5 % ammonium persulfate solution and 0.7 ml of 0.2 M Tricine buffer were added and the solution was well mixed with a magnetic stirrer. After addition of 1 ml of chromatophore solution (protein content 20-30 mg/ml) with stirring, 0.025 ml of TEMED was added. The mixture (4 ml) polymerized in 30 seconds at room temperature. Then the completely polymerized gel block in a beaker was put in ice bath for cooling.

The crushed gel was suspended in 20 ml of 0.2 M Tricine buffer and homogenized in Waring blender for 5 seconds. The gel particles were collected by centrifugation and washed with 20 ml of Tricine buffer and recentrifuged. Finally the gel particles containing entrapped chromatophores were suspended in a total volume of 8 ml with buffer.

In this procedure, the final total monomer concentration (T) was 7.5 % and cross-linking (C) was 12.5 %. Concentration of chromatophores in gel was 5-7.5 mg/ml of gel. The particle size was in the range of 100 to 500 um by microscopic observation.

3.3.2. Determination of diffusion coefficient.

The diffusion coefficient of ADP in polyacrylamide gel was measured by placing a tightly fitting cylinder of gel (0.5 cm) at the bottom of an open tube (1 cm diameter x 7 cm length) and placing 3 ml ADP solution (diffusant) above the gel in the tube. The whole tube was then placed in stirred buffer solution from which aliquots withdrawn at regular intervals and analyzed for ADP concentration.

It was checked that there was no leakage of diffusant, and that a planar diffusion front was evident,by visually following diffusion of a orange dye; the linearity of diffusion with time confirmed this. The diffusion coefficient, D, was then calculated from the formula,

$$\text{diffusion rate} = \frac{AD}{XV} \left([S_o]-[S] \right)$$

where A is the cross-sectional area, X the length of the gel, V the volume of buffer solution, and $[S_o]$ and $[S]$ the concentrations of diffusant. Care was taken to correct for volume changes when the gel was placed in buffer solution.

3.4. Immobilized Chromatophore Reactors.

a. Batch reactor.

A glass tube with flat bottom, 10 cm long and 1.5 cm diameter, was immersed in a constant temperature water bath. The liquid volume was usually 8 ml. The reactant solution was stirred with a small magnetic stirring bar.

b. Continuous reactors.

Two types of reactor, a continuous-flow stirred tank reactor (CSTR) and a packed-bed column reactor, were employed for the continuous regeneration of ATP by immobilized chromatophores. The physical set-up of the reactors and their operation are shown schematically in Figure 5. A continuous flow of the reactant solution into the reactors was maintained by a peristaltic pump (The Zero-Max Co., Minneapolis, MN).

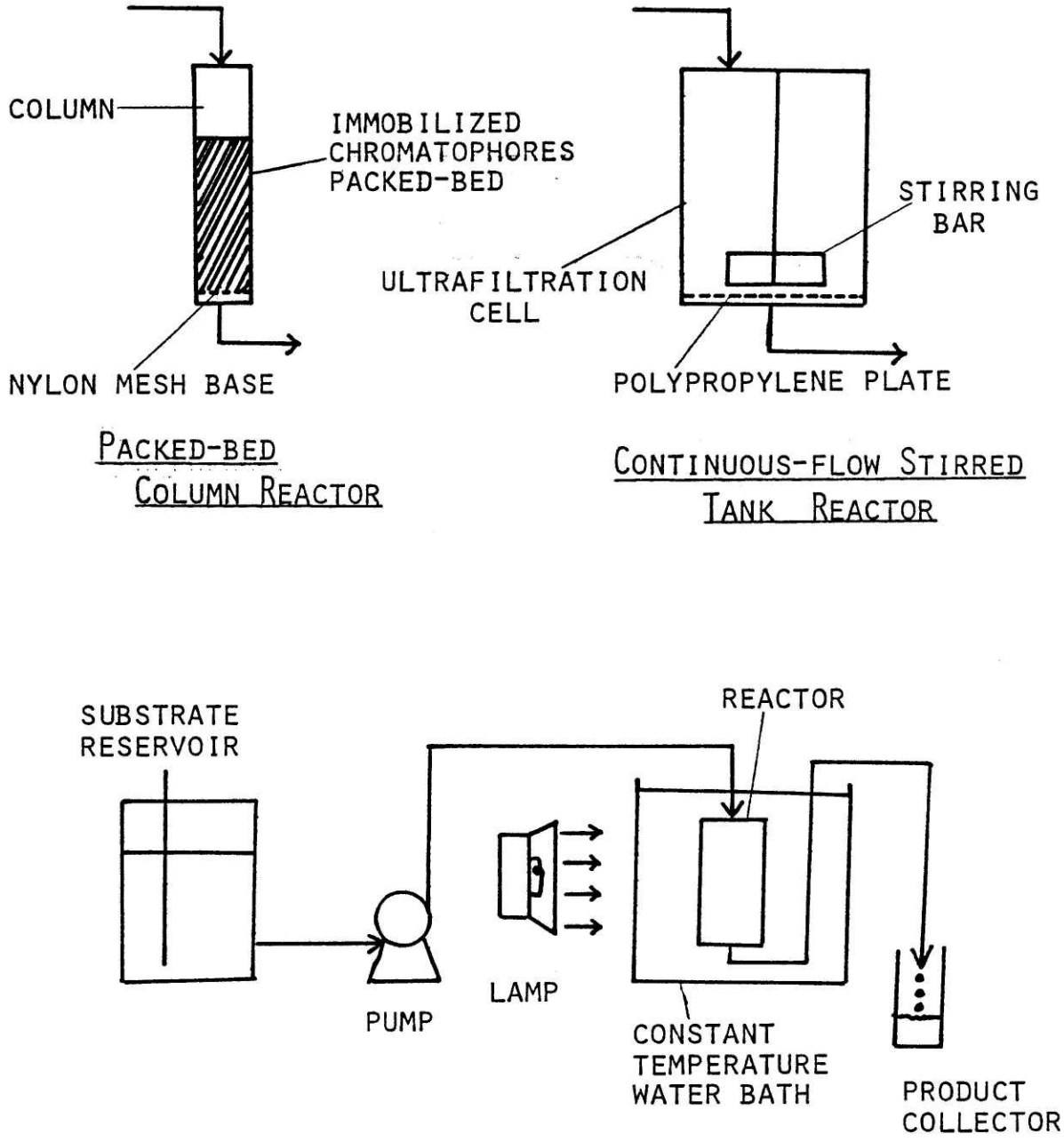


FIGURE 5.

Continuous reactors for immobilized chromatophores and their operation scheme.

CSTR : An ultrafiltration cell (Amicon, Lexington, MA) without the membrane was employed for the CSTR experiments. The immobilized chromatophore particles were retained in the reactor by a polypropylene plate (pore size, 10-30 u). The procedures of the reactor operation were similar to the described in section 3.2. for the U.F. reactor with native chromatophores. The flow was driven by the pump rather than the nitrogen pressure. Stirring at approximately 500 r.p.m. kept the immobilized particles in suspension. The liquid volume of the reactor was 12.5 ml.

Packed-bed column reactor : Beds of immobilized chromatophore particles were made up in a chromatographic column (Pharmacia Fine Chemicals, Upsala, Sweden) - a transparent acrylic plastic tube, 15 cm long and 0.9 cm internal diameter. The bottom of the column was fitted with a nylon net (mesh size, 10 u) which kept the bed of immobilized chromatophore particles in the reactor. During experimental runs the columns were immersed in a constant temperature water bath. Reactant solutions were pumped through the beds from the top of column. A constant flow rate was maintained by a pump. The void fraction of the bed was 0.3-0.4 by a determination using blue dextran.

3.5 Assays.

3.5.1. Photophosphorylation activity of native and immobilized chromatophores.

Native chromatophores.

The reactions were performed in 13 x 100 mm glass tube. The tubes were placed in a transparent water bath with a

constant temperature control.

Unless otherwise specified, the activity assay of chromatophores was carried out as follows. To 4.8 ml of assay solution containing 1.8 mM ADP, 5 mM Pi, 10 mM $MgCl_2$, 1 mM fumarate, 17 mM ascorbic acid, and 50 mM Tricine (pH 7.8) was added 0.2 ml of the chromatophore suspension and the solution was incubated at 25° under illumination with stirring. One ml samples were taken at various time intervals and the reaction was stopped by adding 1 ml of 0.4 M perchloric acid. After precipitates were removed by centrifugation, 0.05 ml of 3 M K_2CO_3 was added to 1 ml of supernatant to neutralize the sample and centrifuged to remove the precipitates. Then the supernatants were assayed for ATP concentration. The activity was expressed in umoles of ATP produced per min per mg chromatophore protein as an initial rate. The rate of ATP production was linear in the range of concentration of chromatophores examined, up to o.05 unit of chromatophores per ml of reactant solution.

Immobilized chromatophores.

The procedures employed for the determination of the photophosphorylation activity of immobilized chromatophores are similar to those for the native chromatophores with the following changes. To 4 ml of assay solution containing 5 mM ADP, 10 mM Pi and other reactants with concentrations described as above was added 2.5 ml of the suspension of immobilized chromatophore particles. The concentrations of reactants in the solutions was sufficient enough to allow optimal activity of immobilized chromatophores. The yield (%) of activity on immobilization was defined as the activity of immobilized chromatophores compared to the activity of the same amount of native chromatophores.

3.5.2. Activity of native and immobilized adenylate kinase.

The activity of adenylate kinase was determined by either a batch or continuous assay. A procedure for the continuous assay of adenylate kinase has been developed and is described in Appendix III. The results of the both assay procedures were in agreement.

Since the reaction of adenylate kinase is reversible, activity can be measured in either direction. The procedure described in Langer(1974) were employed for the batch determination of activity of adenylate kinase. Concentrations in the assay solution were a) for forward reaction 10 mM each AMP and ATP and 20 mM $MgCl_2$ b) for reverse reaction 10 mM ADP and 10 mM $MgCl_2$, both in 0.1 M Tricine, pH 7.8. To 4 ml of the assay solution with 2 ml of additional 2 ml of buffer solution 0.5 ml of native enzyme solution was added. In the immobilized adenylate kinase assay 2 ml of enzyme solution containing about 1 gram of wet gel particles entrapping the enzyme was added to 4 ml of assay solution with addit onal 4 ml of buffer. Concentrations of ATP or ADP were measured as described below.

One unit of activity is defined in this study as an amount of adenylate kinase capable producing one umole of ADP per minute. This activity was 4.2 timess greater than the activity by ATP formation in the reverse reaction. This is due to the differences in stoichiometry and intrinsic rate constant of the reversible reaction.

3.5.3. Adenine nucleotides.

a. Enzymatic methods.

ATP

The coupled enzyme system of hexokinase and glucose-6-phosphate dehydrogenase was used to measure ATP concentration;

$$\text{Glucose} + \text{ATP} \xrightleftharpoons{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP} \xrightleftharpoons{\text{Glucose-6-phosphate dehydrogenase}} \text{6-Phosphogluconate} + \text{NADPH}$$

$$\text{Overall Glucose} + \text{ATP} + \text{NADP} \xrightleftharpoons{} \text{ADP} + \text{6-phosphogluconate} + \text{NADPH}$$

All reactions go to completion. The concentration of NADPH was measured spectrophotometrically at 340 nm.

Chemicals for the ATP assay were obtained in kit form as ATP-Stat Pak from Calbiochem (San Diego, CA).

ADP

The coupled reaction of pyruvate kinase and lactate dehydrogenase was employed;

$$\text{ADP} + \text{Phosphoenolpyruvate} \xrightleftharpoons{\text{Pyruvate kinase}} \text{ATP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightleftharpoons{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}$$

$$\text{Verall, ADP} + \text{Phosphoenolpyruvate} + \text{NADH} \xrightleftharpoons{} \text{ATP} + \text{Lactate} + \text{NAD}$$

NADH absorbs light at 340 nm. The concentration of ADP in samples was measured by the decrease in absorbance at 340 nm.

To 1 ml of assay solution containing 8 unit pyruvate, 17 unit lactate dehydrogenase, 0.1 mg phosphoenolpyruvate, and 0.14 mg NADH in 0.1 M Tricine, pH 7.8, 0.2 ml of samples

was added. All the chemicals were purchased from Sigma (St. Louis, MO). After 10 min of incubation at room temperature the change in absorbance at 340 nm was measured. The extinction coefficient used in the determination was 6.0×10^3 $M^{-1}cm^{-1}$.

b. Chromatographic methods.

For a simultaneous determination of the concentrations of AMP, ADP, and ATP, a liquid chromatographic procedure was developed. The adenine nucleotides were separated using high-performance liquid chromatography (Waters Associates, Framingham, MA) with a strong anion exchange column and the concentrations were monitored by a UV detector (254 nm). Detailed conditions and a chromatogram of standard sample are presented in Figure 6. Determination by the enzymatic method and liquid chromatography were in good agreement.

3.5.4. Protein and bacteriochlorophylls.

Protein content was determined using the Lowry procedures as described by Herbert et al. (1971). Chromatophore samples were heated for 5 min at 100° in 0.5 N NaOH to destory pigments before the assay.

Bacteriochlorophyll content was estimated by measuring the absorption of an acetone-methanol extract at 772 nm following the procedure described in Pace (1975).

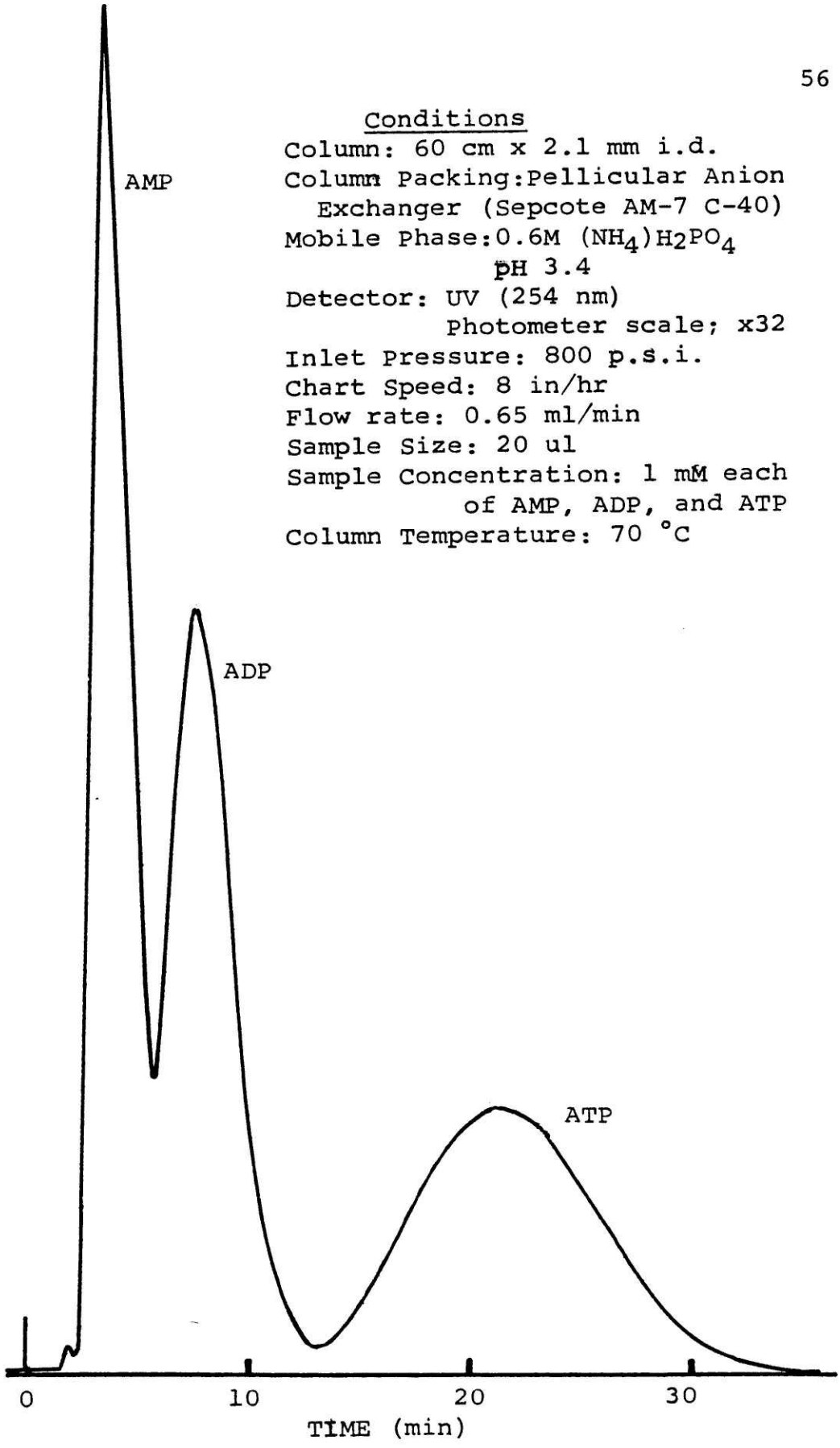


FIGURE 6.
Separation of adenine nucleotides by high-performance liquid chromatography.

3.6. Abbreviations.

AMP ; Adenosine 5'-monophosphate

ADP ; Adenosine 5'-diphosphate

ATP ; Adenosine 5'- triphosphate

Pi ; Inorganic phosphate

Tris ; Tris(hydroxymethyl)aminomethane

Tricine ; N-Tris(hydroxymethyl)methylglycine

BIS ; N,N'-Methylene-bis-acrylamide

TEMED ; N,N,N',N'-Tetramethylethylenediamine

DMSO ; Dimethylsulfoxide

U.F. ; Ultrafiltration cell

CSTR ; Continuous-flow stirred tank reactor

HEPES ; 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

4. RESULTS

4.1. Factors Affecting the Stability of Native Chromatophores in an Ultrafilatration Cell Reactor.

4.1.1. General.

For continuous photophosphorylation, chromatophores were confined in a pressure driven ultrafilatration cell (U.F.) by a membrane. This operated as a Continuous-flow Stirred Tank Reactor (CSTR). Firstly, the performance of the PM 10 membrane (Amicon) employed in the U.F. reactor was evaluated for ATP regeneration. The measurement of protein content showed that chromatophores were all retained by the membrane. A small amount of chromatophores formed a gel layer which was visible on the membrane, particularly at the high pressures and flow rates. The portion of activity lost due to gel layer formation was not significant for photophosphorylation in the reactor.

The pressure drop across the membrane with the chromatophore gel layer limited attainable flow rates to a maximum of 7 ml per hour i.e. 1.4 ml/hr/cm^2 membrane. This limitation obviously restricted the use of residence time shorter than 1.8 hour.

An accumulation of ATP molecules on the membrane was observed,when the outlet concentration of ATP was measured with known concentrations in inlet. This resulted in a lower concentration of ATP in outlet than actually produced in the reactor at the beginning of operation. The fraction of ATP permeated was 0.85 at 3 hours and gradually reached to a steady-state value of around 1 at 4 hours. When used membranes which were washed as described by Pace(1975) were employed, the time necessary to attain the steady state was longer.

Photophosphorylation by chromatophores from R. rubrum in a batch reactor showed Michaelis-Menten kinetics at low

substrate concentrations (up to 1 mM ADP) with fumarate addition. In the U.F. reactor, the volumetric feed rate, concentration of the reactants entering the isothermal constant-volume reactor, and the amount of the chromatophores in the reactor are held constant throughout the operation. Under these conditions, the fractional conversion (X) of the substrate (ADP or Pi), for saturating substrate concentrations ([S]>> Km), is described using the Michaelis -Menten expression and a substrate material-balance as follows:

$$X = k \cdot E \cdot Tr \; / \; Ci$$

where k is a reaction rate constant, E is the concentration of chromatophores (k·E then represents the chromatophore activity), Tr is the residence time, and Ci is the concentration of substrate in the feed stream. This derivation is valid only when the chromatophores in the reactor are stable throughout the run so that the catalytic activity remains constant.

Figure 7 illustrates continuous ATP production in the U.F. reactor under conditions where the substrate concentrations are very much large than the corresponding Km values, the residence time in the reactor is long enough to allow complete conversion of ADP to ATP but where there is chromatophore inactivation. There is a rapid increase in ATP concentration in the outlet stream until a point 'A' is reached at which point all the ADP entering the reactor is converted to ATP. (The outlet ATP concentration of 0.82 mM rather than 1 mM for the complete conversion is due to accumulation of ATP at the membrane as mentioned previously).

In the region A to B, even if there is a decrease in chromatophore activity, there will be corresponding change in the ATP outlet concentration since the activity is high

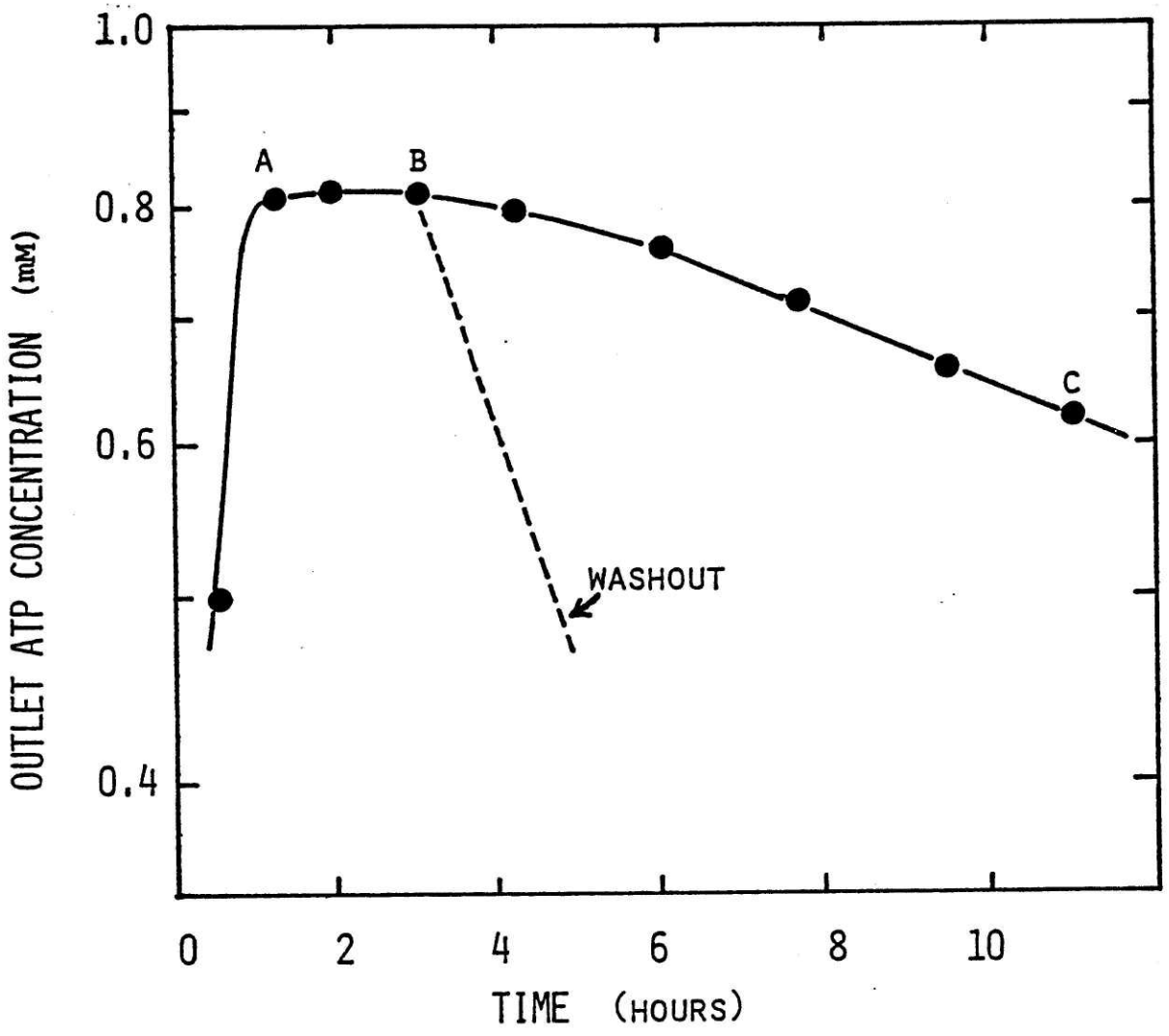


FIGURE 7.

Continuous ATP production from ADP in an ultrafiltration reactor catalyzed by native chromatophores. (outlet ATP concentration in log scale); A, B, and C, see text.

Conditions: 1 mM ADP; 5 mM inorganic phosphate; 1 mM fumarate; 10 mM $MgCl_2$; 17_4mM ascorbic acid; 50 mM Tricine; pH 7.8; light, 4.4 x 10^4 ergs $sec^{-1}cm^{-2}$; stirring speed, 500 rpm; temperature, 25°; residence time, 3 hr; and chromatophores (specific activity, 3 unit(hr)/mg), 1 unit(hr)/ml.

* A unit(hr) is defined as activity of 1 u moles ATP produced per hour.

enough to ensure complete conversion of all ADP. At point B, the chromatophore activity has dropped to a level where all the incoming ADP is not converted to ATP in the time of residence in the reactor. Hence we see a drop in ATP outlet concentration (B to C) which is a reflection of chromatophore inactivation.

This decrease in ATP production followed first order kinetics as shown in the figure. The plot of ln(outlet ATP concentration), or ln(fractional conversion of ADP to ATP), against time was linear with a slope, k_d , which represents the inactivation rate constant for chromatophores. The equation, $\ln([ATP]_2/[ATP]_1) = -k_d\ (t_2 - t_1)$, describes the continuous production of ATP in a system in which the formation reaction is zero order (substrate saturated) and the inactivation of catalyst is first order (see Appendix I for details of derivation of this equation).

In many of the cases reported in this study the rate of chromatophore inactivation was so rapid that the portion of the curve A to B in figure 7 was either very short or completely absent. In these cases, the ATP concentration in the outlet stream rapidly increased to a maximum, which may represent equilibrium conversion, and then decreased from point A reflecting the inactivation kinetics.

In the absence of any chromatophore activity, ATP would be simply washed out of the reactor. The wash-out curve is $\ln([ATP]/[ATP]_o) = -Dt$ where $[ATP]_o$ is the ATP concentration at zero activity, D is dilution rate (1/Tr), and t is time elapsed since reaching zero catalytic activity. This is represented by the dotted line in figure 7 . If ATP is consumed by reactions such as the reverse of phosphorylation, then the ATP concentration will decrease faster than

the wash-out curve. This was observed with chromatophores in which ATPase was uncoupled by use of Tris as the pH buffer (Pace, 1975). None of the results reported here showed the presence of an ATPase activity, as will be dicussed later.

Figure 8 illustrates in greater detail chromatophore activity during continuous photophosphorylation. The solid line (BC) represents the chromatophore activity calculated from the measured ATP outlet concentration. When this line is extrapolated to zero time, however, the computed initial chromatophore activity (Ai) is much lower than the actual activity of the chromatophores added to the system (Ao). Thus, there appears to be a two-phase inactivation process that includes an initial rapid inactivation in which 70-80 % of the activity is lost in about 3 hour (corresponding to an inactivation constant of 0.4 hr^{-1}) followed by a slow inactivation in which a further 10 % of the activity is lost in the next several hours (corresponding to an inactivation constant of 0.04 hr^{-1}). A direct measurement of activity of the chromatophores from the U.F. reactor could not be achieved because of the reduction in the activity of chromatophores due to disintegration during the recovery procedures. A possible mechanism for this two-phase inactivation will be discussed later.

4.1.2. Flow Rate.

ATP production in continuous reactors depends on the flow rate of the reactant solution which, in turn, affects the residence time of reactants. The effect of various flow rates on chromatophore stability in the U.F. reactor is shown in figure 9. There was no significant difference in

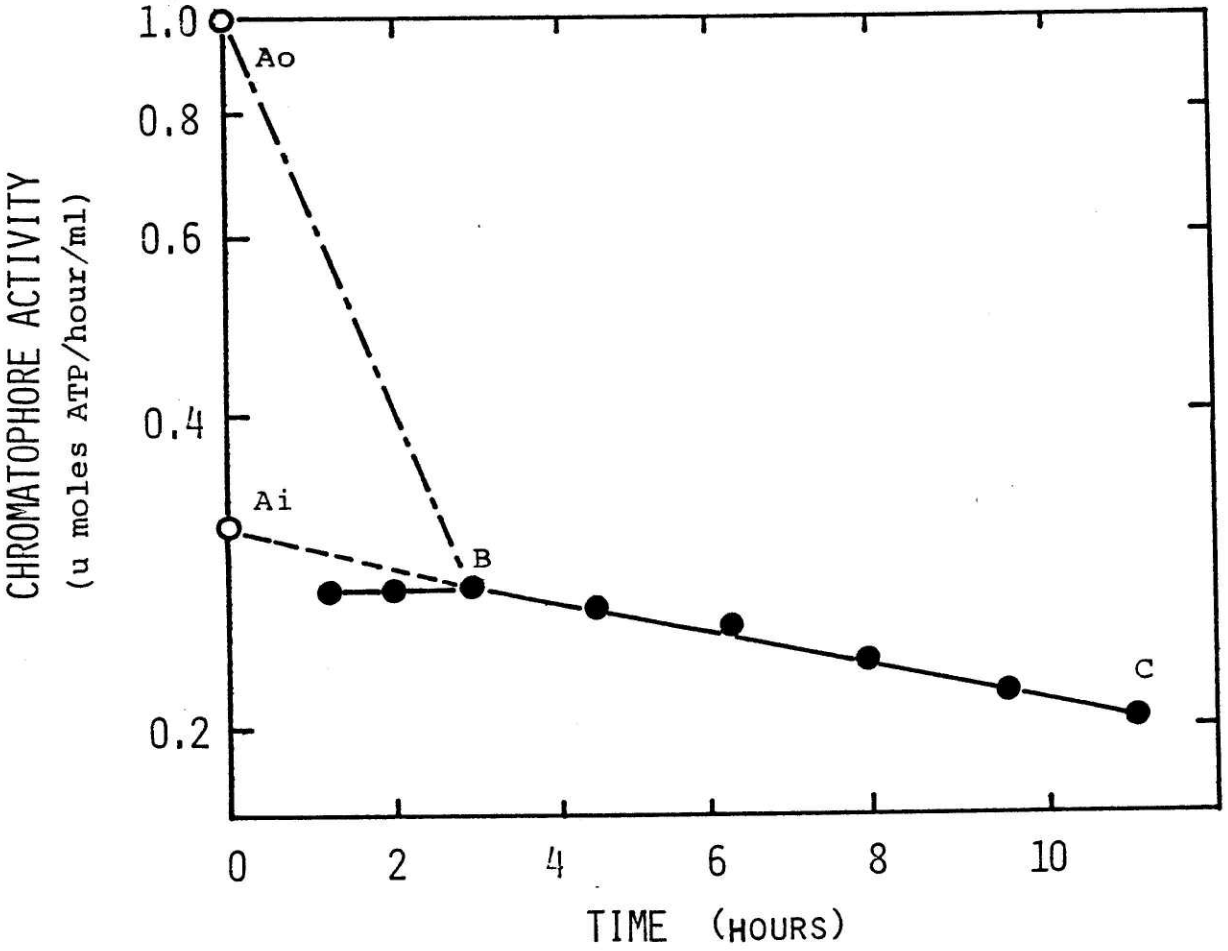


FIGURE 8.
Analysis of chromatophore inactivation kinetics.
Data taken from Figure 7. see text for Ao, Ai, B, and C.

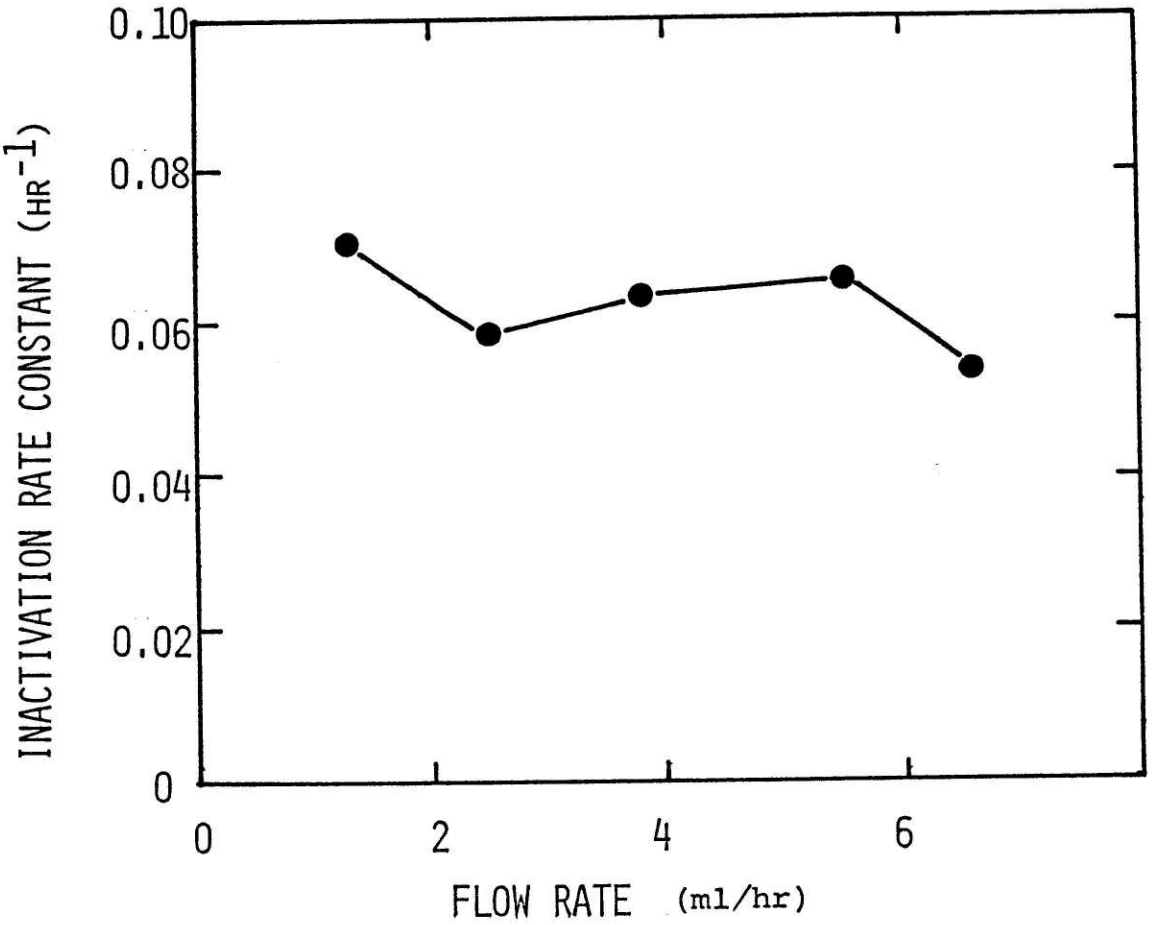


FIGURE 9.

Inactivation rates of chromatophore activity in the U.F. reactor at various flow rates.

Conditions: same as in Figure 7 except residence time. Reactor volume, 12.5 ml and flow rates as indicated in the figure.

the inactivation rate constants of the chromatophores at the flow rates within the range attainable with the U.F. reactor.

4.1.3. Substrate concentrations.

ADP

Inlet concentrations of ADP were varied from 1 to 10 mM using 10 mM inorganic phosphate. As the inlet ADP concentration increased the inactivation rate of chromatophores increased (Figure 10). The high concentrations of ADP led to increased concentrations of ATP in the outlet stream at the residence time employed (3 hours). Thus, the increased inactivation rate of chromatophores may be caused by either an inactivation effect of ADP itself or an inactivation effect associated with an increased amount of photophosphorylation.

The stability of chromatophores in a batch reactor in the presence of 10 mM ADP was determined to find whether ADP itself increases the inactivation of chromatophores. A small amount of ATP which was produced in the incubating solution even without addition of inorganic phosphate, indicating the presence of endogeneous phosphate (Yamamoto et al., 1972), was taken into account for the activity determination. The stability of chromatophores with and without ADP was similar. This suggests that ADP itself does not directly affect chromatophore inactivation.

To investigate the change in activity of chromatophores during active photophosphorylation at various concentration of ADP, the full time-course of the reaction was followed in batch reactors (Figure 11). The initial rates were the same at various ADP concentrations as expected since the

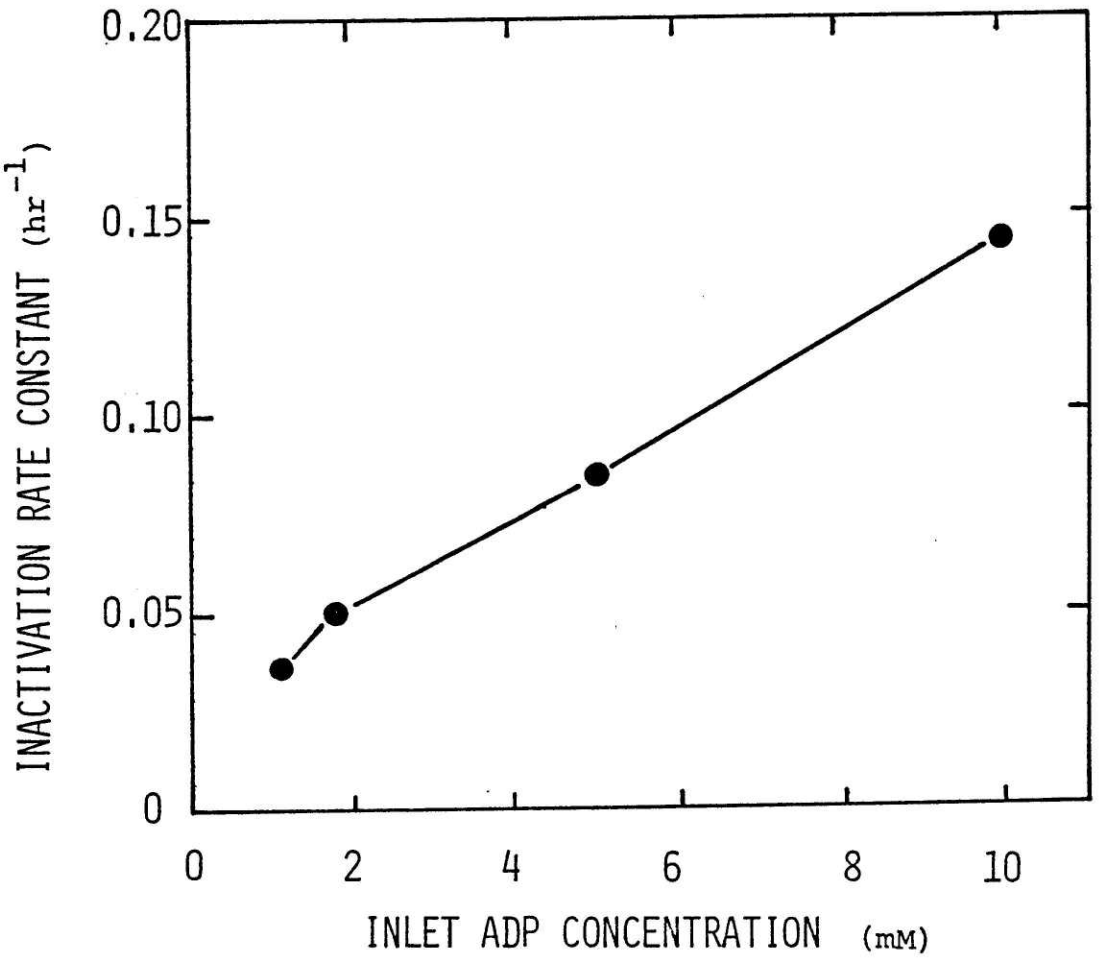


FIGURE 10.

Inactivation rates of chromatophore activity in the U.F. reactor at various inlet ADP concentrations.

Conditions were the same as in figure 7 except inorganic phosphate, 10 mM and ADP, as indicated in the figure.

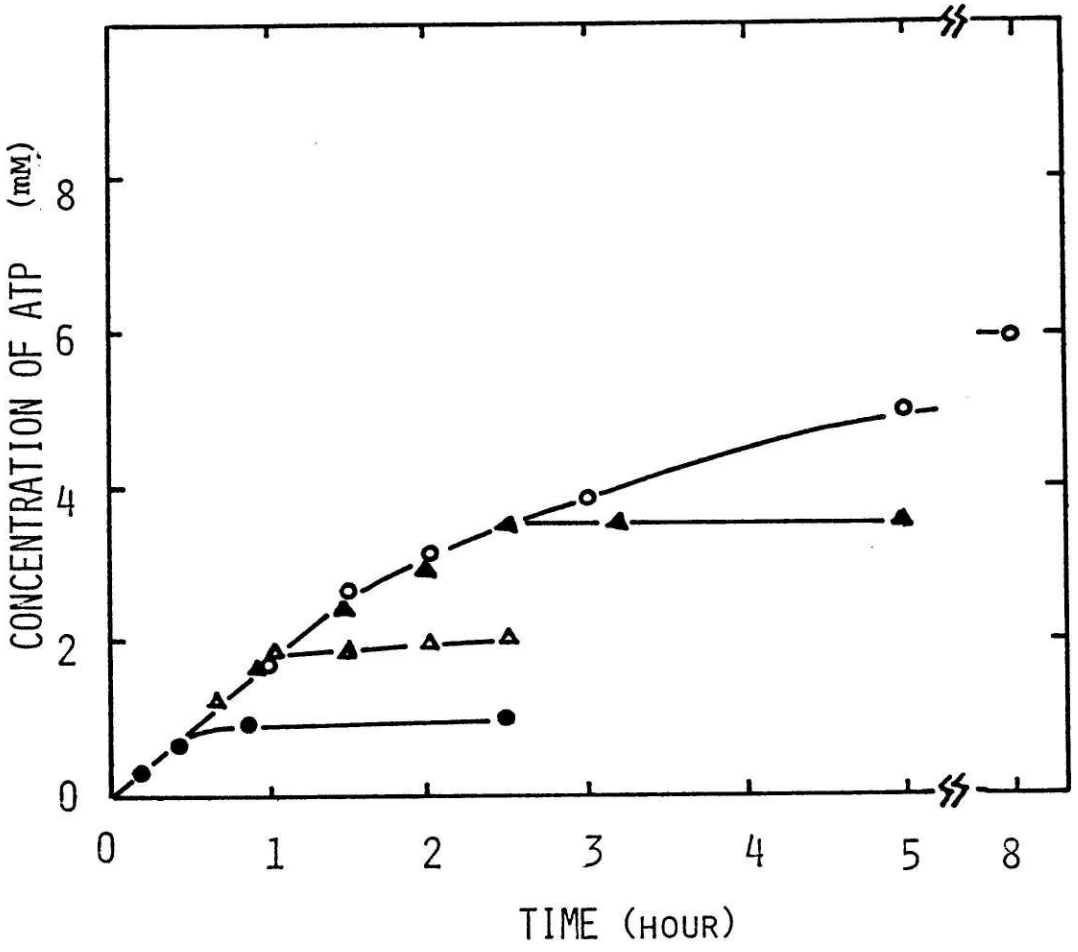


FIGURE 11.

Photophosphorylation of ADP by native chromatophores in a batch reactor at various ADP concentrations.

ADP ; 1 (●), 2.5 (△), 5 (▲), and 10 mM (●).

Conditions: ADP as indicated; 10 mM inorganic phosphate; 1 mM fumarate; 10 mM $MgCl_2$; 17 mM ascorbic acid; 50 mM Tricine; pH 7.8; temperature, 25°; light, 4.4 x 10^4ergs $sec^{-1}cm^{-2}$; chromatophores, 1.8 units(hr)/ml.

substrate concentrations were well above Km values and the inhibition by ADP was not significant within the ADP concentrations employed. At the higher ADP concentrations, however, the rate of reaction decreased with time and the reaction eventually stopped completely at a lower ATP concentration than anticipated. The final conversions were 91, 76, 69, and 60 % at 1, 2.5, 5, and 10 mM ADP, respectively. Thus, the increase in ATP concentration with time at high initial ADP concentrations appeared to lower the chromatophore activity as the reaction progressed. The results of above experiments suggest that the amount of photophosphorylation influences the chromatophore activity.

To determine whether inhibition by ATP, the product of the reaction, is responsible for the loss in activity of chromatophores, the reaction was carried out in batch reactors using various initial concentrations of ATP. The time course of the reaction was followed and the initial rates and the final conversion at apparent equilibrium were determined. The results are shown in Table 1. ATP inhibited the chromatophore activity as observed by decreased initial rates of the reaction. As a result of the decreased activity, for example, it took 55 minutes for chromatophores in 3.8 mM ATP to convert 1 mM ADP to ATP, whereas the same reaction took 40 minutes in the absence of added ATP. However, there was no significant difference in the final conversion (approx. 88 %) with various concentrations of ATP. The inhibited chromatophores increased the time required for conversion of substrates but did not completely stopped the reaction to lower overall conversions. Higher concentr-

TABLE 1

Effect of Concentration of ATP on Initial Rate and Final Conversion at Apparent Equilibrium of Photophosphorylation.

Conc. of ATP Initially Added	Initial Rate of Reaction		Final Conversion at App. Equil.	
mM	u moles ATP per ml /min	%	mM	% *
0	0.029	100	1.7	85
0.5	0.026	90	2.2	88
1.0	0.025	86	2.7	90
2.0	0.021	72	3.5	88
3.8	0.020	69	5.1	88

* Final concentration of ATP over concentration of initial ATP plus ADP (2 mM).

Initial reaction conditions : ATP as indicated, 2 mM ADP, 5 mM Pi, 10 mM Mg^{++}, 1 mM Fumarate, 17 mM ascorbic acid, 50 mM Tricine, pH 7.8, 25°.

ations of ATP added initially did not lower the apparent equilibrium conversion indicating that ATP is not responsible for inactivation of the chromatophore activity at high ADP concentrations.

Inorganic phosphate

As the concentration of inorganic phosphate in reactant solution increased the rate of inactivation of chromatophores increased (Figure 12). The phosphate concentration was in excess and ADP was the rate limiting substrate (1 mM). (Thus increasing the phosphate concentration above 1 mM can not in this case increase photophosphorylation yield.) The inactivation caused by high phosphate concentrations is probably due to the effect of phosphate ions on chromatophore aggregation. A visible aggregation of chromatophores was indeed observed in a batch reactor when the chromatophores were incubated in a medium with 100 mM phosphate. Gibson (1965) reported that a high (unspecified by the author) concentration of phosphate or metal ions causes an aggregation of chromatophores.

4.1.4. Other Reactants in Reaction Solution.

Fumarate

Addition of 1 mM fumarate completely relieved the inhibition affecting the overall conversion of 1 mM ADP to ATP in batch reactors.(Orginally described by Pace, 1975). However, when the ADP concentration was increased to 2.5 mM or 5 mM, conversions to ATP were not complete in the presence of 1 mM fumarate. This indicates that fumarate is effective in relieving the inhibition only up to a certain level of photophosphorylation.

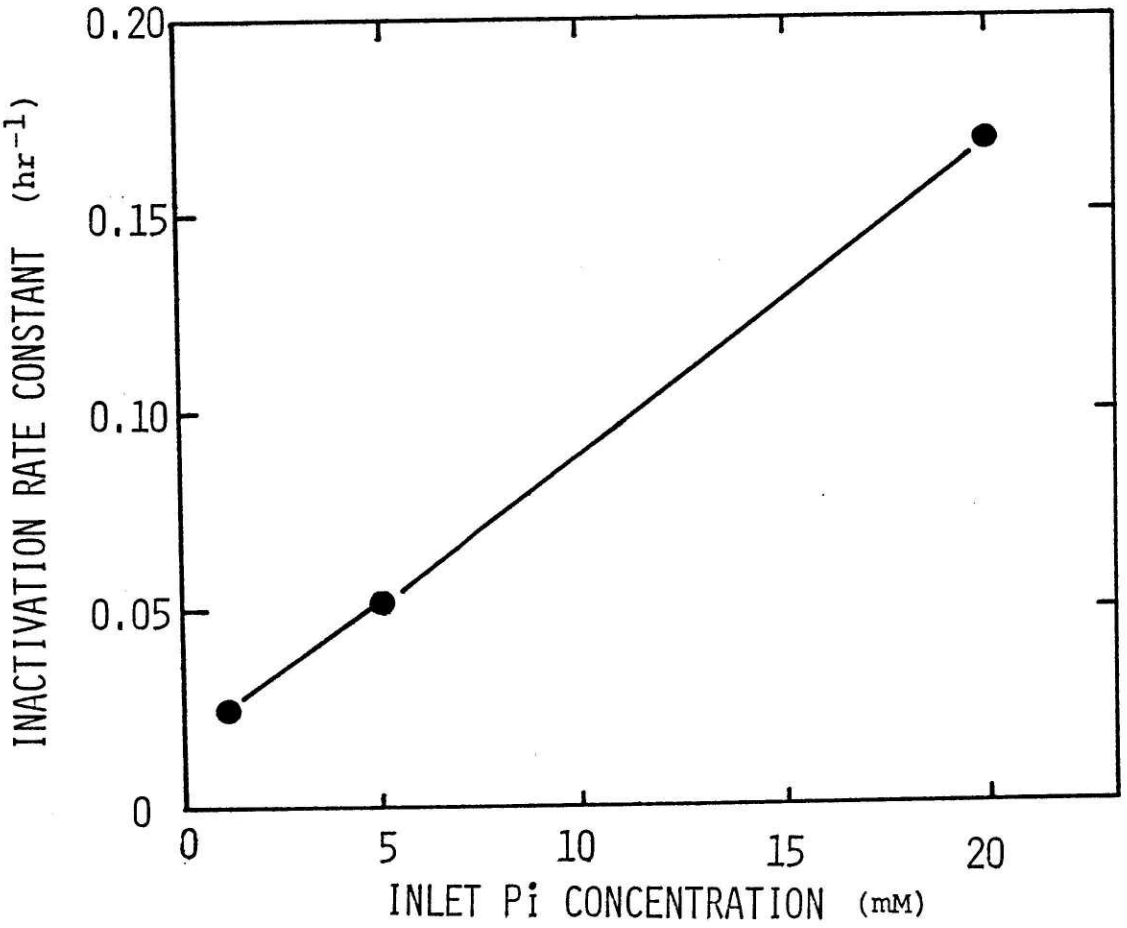


FIGURE 12.

Inactivation rates of chromatophore activity in a U.F. reactor at various inlet concentrations of inorganic phosphate (Pi).

Conditions were the same as in Figure 7 except 1 mM ADP and inorganic phosphate as indicated.

The effect of fumarate concentration on continuous photophosphorylation in the U.F. reactor was investigated and the results are shown in Figure 13. ATP production in the early phase was not affected by the presence or absence of fumarate in the inlet stream. After the first 4 hours of reaction, however, the chromatophore activity in reactors containing no fumarate decreased to zero as indicated by the ATP concentration paralleling the wash-out curve. Addition of 1 mM fumarate, however, resulted in maintenance of chromatophore activity over extended periods of time, illustrated in the figure, for up to 10 hours. An increase of fumarate concentration to 10 mM did not affect the performance of the chromatophores under these conditions.

Ascorbic acid

Ascorbic acid was added to maintain the optimal red-ox potential for the photophosphorylation. ATP production in the U.F. reactor was slightly higher with 60 mM ascorbic acid than with 17 mM which was the optimal concentration determined in the batch reactor by Pace (1975). The increased ascorbic acid concentration did not improve the stability of chromatophores during continuous photophosphorylation (Figure 14).

Magnesium ions

An increase in Mg^{++} concentration from 10 mM to 20 mM in continuous ATP production in the U.F. reactor resulted in a slight increase (less than 10 %) in conversion when both ADP and inorganic phosphate were 10 mM. The stability of chromatophores was not affected by change of Mg concentr-

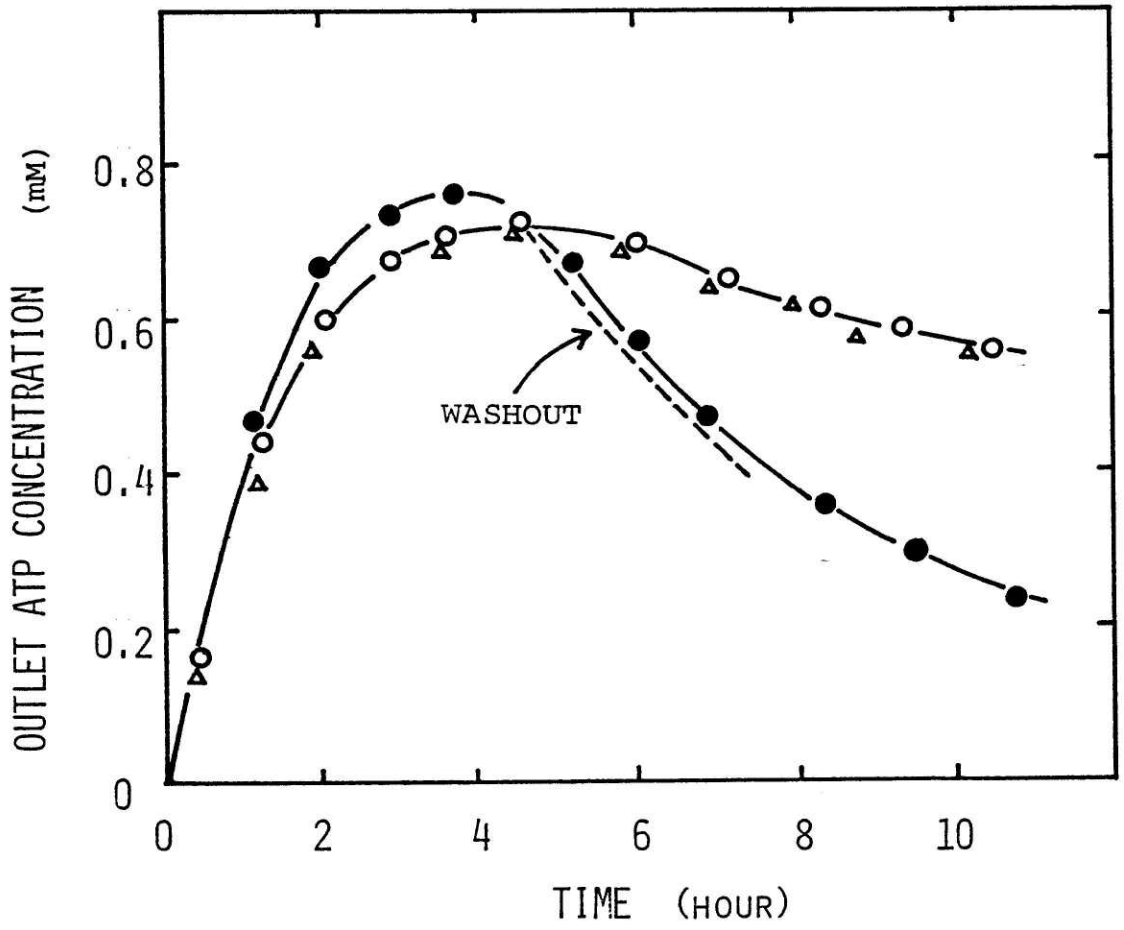


FIGURE 13.

Effect of fumarate concentration on continuous ATP production by native chromatophores in a U.F. reactor. Fumarate; none (●), 1 mM (○), and 10 mM (△).

Conditions were the same as in Figure 7 except fumarate as indicated.

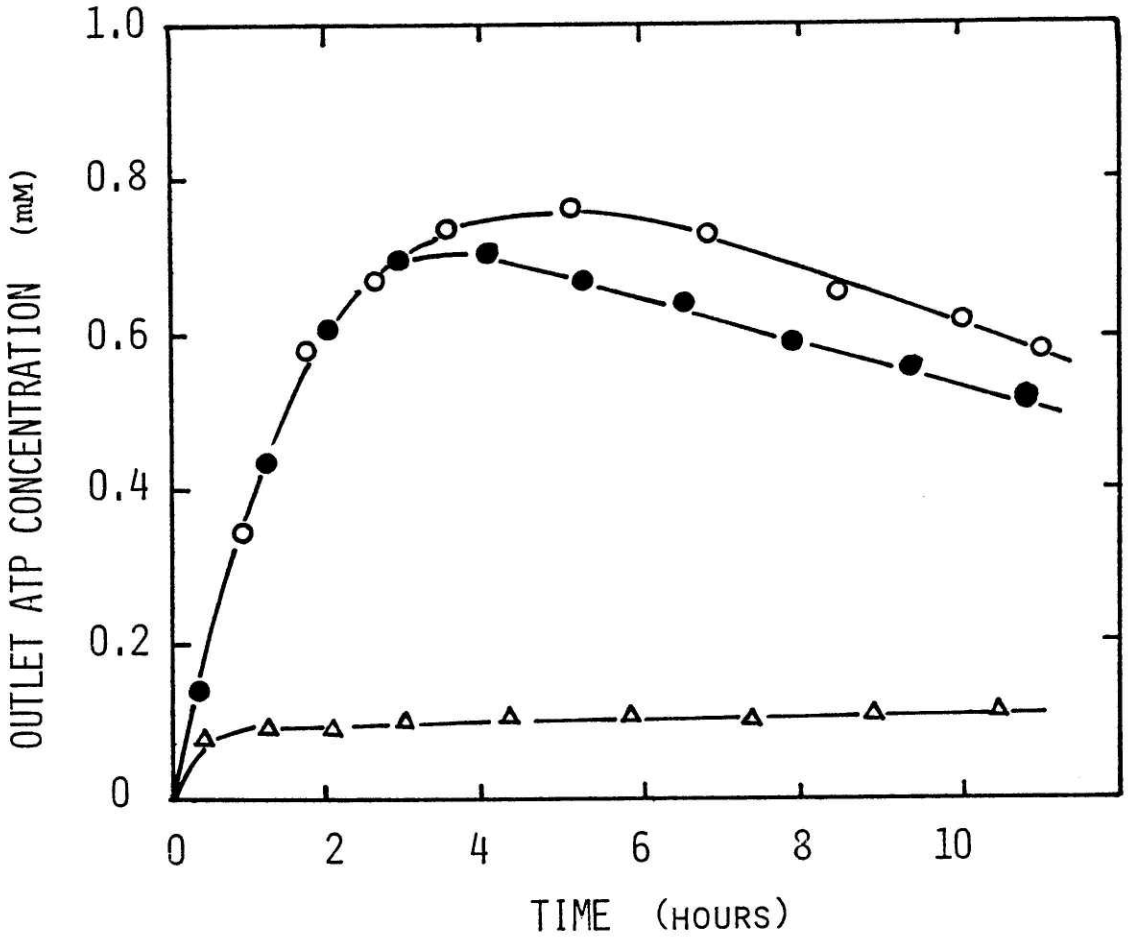


FIGURE 14.

Effect of ascorbic acid concentration on continuous ATP production by native chromatophores in a U.F. reactor. Ascorbic acid; none (Δ), 17 mM (●), and 60 mM (O).

Conditions were the same as in Figure 7 except ascobic acid as indicated.

ation in this range.

In a batch study 10 mM magnesium gave optimal activity of the chromatophores under the conditions of interest in this study. The role of magnesium ions in photophosphorylation is not clear. An excess of Mg ions was required for optimal chromatophore activity above the Mg-ADP complex level (Nishimura, 1962). The Mg-ADP complex has been shown in general to be the actual substrate for kinase reactions. whether Mg-ADP and/or Mg-Pi complexes serve as active substrate in photophosphorylation is not known. Recently Blodin and Green (1976) have suggested in their new theory of energy coupling that magnesium ion is required in the separation of the anion ($HPO_4^{=}$) by ionophores. In the experiments reported in this thesis, Mg^{++} is usually 10 mM, and ADP 1 mM. Thus ADP is completely in the Mg-ADP complex form.

pH buffers

The optimal pH for photophosphorylation by chromatophores from R. rubrum was 7.8 determined by an initial rate study (Yamashita et al., 1967). To select the best buffer system for continuous ATP production the effect of various buffers on the initial rate of photophosphorylation (Table 2) and the time-course of the reaction was investigated.

Tris, which is a common buffer employed in biological experiments, initially stimulated the reaction, but inactivated the chromatophores rather rapidly. Furthermore, Tris caused inactivated chromatophores to degrade the ATP produced, indicating the appearance of ATPase activity. Tris has been reported to be an uncoupler of electron transport in chloroplasts during photophosphorylation (Good and Izawa, 1972). During continuous photophosphoryl-

TABLE 2

Effect of pH Buffers on the Initial Rate of Photo-phosphorylation.

Buffers	pKa*	Initial Rate	Relative Rate
		u moles ATP per min/ml	%
Phosphate	6.8	0.21	100
Tris	8.3	0.40	190
Glycine	9.9	0.19	90
Glycylglycine	8.4	0.20,	95
Tricine	8.15	0.22	104
Hepes	7.55	0.14	67

Reaction Conditions : pH 7.8, 30°, 2 mM ADP, 20 mM Pi, 10 mM $MgCl_2$, 17 mM ascorbic acid, and 100 mM of each buffer above.

* from Good and Izawa (1972).

ation in the U.F. reactor (Pace, 1975) a decrease in the outlet ATP concentration faster than the wash-out was observed with chromatophores in which ATPase was uncoupled by use of Tris as the pH buffer.

Tricine and phosphate gave good results as the buffer. However, phosphate is one of the substrate in the reaction, which limits its use as the buffer. Tricine was therefore selected as the buffer for continuous ATP production by photophosphorylation with chromatophores.

Additives

Protective agents are often added to biological system to prevent denaturation during reaction as well as storage. Ethylene glycol was effective as cryotective agent for the storage of the frozed chromatophores (Geller and Lipmann, 1960). Sucrose was added to the isolated medium of chromatophores to enhance the stability (Cusanovich and Kamen, 1967).

Addition of ethylene glycol, however, did not enhance chromatophore stability in the U.F. reactor; rather , it resulted in an adverse effect at high concentration (Fifty percent ethylene glycol made the reactant solution cloudy and the low reaction rate was probably due to the interference of light transfer.)

Addition of 10 % sucrose did not result in a protective effect.

4.1.5. Light Intensity.

Light intensity was varied by adjusting the voltage to floodlamps (150 watt, Sylvania). Light intensity was determined using a calibration curve of Pace (1975).

The light intensities employed were well above the Km value for photophosphorylation i.e. light was saturating and hence ATP production in the early phase was unaffected.

With increasing time, however, the rate of chromatophore inactivation increased with increasing light intensity in the continuous reactor. The rate constant for the decrease were calculated at various light intensity levels and the results are shown in Figure 15.

Stability of chromatophores in batch reactors (Figure 16) indicated that light caused an increase in inactivation of chromatophore activity even in the absence of photophosphorylation. The inactivation rate constant was 0.18 hr^{-1}. The addition of components of the reactant solution (but not ADP and inorganic phosphate) did not influence the stability.

4.1.6. Temperature.

The effect of operating temperature on continuous ATP production is shown in Figure 17. As expected, chromatophore stability decreased with increasing temperature. At 40° ATP production stopped completely after less than one hour of operation (ATP washed out of the reactor). These results could not be plotted as an Arrhenius function since the high inactivation rate at the higher temperature invalidated the pseudosteady-state assumption (see Appendix I).

The temperature optimum for the initial rate of photophosphorylation was around 50°. High inactivation rates, however, would preclude operation of the raactor at high temperatures. The activstion energy for the chromatophore

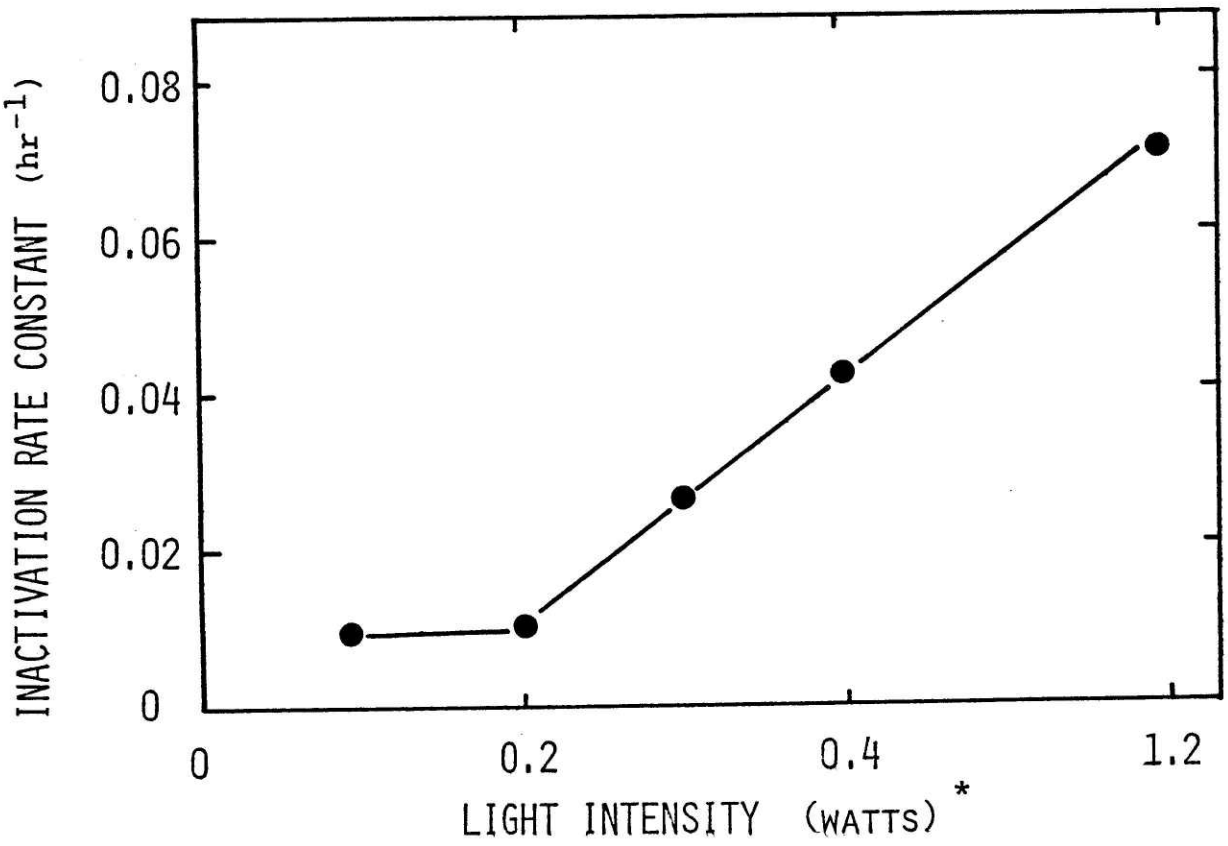


FIGURE 15.

Inactivation rates of chromatophore activity in a U.F. reactor at various light intensities.

Conditions were the same as in Figure 7, except light intensity as indicated.

* One watt is equivalent to 4.5 x 10^4ergs $sec^{-1}cm^{-2}$under the experimental conditions employed.

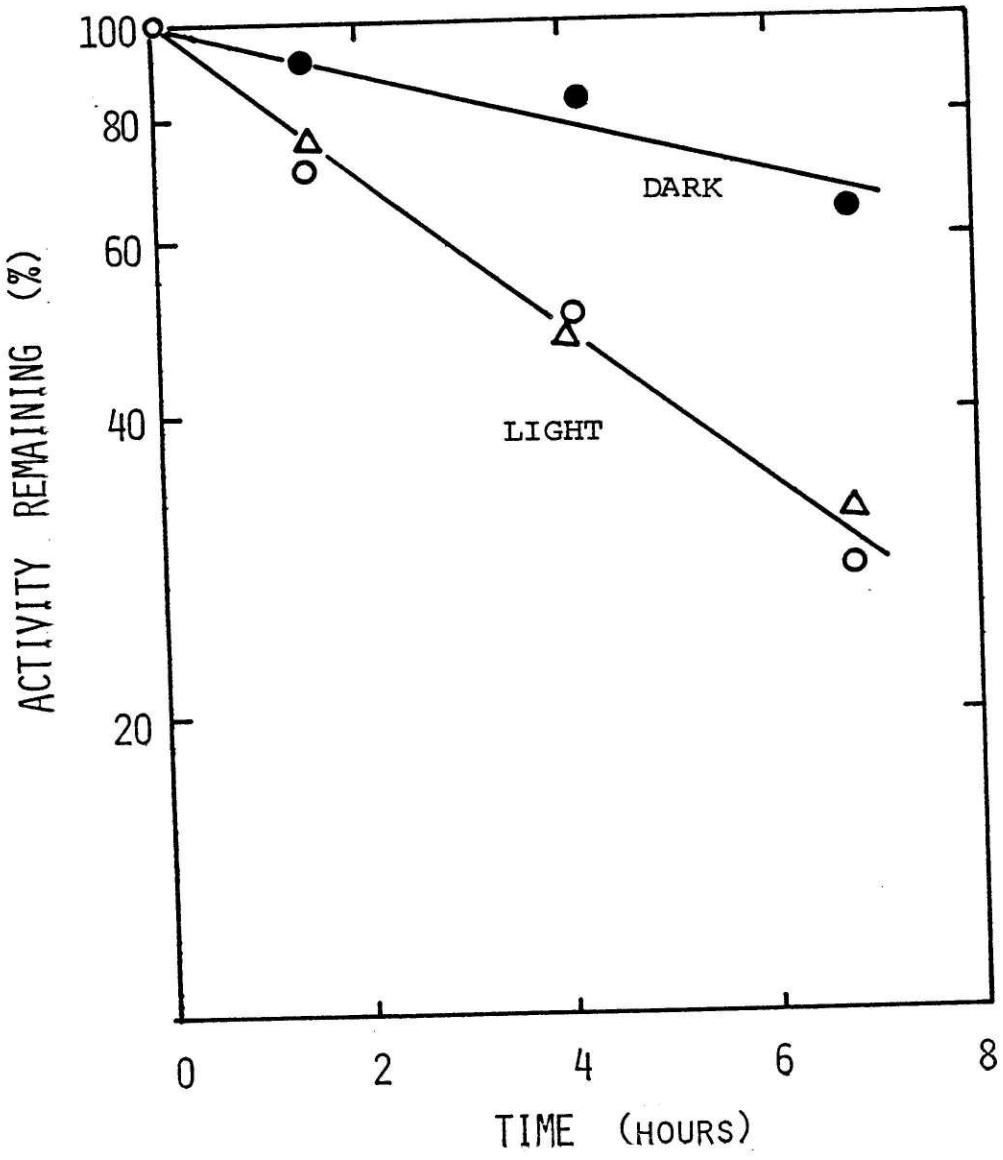


FIGURE 16.
Effect of light on chromatophore inactivation.

Chromatophores were incubated in 50 mM Tricine, pH 7.8 with (O) or without (●) light, or in a solution (Δ) of 10 mM $MgCl_2$, 1 mM fumarate, 17 mM ascorbic acid, and 50 mM Tricine, pH 7.8, at 25°. the light intensity was 4.4×10^4 ergs $sec^{-1} cm^{-2}$. Then samples were taken at various times and assayed for the photophosphorylation activity.

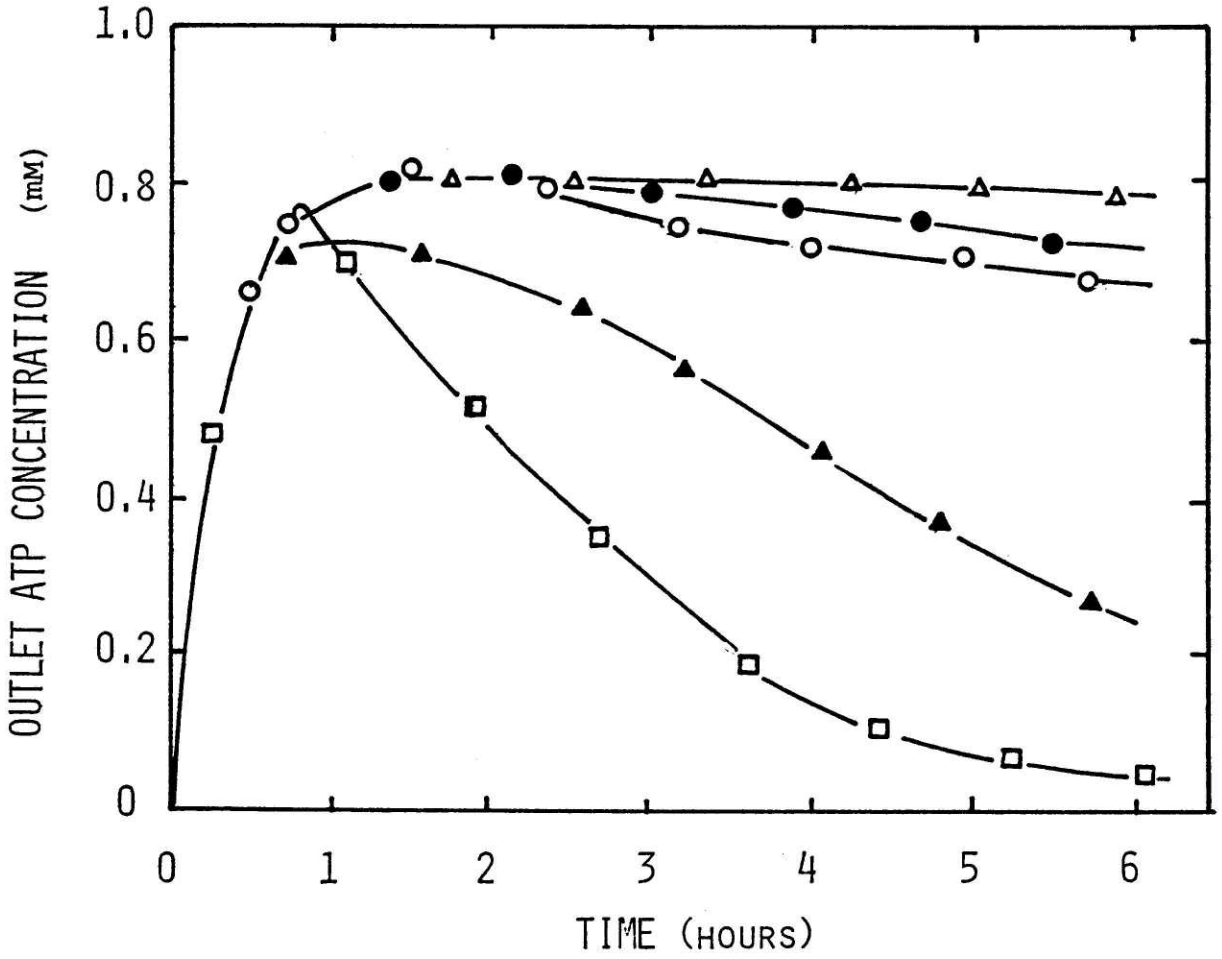


FIGURE 17.

Effect of temperature on continuous ATP production by native chromatophores in a U.F. reactor.

Temperature; 20° (△), 25° (●), 30° (○), 35° (▲) and 40° (□).

Conditions were the same as in Figure 7 except temperature as indicated.

inactivation in a batch system was 35.3 kcal/mole/°K (Figure 18). The reaction conditions were the same as in the U.F. system except ADP was ommited. Under similar conditions, the activation energy for photophosphorylation was 10.6 kcal/mole/°K, measured from initial rates (Figure 19).

Operation of the reactor at lower temperature would seem to be advantageous for ATP production. However, a decrease in flow rate at lower temperture may limit reactor operation. The temperature-flow relationship across the U.F. membrane has been reported (Pace, 1975).

The experiments in this study have been carried out at 25°, unless otherwise specified.

4.1.7. Changes in Chromatophores during Photophosphorylation.

An attempt was made to investigate any changes in chromatophore biochemistry or morphology which developed during the photophosphorylation. This would help to eluciadate the mechanism of the chromatophore inactivation during continuous use.

Pace(1975) reported that the decrease in ATP production in the U.F. reactor could be due to an increased ATPase activity in chromatophores resulting from thermal denaturation. However, when 1mM ATP was fed to the U.F. reactor containing chromatophores and all other reactants except ADP, and in the presence of light, the outlet ATP concentration remained constant at 1 mM. No ATPase activity had thus developed in the chromatophores during this treatment.

A similar experiment in a batch reactor showed that there was no significant ATPase activity during a period

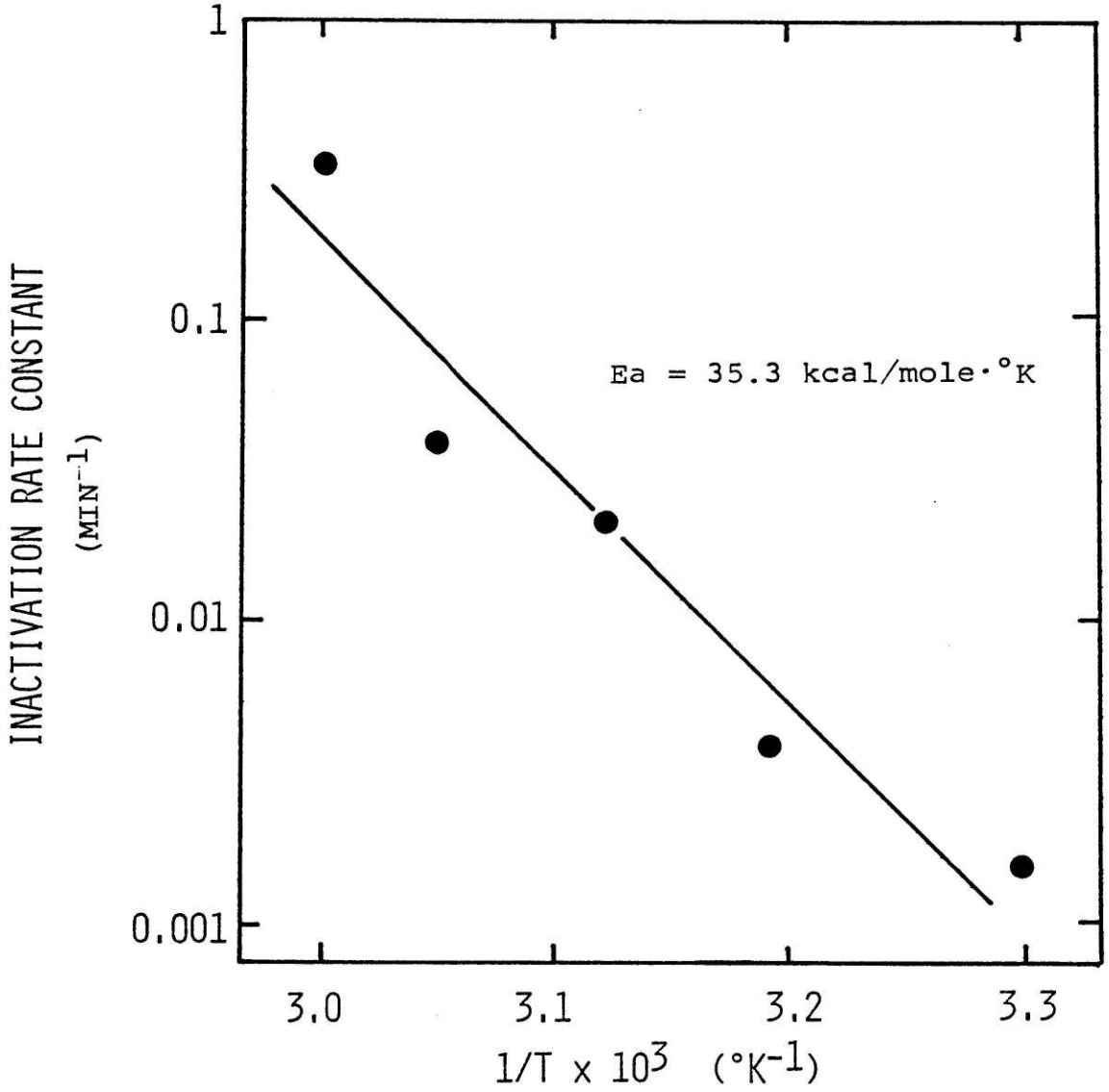


FIGURE 18.
Thermal inactivation of chromatophores (Arrhenius plot).

Incubation conditions; 5 mM Pi, 10 mM $MgCl_2$, 1 mM fumarate; 17 mM ascorbic acid; 50 mM Tricine; pH 7.8, light, 3.2 x 10^4ergs $sec^{-1}cm^{-2}$, and temperature as indicated.

Activation energy of chromatophore inactivation (Ea) is calculated from a arrhenius equation, ln k = Ea/R (1/T).

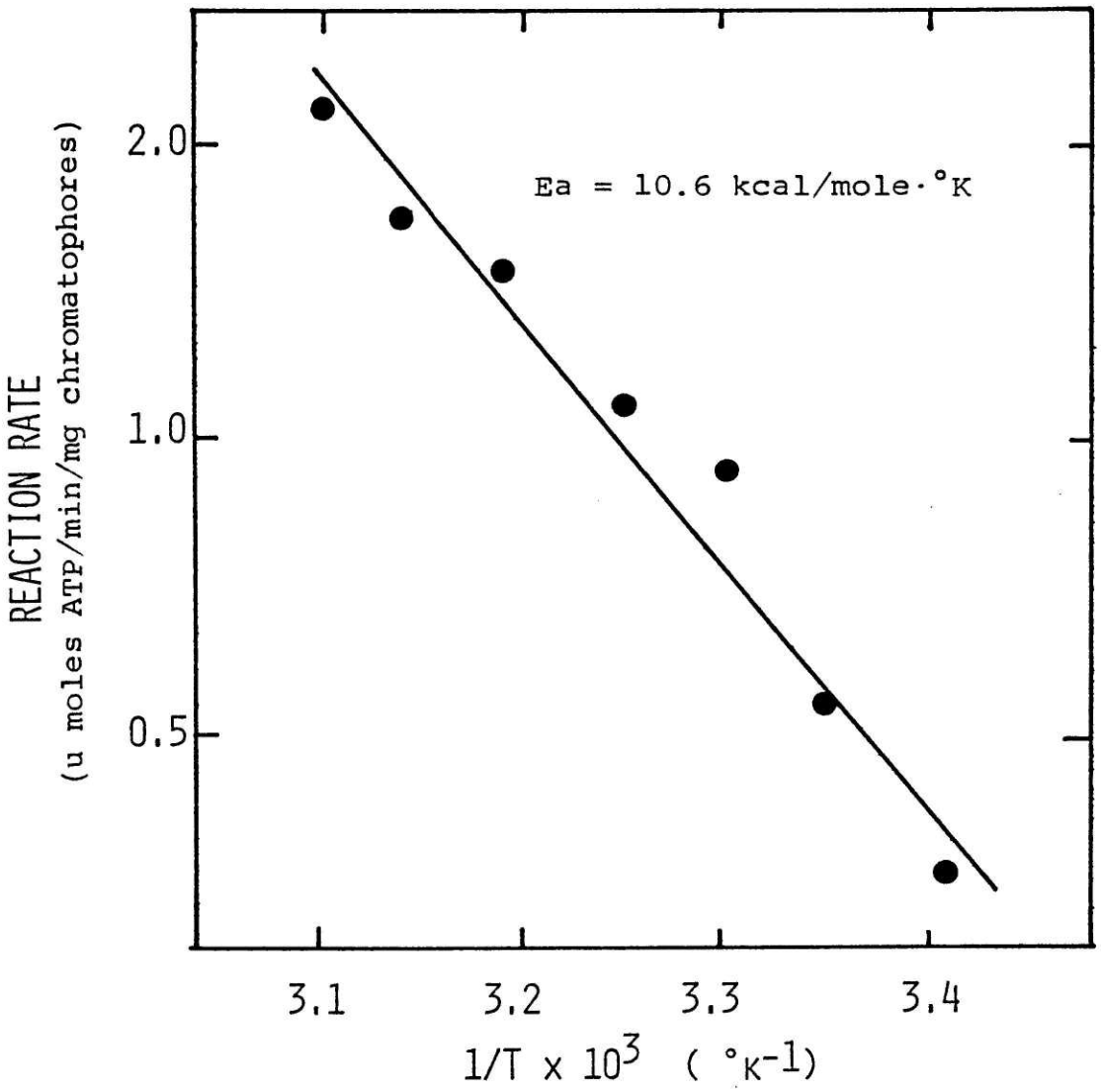


FIGURE 19.

Photophosphorylation rate of chromatophores at various temperature (Arrhenius plot).

Reaction conditions were the same as the incubation conditions in Figure 18 except temperature was 25° and 1 mM ADP was added.

Activation energy of reaction (Ea) is obtained from a Arrhenius equation, $\ln k = Ea/R\ (1/T)$.

of up to 5 hours (Figure 20). Also, in the presence of ADP, after 4 hours of photophosphorylation the concentration of ATP remained constant up to at least 8 hours but decreased when the light was switched off indicating the development of ATPase activity. These results suggest either that the chromatophores only develop an ATPase activity after a period of active photphosphorylation and when the light is switched off, or that ATPase develops during active photophosphorylation but is then compensated an equivalent amount of ATP synthesis to yield no net change in ATP concentration. An alternative explanation is that ATPase is inhibited in light as observed by Nishimura (1962).

To determine whether inactivation of chromatophores during continuous ATP production was caused by a change in the morphology of the chromatophores, experiments were conducted using a sucrose density gradient. The chromatophores, a) freshly prepared, b) frozen-stored with and without addition of 5 % dimethylsulfoxide (DMSO), and c) from U.F. reactor after 15 hours of reaction, were layered on a sucrose density gradient (0.4 - 2.0 M) and centrifuged at 25,000g for 90 minutes. The results are shown in Figure 21. The fresh and the frozen-stored with DMSO showed only one light band while the frozen-stored without DMSO and the ones from U.F. reactor showed two bands, light and heavy. The light band was pinker and more homogeneous than the heavy band which appeared faded and opaque.

The fractions were eluted from the gradient and their photophosphorylating activity and protein content were assayed. The results are presented in Table 3. Freezing and storing the chromatophores without addition of DMSO

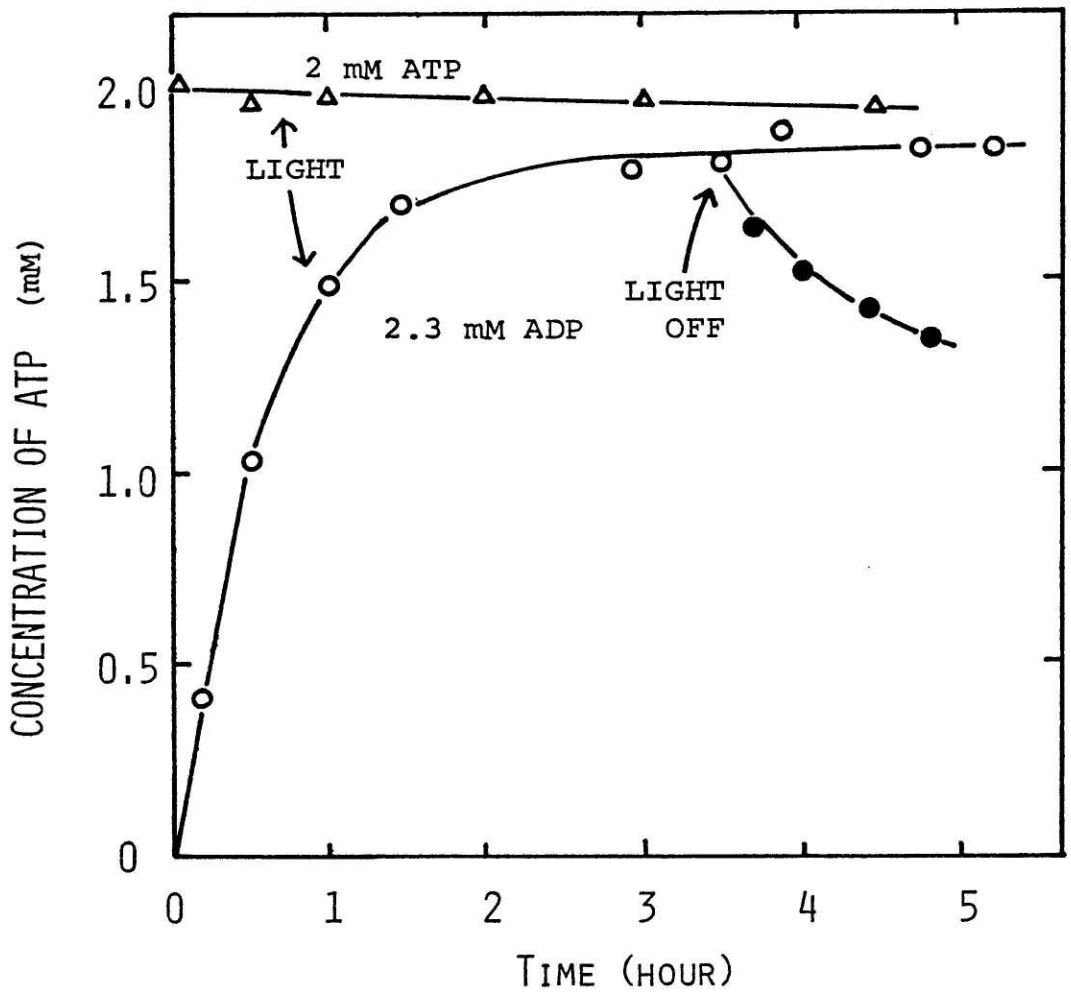


FIGURE 20.

ATPase activity of chromatophores in batch reactors.

(▲); ATPase activity of chromatophores without photophosphorylation. (O); photophosphorylation of ADP by chromatophores. (●); ATPase activity in the dark of chromatophores after photophosphorylation.

Conditions; ATP or ADP as indicated; 5 mM Pi, 10 mM $MgCl_2$; 1 mM fumarate, 17 mM ascorbic acid; 50 mM Tricine; pH 7.8; light, 4.4×10^4 ergs $sec^{-1}cm^{-2}$; 25°.

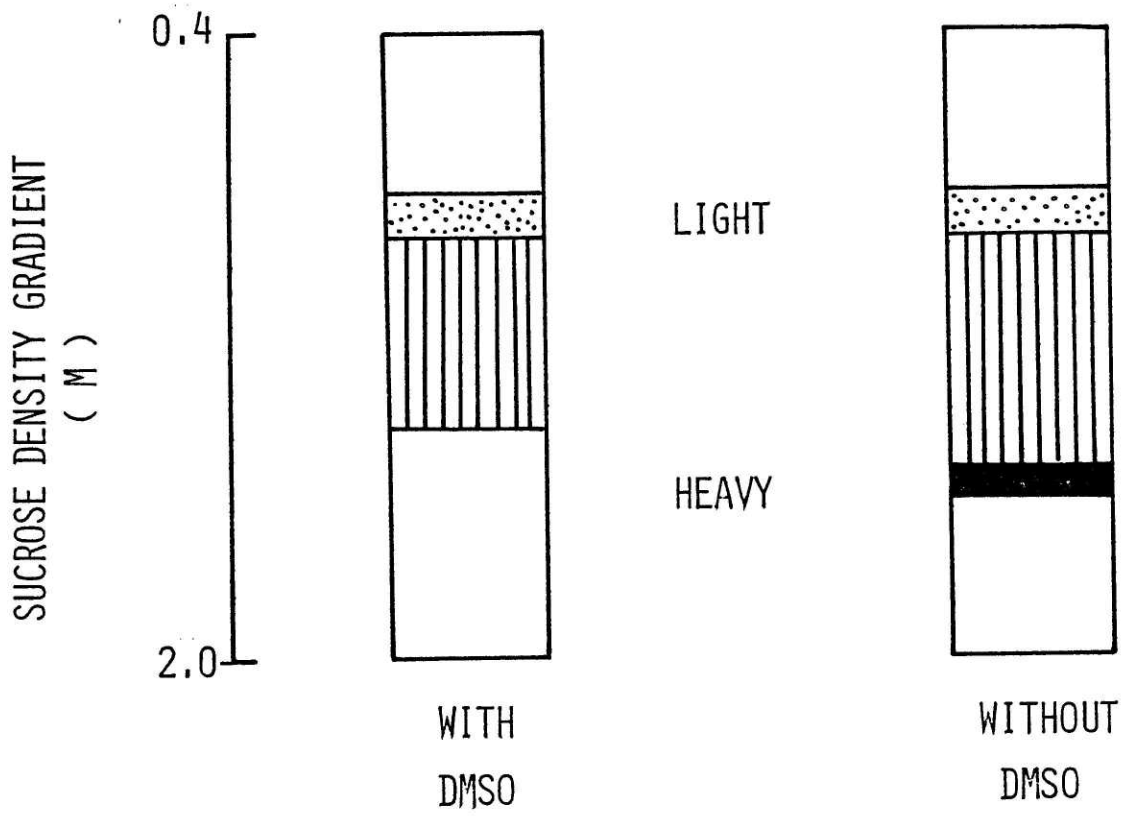


FIGURE 21.

Fractionation of chromatophores frozen-stored with or without addition of dimethylsulfoxide (DMSO) by centrifugation on a sucrose density gradient.

Conditions: volume, 20 ml; sucrose gradient, 0.4-2 M, centrifugation (SW 25 rotor, Beckman), 25,000 g for 90 min.

TABLE 3

Activity and Protein Concentration of Fractions on Sucrose Density Gradient of Chromatophores Frozen-stored with and without Addition of Dimethylsulfoxide (DMSO).*

	With DMSO			Without DMSO		
	Protein	Act.	Spec. act.	Protein	Act.	Spec. act.
	mg/ml	units per ml	units per mg	mg/ml	units per ml	units per mg
Before separation	8.4	1.17	0.140	7.9	0.57	0.072
Fractions separated,						
Light	0.7	0.97	0.141	0.50	0.035	0.070
Medium	0.42	0.03	0.078	0.42	0.027	0.061
Heavy	0	0	0	0.67	0.048	0.041

* see Figure 21 for separation on sucrose gradient.

decreased the activity to about one half of the ones with DMSO. The heavy fractions were lower in specific activity than the light fractions. The activity of the chromatophores from U.F. reactor could not be determined because of their low concentration. These results indicated that the decrease in chromatophore activity was accompanied by appearance of the heavy fraction with low specific activity. The nature of the heavy fractions was not clear but could be due to the aggregation of chromatophore particles.

4.2. Regeneration of ATP from AMP by Chromatophores Coupled to Adenylate Kinase.

4.2.1. General.

ATP formation via photophosphorylation utilizes ADP as the phosphate acceptor. ATP may be regenerated from AMP, however, by addition of the enzyme adenylate kinase, which catalyzes the reaction of a molecule of AMP with a molecule ATP to yield two molecules of ADP:

$$\text{AMP} + \text{ATP} \xrightleftharpoons{\text{Adenylate kinase}} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\text{Pi} \xrightarrow[\text{light}]{\text{Chromatophores}} 2\ \text{ATP}$$

$$\text{net,}\quad \text{AMP} + 2\text{Pi} \xrightarrow[\text{Light}]{} \text{ATP}$$

The coupled enzyme/chromatophores system was first studied in a batch reactor and then employed for a demonstration in a continuous U.F. reactor.

Rate equations for the coupled reaction were derived in terms of the enzyme activities and the substrate concentration in a batch and a continuous reactor. Changes of the steady-state concentrations of each adenine nucleotide in the batch reactor during the time course was also derived. Details of the derivation is given in Appendix II. The following equation for ATP represented the actual ATP production at the steady state,

$$[\text{ATP}] = A/2\ (t-tr) + [\text{ATP}]_o \qquad \text{Eq (1)}$$

where A is activity of adenylate kinase (or the limiting enzyme), t is time, tr is the trasient time but close to zero under the conditions employed, and $[\text{ATP}]_o$ is the initial ATP concentration (added).

Properties of adenylate kinase from Baker's yeast and chromatophores from R. rubrum are presented in Table 4.

TABLE 4

Properties and Optimum Conditions for Reactions of Adenylate Kinase from Baker's yeast and chromatophores from R. rubrum.

	Adenylate kinase	Chromatophores
Apparent equilibrium	$\frac{(ADP)^2}{(AMP)(ATP)} = 2.1$	90-95 % conversion with 1 mM fumarate at 1 mM ADP.
pH	7.8	7.8
Temperature		45°
Red-Ox potential	*	0.0±0.1 volts
Light; Km	not required	1.5×10^3 erg/cm^2/sec
Km for substrates		
AMP	0.058 mM	*
ADP	*	0.015 mM
ATP	0.054 mM	*
Pi	*	0.11 mM
Ki for inhibition		
AMP	competitive for ATP (10.96 mM)	rate decreased by 20-30% at 5mM AMP.
ADP	competitive for ATP(4.7mM),AMP(0.61mM)	K'm = 50 mM
ATP	competitive for AMP (0.078mM).	uncompetitive for ADP(25mM);competitive for Pi(0.5M).
Pi	not significant	none upto 30 mM
Magnesium ion	K_{MgATP}=10mM^{-1};Mg,1mM	10 mM,optimal
Stability		
Buffers		Tris,inhibitory.
Half-life at 25°	10 days in 0.2M Tris buffer	3 hours in reactant solution with light (two-phase inactivation during use).

* Values are not required for reactions.

ATP production from AMP and inorganic phosphate by chromatophores from R. rubrum and adenylate kinase from Baker's yeast in an illuminated batch reactor is shown in figure 22. A trace amount of the ATP (0.1 mM) was added to "prime" reaction of adenylate kinase. The conversion of AMP to ATP followed zero-order kinetics during most of the time course, as predicted in the rate equations, since the substrate concentrations were much greater than the Kms. In the presence of excess phosphate, AMP was completely converted to ATP by the coupled reaction (at 1 mM AMP). Though the equilibrium constant for the adenylate kinase reaction is not very favorable; $K_{eq} = (ADP)^2/(AMP)(ATP) = 2.26$ (pH 7.4, 25°C, 10^{-2} M $MgCl_2$) (Langer, 1974), the equilibrium of photophosphorylation strongly favors ATP formation, and thus drive the adenylate kinase reaction towards completion.

4.2.2. The Concentrations of Adenylate kinase and Chromatophores and Their Ratio.

Steady-state rates of ATP production at various ratios of chromatophore activity to adenylate kinase activity (C/A) were obtained at a number of enzyme concentrations. The rate of ATP production at each C/A increased as the concentration of adenylate kinase (and hence chromatophores) was increased. A plot of steady-state ATP production rate at various C/A at a fixed enzyme concentration is shown in Figure 23. The production was entirely controlled by the chromatophore reaction when C/A was below about 1; above a C/A of 1 the adenylate kinase reaction become rate limiting in ATP production by the coupled reaction. It should be noted that the steady-state rate of ATP production at the

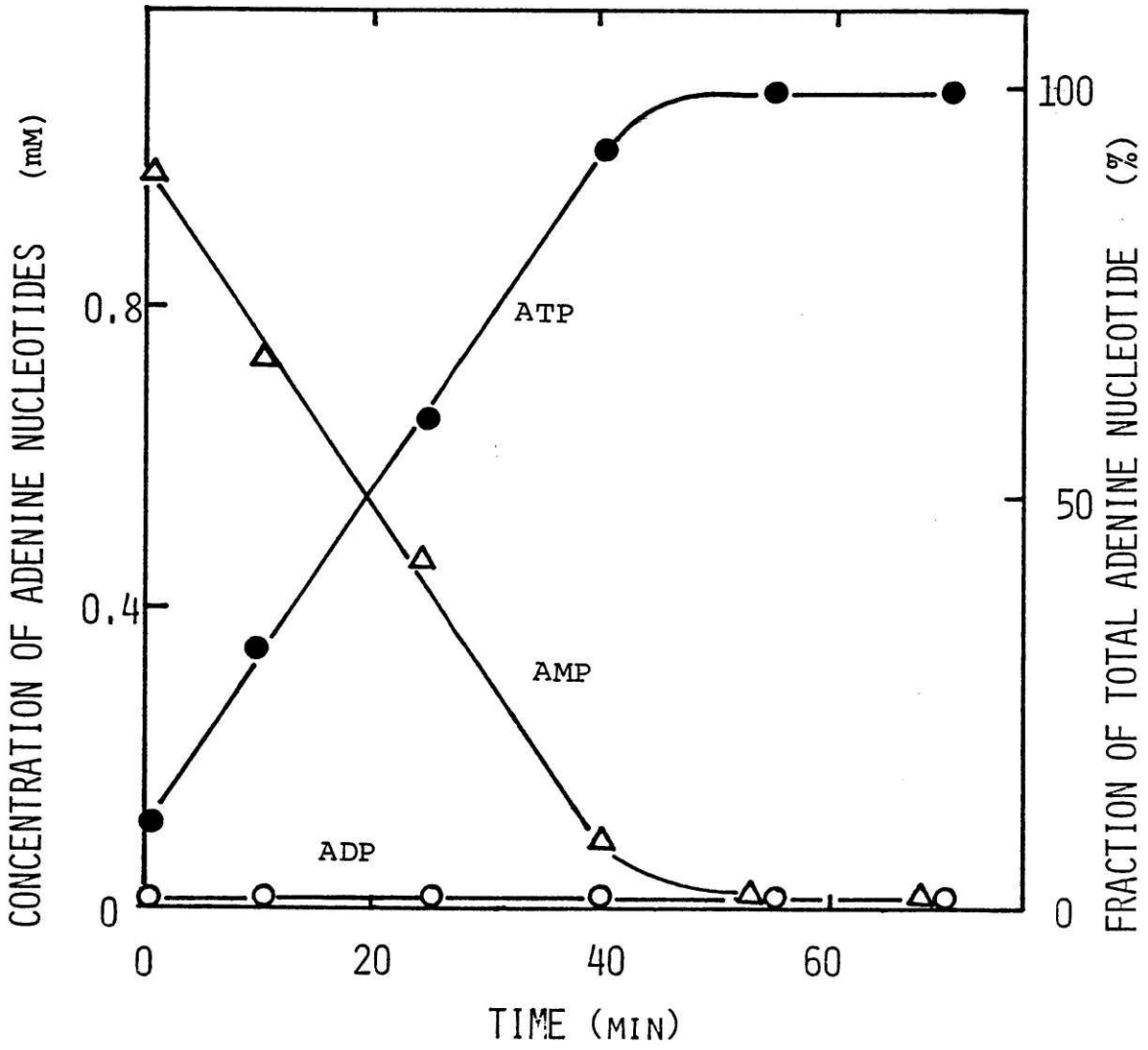


FIGURE 22.

Change in concentrations of adenine nucleotides during ATP production from AMP by the coupled reaction of adenylate kinase and chromatophores in a batch reactor.

Initial conditions: 1 mM AMP; 5 mM inorganic phosphate; 0.1 mM ATP; 10 mM $MgCl_2$; 1 mM fumarate; 17 mM ascorbic acid; 50 mM Tricine, pH 7.8; light, 4.4×10^4 ergs sec^{-1} cm^{-2}; temperature, 25°; and adenylate kinase, 0.043 unit/ml; and chromatophores, 0.06 unit/ml.

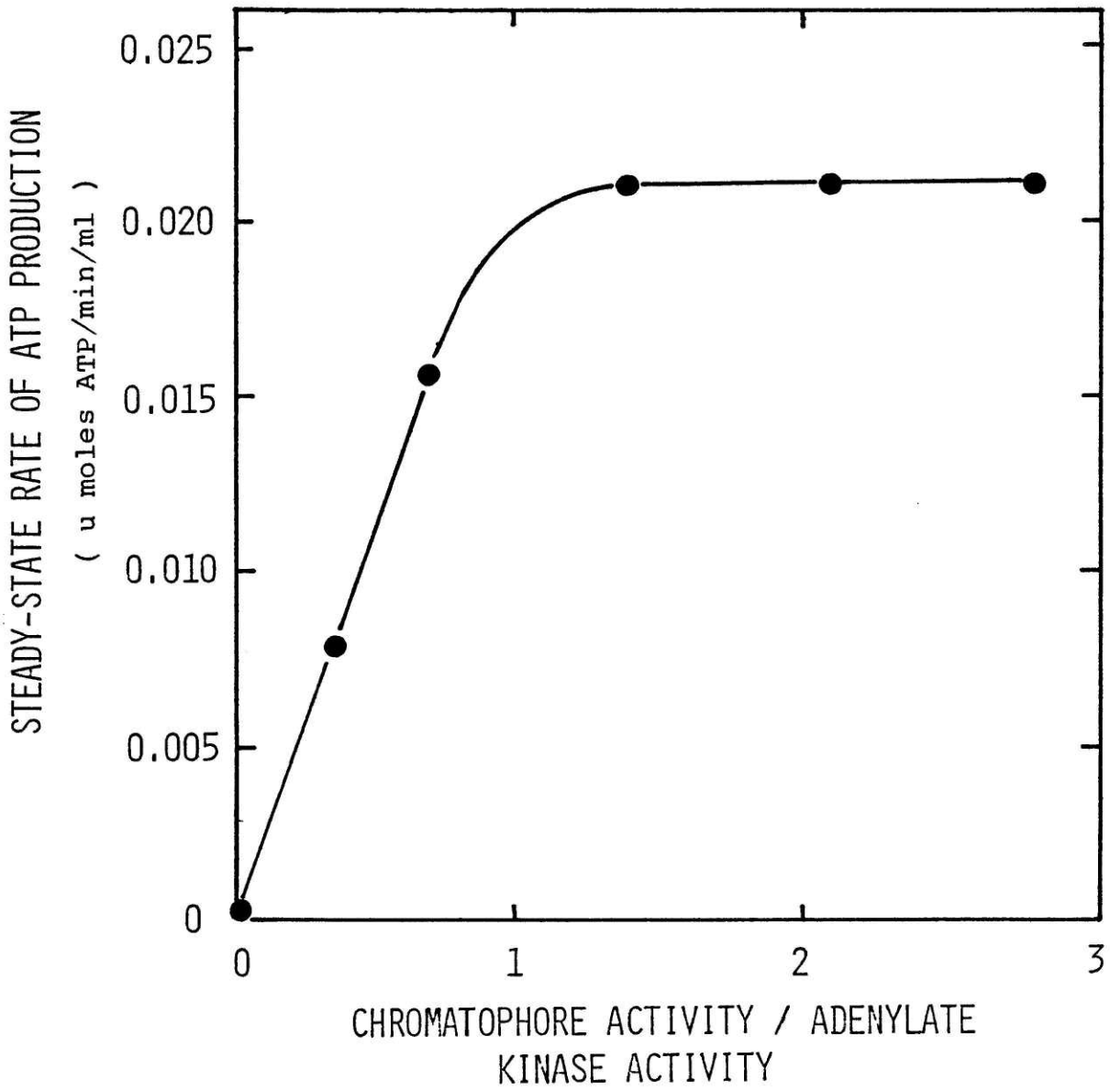


FIGURE 23.

ATP production from AMP at various ratios of activity of chromatophores to adenylate kinase in a batch reactor.

Conditions were the same as in Figure 23 except adenylate kinase 0.043 unit/ml and chromatophores as indicated.

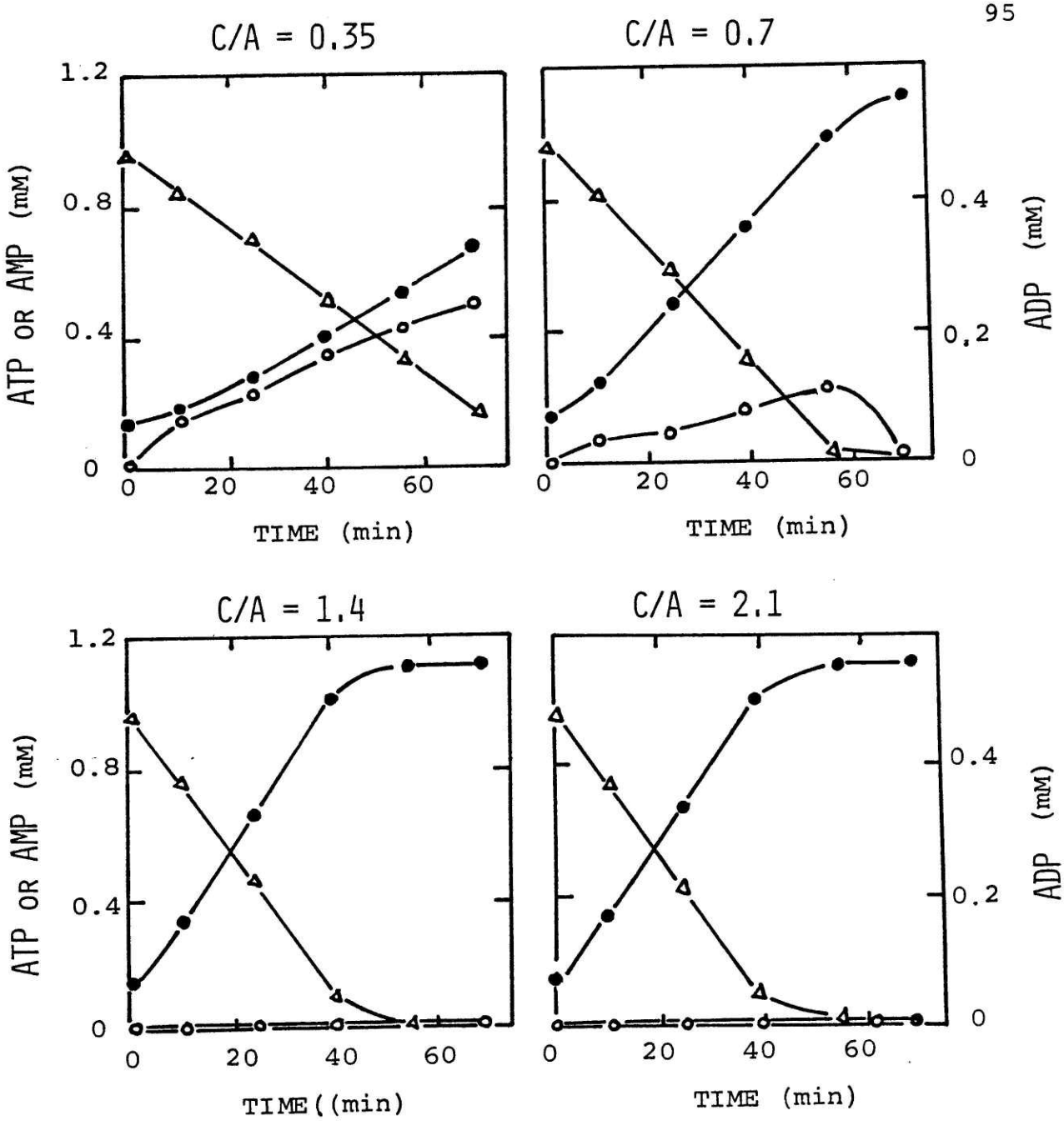


FIGURE 24.
Concentrations of AMP (Δ), ADP (O), and ATP (●) during the course of coupled reaction for ATP production at various ratios of activity of chromatophores to adenylate kinase (C/A).

Adenylate kinase was 0.043 units/ml and chromatophores were as indicated, and other conditions are the same as described in Figure 22.

optimal C/A, which is 1, is close to 0.5 umoles per unit of enzyme as predicted by equation (1).

The individual concentration of AMP, ADP, and ATP during the course of reaction is shown in Figure 24 at various C/A. The nucleotide concentrations were measured by high performance liquid chromatography. As expected, at high C/A (above 1), a very low steady-state concentration of ADP (the intermediate) was observed. At C/A less than 1, however, ADP increased in the reactor until AMP was exhausted. Also at low C/A the consumption of ATP by adenylate kinase until the concentration of ADP is increased for a rapid phosphorylation by chromatophores resulted in a lag phase in ATP production at the beginning.

4.2.3. Substrate concentrations.

The substrates for the coupled system are AMP and inorganic phosphate. An excess concentration of phosphate (approximately 100 x Km which is 0.11 mM) was added throughout the experiments and hence AMP was the only limiting substrate.

A catalytic amount of ATP was necessary to initiate the adenylate kinase reaction as mentioned previously. The effect of initial concentration of ATP on ATP production (at C/A of 1) is presented in Table 5. Addition of a low concentration of ATP resulted in a low rate at the beginning of the reaction. This lag phase is due to the slow build-up of ADP, the substrate for chromatophores. No difference in the rate was observed as the reaction further progressed.

ATP production at concentrations of 1, 5, and 10 mM AMP are shown in figure 25. There was no difference in the

TABLE 5

Effect of initial concentration of ATP on ATP regeneration from AMP.

Initial conc. of ATP	ATP produced		Conc. of ATP at 70 min
	0 - 30 min	30 - 60 min	
mM	u moles / ml		mM
0	0.27	0.52	0.95
0.02	0.34	0.53	1.01
0.10	0.41	0.53	1.10
0.50	0.62	0.60	1.37

Initial conditions of reaction: 1 mM ADP, 5 mM Pi, 10 mM $MgCl_2$, 1 mM fumarate, 17 mM ascorbic acid, 50 mM Tricine, pH 7.8, 4.4 X 10^4 ergs/cm^2/sec, 25°C.

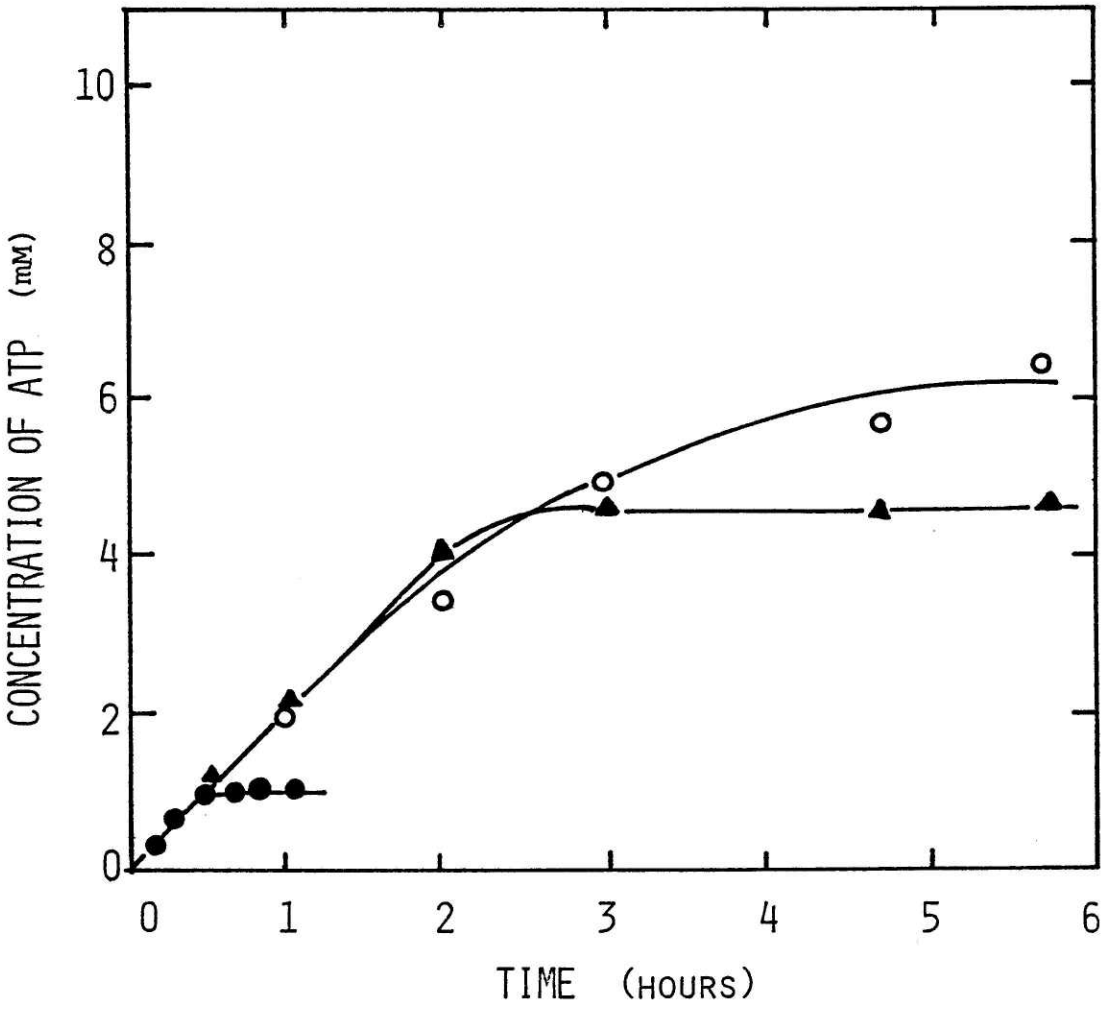


FIGURE 25.

ATP production at various substrate concentrations by the coupled reaction of adenylate kinase and chromatophores in a batch reactor.
AMP concentrations are 1 (●), 5 (▲), and 10 mM (○).

Initial conditions; AMP as indicated; 20 mM Pi, initial ATP/AMP, 0.1; adenylate kinase 0.086 units/ml; chromatophores, 0.06 units/ml; and other conditions are the same as described in Figure 22.

initial rate of the conversion of AMP to ATP. At 1 and 5 mM AMP the substrate was almost completely (approx. 95 %) converted to the product. However, at 10 mM the production rate decreased as time progressed.

The decrease in conversion at 10 mM could be due to substrate or product inhibition and/or stability of chromatophores and adenylate kinase. Inhibition of the adenylate kinase reaction is under extensive investigation by the Chemical Engineering group at M.I.T.. ADP was a strong inhibitor (competive for ATP, Ki = 4.72 mM and for AMP, Ki = 0.61 mM) of the adenylate kinase reaction. Inhibition by ATP was severe at low AMP but it was eliminated at high AMP (20 mM), Ki = 0.078 mM (competive for AMP). Inhibition by AMP was competitive for ATP (Ki = 10.96 mM) and dependent on MgATP. Concentrations of inorganic phosphate up to 10 mM gave no significant inhibition of adenylate kinase. In the chromatophore reaction ADP was an inhibitor with K'm (substrate inhibition constant) of 50 mM. ATP was competitive for Pi (Ki = 0.5 mM) and uncompetitive for ADP (Ki = 25 mM) (Pace, 1975). At low ADP concentrations the initial rate of photophosphorylation was decreased by 30 % in the presence of 5 mM ATP (See Table 1).

In the coupled reactions the concentration of ADP is low so that the inhibition by AMP and ATP only are significant. Effect of AMP on yhr initial rate of photophosphorylation of ADP by chromatophores alone were studied. The results showed that there was no inhibition at 1 mM AMP and that the rate was decreased by 10 % at 10 mM AMP. Thus for the majority of experiments of coupled reactions reported here in which AMP levels were 1 mM and, hence, the ATP did

not exceed 1 mM, the inhibition by substrate and product was not significant.

Adenylate kinase was stable, having a half-life of more than 10 days during storage in 0.2 M Tris buffer, pH 7.4 at 25°C (Nemet, 1976). The operational stability of adenylate kinase has been reported to be similar to the storage stability (Quintero-ramirez, 1974). The decrease in rate of production of ATP with time at high AMP concentration is therefore a result of chromatophore inactivation. Thus stability of chromatophores appeared to be the limiting factor in use of the coupled system for ATP regeneration.

4.2.4. Continuous Regeneration of ATP from AMP Using a U.F. reactor.

Continuous regeneration of ATP from AMP in a U.F. reactor is shown using the coupled chromatophores/adenylate kinase system (Figure 26). The membrane employed (PM 10, Amicon) completely retained the adenylate kinase as well as chromatophores in the reactor. The first-order rate constant of the decrease in the continuous production of ATP from AMP was 0.032 hr^{-1} which closely resembles the inactivation rate constant for chromatophores under the same conditions. When the continuous production of ADP from AMP and ATP was performed by adenylate kinase in a U.F. reactor the outlet ADP concentration remained constant for at least 24 hours. This indicates that adenylate kinase is stable during continuous use. Thus the decrease in ATP production in the continuous reactor is due to the inactivation of chromatophores.

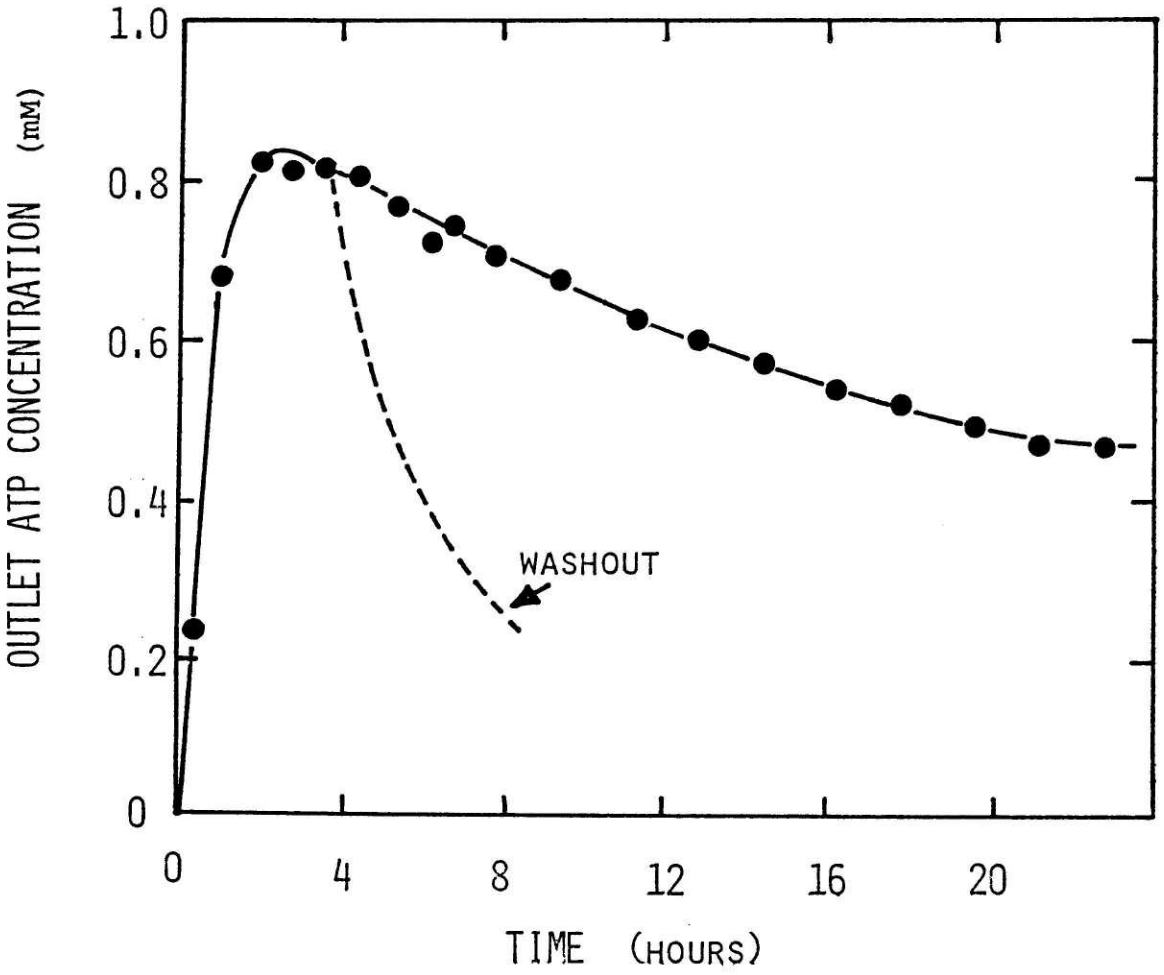


FIGURE 26.

Continuous ATP production from AMP in an ultrafiltration reactor catalyzed by native chromatophores and adenylate kinase.

Conditions: adenylate kinase, 0.043 units/ml; chromatophores, 0.035 units/ml; stirring speed, 500 rpm; residence time, 3.4 hr; and other conditions are the same as in Figure 22.

4.3. Immobilization of Chromatophores.

4.3.1. Preparation of Immobilized Chromatophores by Entrapment in Polyacrylamide Gel.

The effects of using varing conditions in the procedure of entrapment were investigated to achieve a preparation of immobilized chromatophores with high photophosphorylating activity.

Monomer concentration.

The effect of varying the concentration of acrylamide monomer and BIS on the immobilization is shown in Table 6. The highest activity was obtained between 7.5 to 10 % monomer concentration.

Cross-linking degree.

By changing the ratio of concentration of BIS to total monomer the degree of cross-linking in polyacrylamide was varied. The results are shown in Table 7. The activity was highest at a cross-linking of 12.5 %. At higher cross-linking, chromatophores were drived to leak out of the gel matrix during the washing of gel particles. The transparency of polyacrylamide gel is dependent on the total monomer concentration, cross-linking degree, and polymerization temperature (Lamotte, 1974). It was observed that the gels became opaque at cross-linking greater than 7.5 % and those made at room temperature were more transparent than those made at 0°. However, the activity seemed not to be affected by the opaqueness at light intensity used. This point will be discussed further in subsequent section (4.3.2.). The texture of gels was more brittle with less cross-linking and became softer and more fragile with greater cross-linking.

TABLE 6

Effect of Concentration of Acrylamide Monomers on Immobilization of Chromatophores.

Acrylamide Conc. in Gel	Activity Yield	
	Exp. I	Exp. II
%	%	
5		10.0
7.5	9.5	10.5
10	8.5	11.8
15	5.2	2.8

Conditions : C = 5 %, Potter Homogenizer.

TABLE 7

Effect of Extent of Cross-linking on Immobilization of Chromatophores.

Cross-linking	Activity Yield
%	%
5.0	26
7.5	21
10.0	32
12.5	40
15.0	32
25.0	31

At 7.5 % total monomer concentration.

Temperature and pH

Orginally, it was suspected that the activity of chromatophores might be affected by high temperature encountered during polymerization, and the high pH (9.5) resulting from mixing of TEMED and persulfate. A short period of exposure at the high temperature and pH, however, did not diminish chromatophore activity. Rather, an adjustment of pH with concentrated HCl resulted in a lower chromatophore activity presumably caused by high local concentration of hydrogen ions as the acid droplets fall into the bulk solution. Since a transparent gel was obtained at room temperature, the polymerization was carried out at room temperature until completion (approx. 1 minute) and then the resulting gel was kept on ice.

Chromatophore concentration

The effect of varying the concentration of chromatophores in the gel was investigated as shown in Table 8. The yield of activity was not changed up to 10 mg chromatophore protein per ml gel. Further increases in chromatophore concentration in the gel produced a heavy leakage of chromatophores and was accompanied by lower retention of activity.

Breaking of gel

Breaking the gel into small particles is necessary for efficient transfer of substrate and product (and possibly light) to chromatophores inside the gel matrix. Several methods of mechanical disruption of the gel have been attempted and the results are shown in table 9. It

TABLE 8

Effect of Concentration of Chromatophores in Gel on Immobilization of Chromatophores.

Exp. I		Exp. II	
Chromatophore concentration	Yield of activity	Chromatophore concentration	Yield of activity
mg protein/ml	%	mg protein/ml	%
1.2	20.7	3.0	23.6
2.5	19.8	7.5	23.7
3.8	21.5	15	17.9
5.0	21.2	21	13.9
7.5	19.4		

Conditions : T = 7.5 % , C = 7.5 % (Exp.I) or 12.5 % (Exp.II).

TABLE 9

Effect of Method of Gel Disruption on Immobilization of Chromatophores.

Method	Activity yield
	%
Potter, 10 passes	7.0
2 passes	10.7
Polytron	21.9
Waring, 5 sec.	22.2
Manual	29.8

Conditions : T = 7.5 %, C = 5 %.

seems that high mechanical shearing forces hinder chromatophore activity as observed in a Potter homogenizer. Breaking the gel with a spatula by hand, which would in a lower shear force, showed the best retention of activity (measured at high concentration of ADP) but the large particle sizes produced by this method would resist mass transfer at lower substrate concentrations. Homogenation by a Waring blender showed relatively good results. The particle sizes after a 5-second blending period were in the range of 100 to 500 um. Control of particle size was not possible with the methods attempted.

Drying of chromatophore-entrapping gels

Sieving the dried gel particles in a sonic siever with screens of different mesh size allowed the separation of various sizes of immobilized particles. The retention of activity was 12 % for freeze-dried immobilized chromatophores, and 9 % for the vacuum-dried. Freezing and thawing of chromatophores in gel caused about a 50 % decrease in activity. The retention of activity by dried immobilized chromatophores was low compared to 40 % retention by wet-homogenized immobilized chromatophores. The low specific activity by unit weight of the freeze-dried immobilized chromatophores prevents their use in photosynthetic ATP regeneration in spite of their easy handling and storage in the dried state.

Conclusion of the investigation of the preparation procedure

The following condition were chosen for optimal reproducible immobilization of chromatophores:

Total acrylamide monomer (T), 7.5 %; cross-linking degree, 12.5 %; temperature,25° for 1 minite and then cooling at 0°; pH, 7.6 with 0.2 M Tris; 10 mg chromatophore protein per ml gel; homogenation, Waring blender for 5 second; and catalysts for polymerization, 0.2 ml pf 5 % ammonium persulfate and 0.025 ml of TEMED per 4 ml gel. Under these conditions yield of activity was 35 ± 5 %.

4.3.2. Properties of Immobilized Chromatophores.

Immobilized chromatophores were prepared by entrapment in polyacrylamide gel under optimal conditions for high yield as described in previous section and their properties were studied and compared to those of native chromatophores.

pH profile

The pH profile for photophosphorylation activity of immobilized and native chromatophores is shown in Figure 27. The pH optima and profiles were identical,as expected, since the polyacrylamide gel is uncharged. Charged matrices often alter pH optima and profiles of immobilized enzymes because of the change of hydrogen ion concentration in the micro-enviroment of the fixed enzymes (Zaborsky, 1973).

Temperature

Relative activities of the immobilized and native chromatophores at various temperatures are shown in Figure 28. The optima were the same (45 - 50°). There was a slight down shift of the profile for immobilized chromatophores suggesting a temperature difference in the microenviroments.

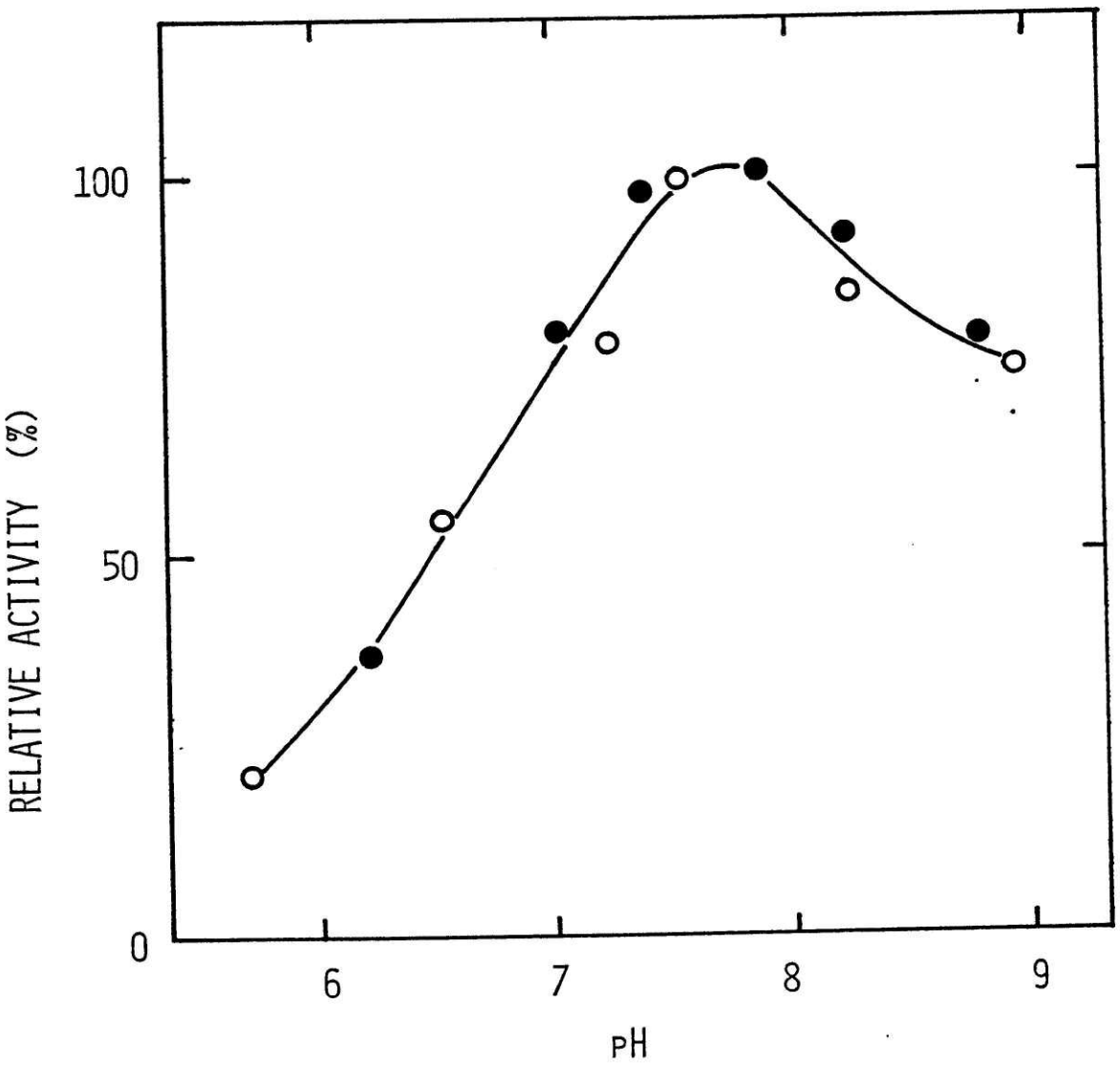


FIGURE 27.

Photophosphorylation by immobilized (O) and native (●) chromatophores at various pHs. The activity at the pH optimum was arbitrarily set at 100 %.

Reaction conditions were as described in the text.

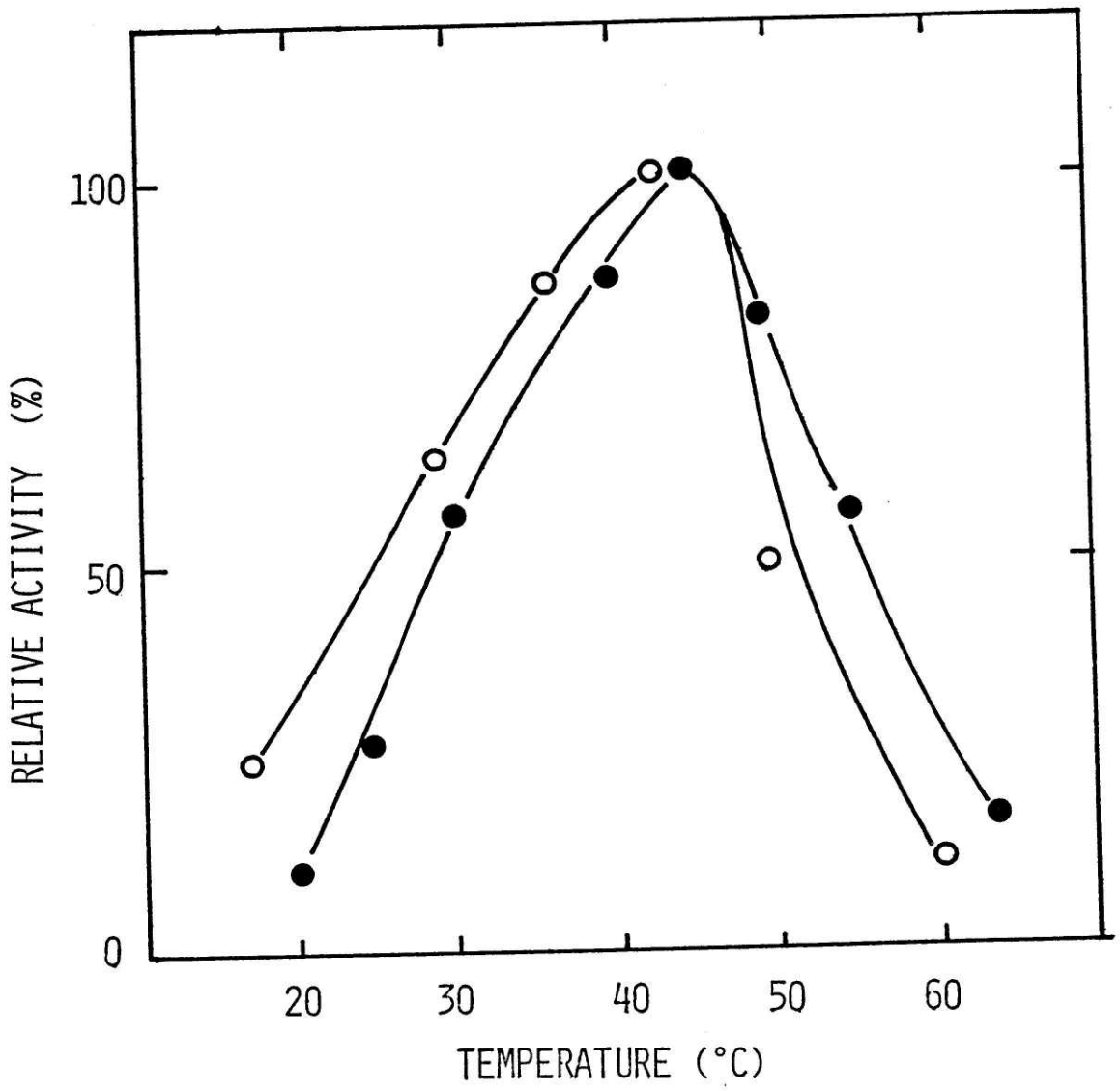


FIGURE 28.

Photophosphorylation by immobilized (○) and native (●) chromatophores at various temperatures. The activity at the temperature optimum was arbitrarily set at 100 %. Reaction conditions were as described in the text.

Substrate concentrations

Photophosphorylation by chromatophores follows Michaelis-Menten kinetics when light is saturating (Nishimura, 1962). With native chromatophores Km for ADP was 0.015 mM. Effects of immobilization on photophosphorylation rate at various ADP concentrations were investigated. Figure 29 is a Lineweaver-Burk plot that shows the reciprocal of the rate of ATP fromation as a function of the reciprocal of the ADP concentration. Vmax for the immobilized chromatophores was 0.54 u moles of ATP/hr/mg of chromatophore protein, compared to 1.32 u moles of ATP/hr/mg of protein for native chromatophores. Thus, in this preparation the yield of immobilized chromatophores was 40 %. Km for ADP was 0.28 mM, or 18 times higher than the Km for native chromatophores. This high Km value indicates that the reaction is controlled by the diffusion of substrate into the gel particles.

Bunting and Laidler (1972) reported that rates of reaction with gel-entrapped enzymes are influenced by particle size as well as composition of gel. The acrylamide concentration in the gel affects the diffusion coefficient of substrate. Gel compositions was optimized for retention of chromatophore activity during the immobilization procedures. A diffudion coefficient was measured by the procedures similar to Bunting and Laidler (1972) using the polyacrylamide gel memebrane between diffusant (ADP) and buffer solution. Diffusion of ADP molecules through the polyacrylamide gel was linear with time. The diffusion coefficient was determined to be 3.0×10^{-6} $cm^2 sec^{-1}$. This value is close to that of reported by Hinberg et al.(1974),who observed a 10 fold increase in Km for the molecule with a similar molecular weight to ADP when the size of gel particle entrapping

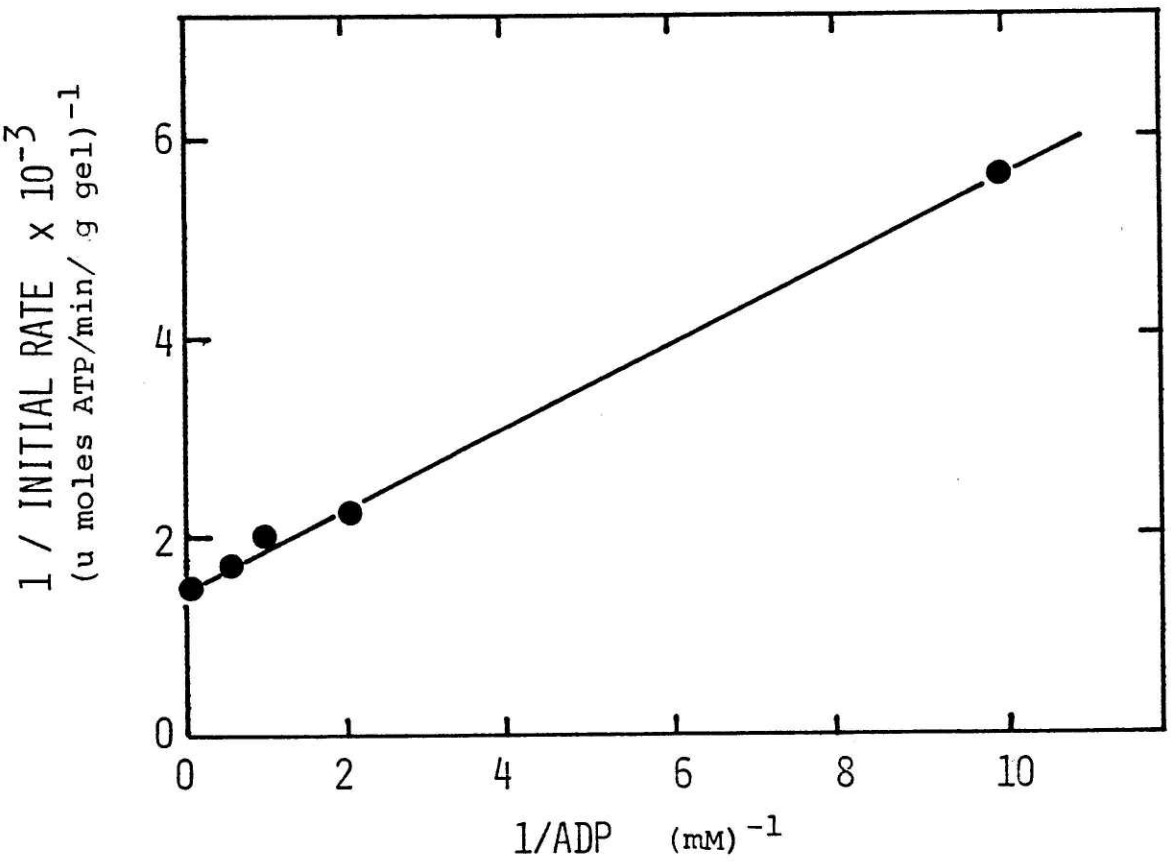


FIGURE 29.

Photophosphorylation rate by immobilized chromatophores at various ADP concentrations (Lineweaver-Burk plot).

Conditions: ADP as indicated ; 10 mM inorganic phosphate; immobilized chromatophores, o.12 gram /ml; and other conditions were as described in the text.

enzyme was 231 um.

As particle size increased the rate of reaction at low ADP concentration (0.5 mM) decreased (Figure 30). This indicates that internal diffusion of substrate becomes rate limiting and is more significant at larger particle sizes. As diffusion control increases in importance the reaction rates at low substrate concentrations decrease and therefore the apparent Km increases with immobilized chromatophores.

Light intensity

Photophosphorylation by immobilized chromatophores requires transfer of light through the reaction medium and the immobilizing material surrounding the chromatophores.

Before the study of light transfer on immobilized chromatophores, effects of concentration of chromatophores in the reaction solution on photophosphorylation rate were investigated at various light intensities. The rate of reaction at various light intensity followed apparent Michaelis-Menten kinetics and Vm and Km for light were determined using a Lineweaver-Burk plot (Figure 31). Vmax was a linear function of concentration of chromatophores within the range employed, 0.24 - 2.0 mg chromatophore protein per ml. Km for light at 0.12 mg/ml was 1.0 x 10^{3} ergs cm^{-2} sec^{-1}, which is close to the Km calculated from a plot by Batscheffsky and Batscheffsky (1971).

Since the absorption of light by chromatophores in the solution was significant (70 % of light at 890 nm admitted is absorbed at the 1 cm of light path) the change in Km for light at various chromatophore concentrations was

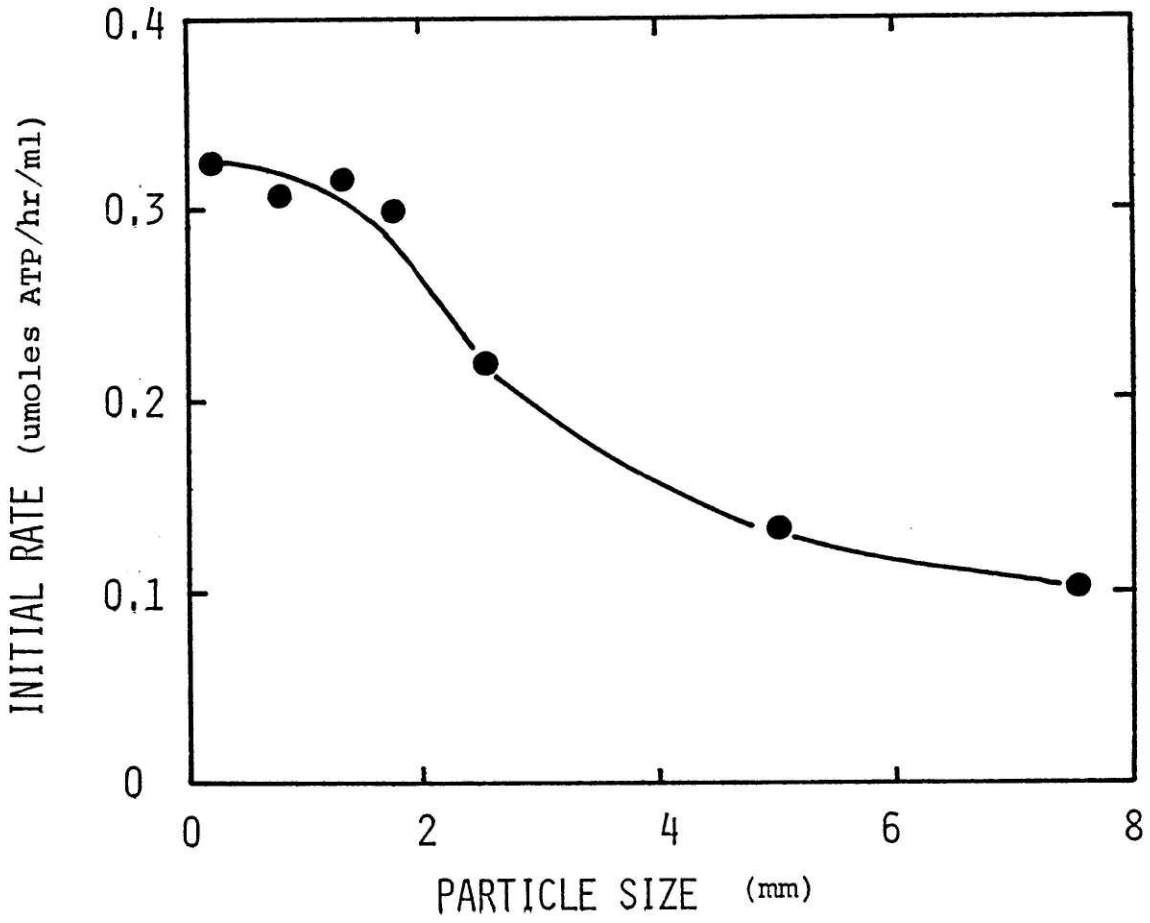


FIGURE 30.

Photophosphorylating activity of immobilized chromatophores with various average particle sizes.

Immobilized chromatophore gel particles were provided by forcing through the syringe needles of various sizes.

Conditions: 0.5 mM ADP; 2.5 mM Pi; immobilized chromatophores, 0.12 gram/ml; and other conditions were same as described in the text.

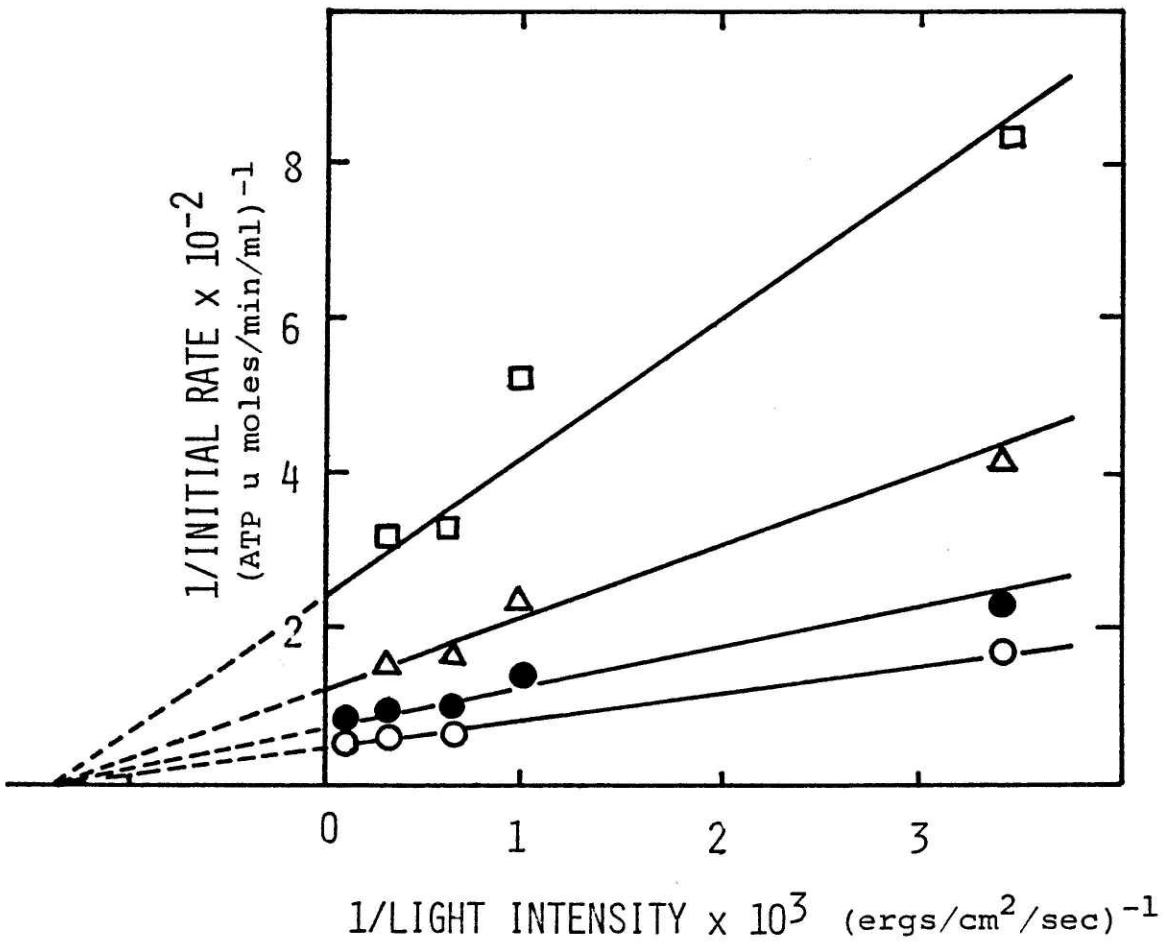


FIGURE 31.

Initial rates of photophosphorylation at various light intensities with different concentrations of native chromatophores.

Chromatophores; 0.24 (□), 0.5 (△), 1.0 (●), and 2.0 mg/ml (○).

Other conditions were as described in the text.

expected. However, Change in the Km could not be detected with the reactor employed within the chromatophore concentrations. The reduction in light due to absortion by chromatophores at high concentrations will increase importance when the size of reactors and thus the light path is increased. This will be dicussed in depth later in Section 5.3.

In photophosphorylation with immobilized chromatophores light has to be transferred through immobilizing materials to chromatophores inside. Thus light transfer depends on transparency of gel and light scattering by gel particles as well as absorbance by chromatophores themselves.

A Lineweaver-Burk plot(Figure 32) of the reciprocal of the rate of photophosphorylation by immobilized chromatophores as a function of the reciprocal of light intensity yielded a Km value of 1.7×10^{3} ergs cm^{-2} sec^{-1}, which is approximately the same as the Km for native chromatophores. This result suggests that light is not hindered in its transfer through the gel suspension.

Transparency was one of the criteria used in selection of supporting material for immobilization of chromatophores. Polyacrylamide can produce transparent gels when prepared under proper conditions (See Section 4.3.1.). However, immobilization conditions which yielded high chromatophore activity produced an opaque gel. Variable transmittance of light through the gel was observed at different wave lengthes (Figure 33.). A high transmittance in the red region of the spectrum was observed even though the gel was visibly opaque.

Thus under the conditions of illumination and particle preparation employed in these experiments, the light intensity

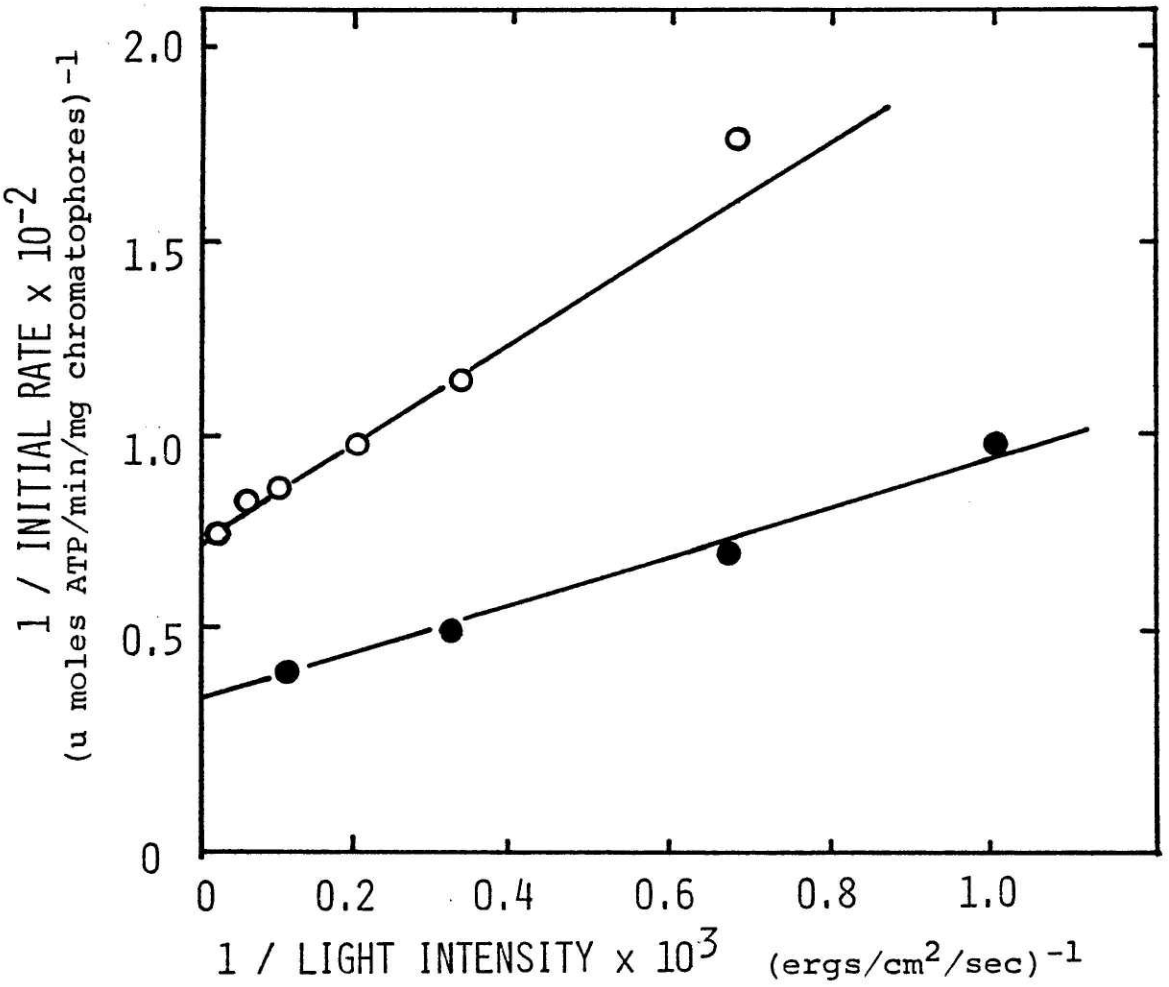


FIGURE 32.

Photophosphorylation by immobilized (O) and native (●) chromatophores at various light intensities.

Conditions; light and chromatophores as indicated, and other conditions were as described in the text.

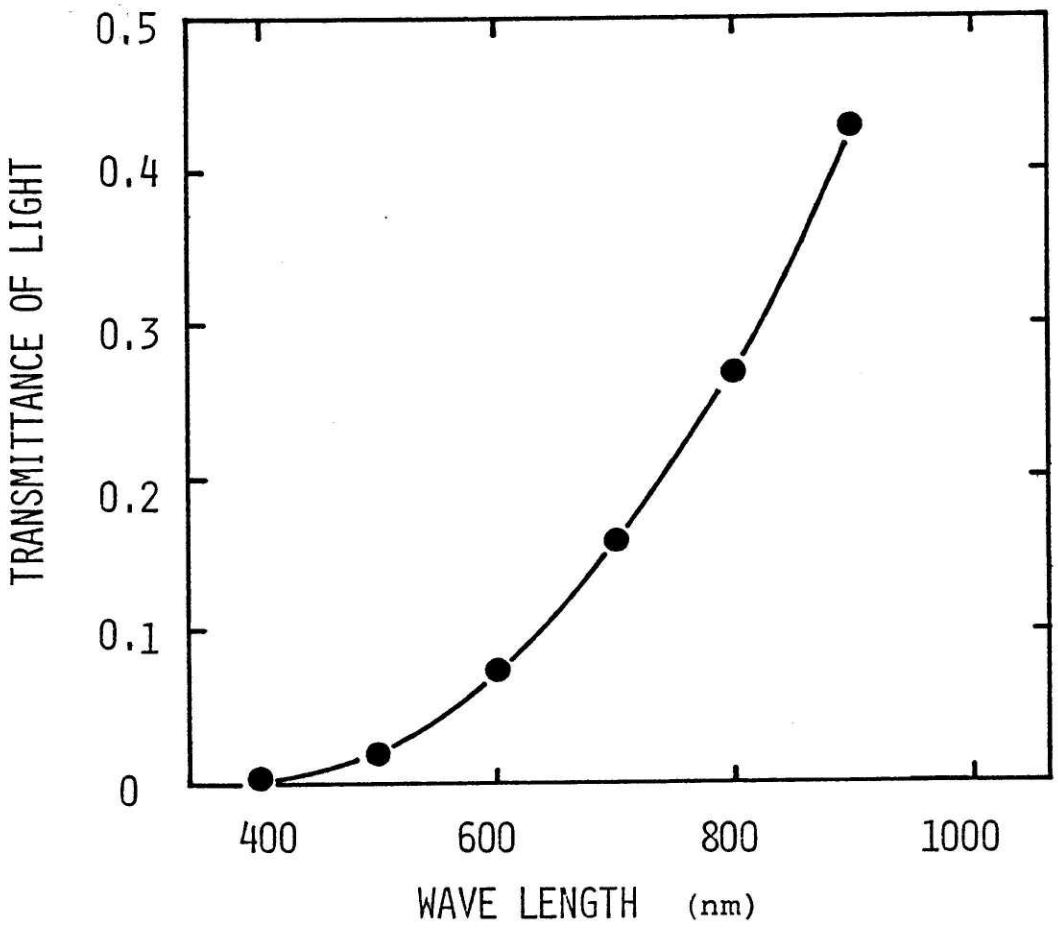


FIGURE 33.

Light teansmittance of polyacrylamide gel at various wavelength.

Conditions: gel was prepared in a cuvett of 1 cm path; chromatophores are not included; and other conditions were as described in the text.

at the optimum wavelength for photophosphorylation (890 nm) was saturating even in gels that visually appeared translucent or opaque.

Storage stability

Immobilized and native chromatophores were stored in 0.1 M Tricine buffer, pH 7.8 for various lengthes of time at 3° and 25° in the dark. Figure 34 shows that the decrease in activity followed first-order kinetics. The half-life of immobilized chromatophores at 25° was 72 hours compared to 12 hours for native chromatophores. Immobilization thus decreased the rate of activity loss by a factor of six. At 3°, immobilized chromatophores lost less than 5 % of their initial activity after 3 days.

4.3.3. Use of Immobilized Chromatophores in Photosynthetic ATP Regeneration.

The immobilized chromatophores were investigated for their use in photosynthetic regeneration of ATP. Of particular concern was the problem of inhibition of photophosphorylation under certain conditions as observed with native chromatophores.

Reactors

Two reactors, a Continuous-flow Stirred Tank Reactor (CSTR) and a packed-bed column reactor, were studied for continuous photophosphorylation by immobilized chromatophores in addition to the batch reactor employed previously. Continuous ATP production by immobilized chromatophores in the CSTR and the column reactor are shown in Figure 35. The

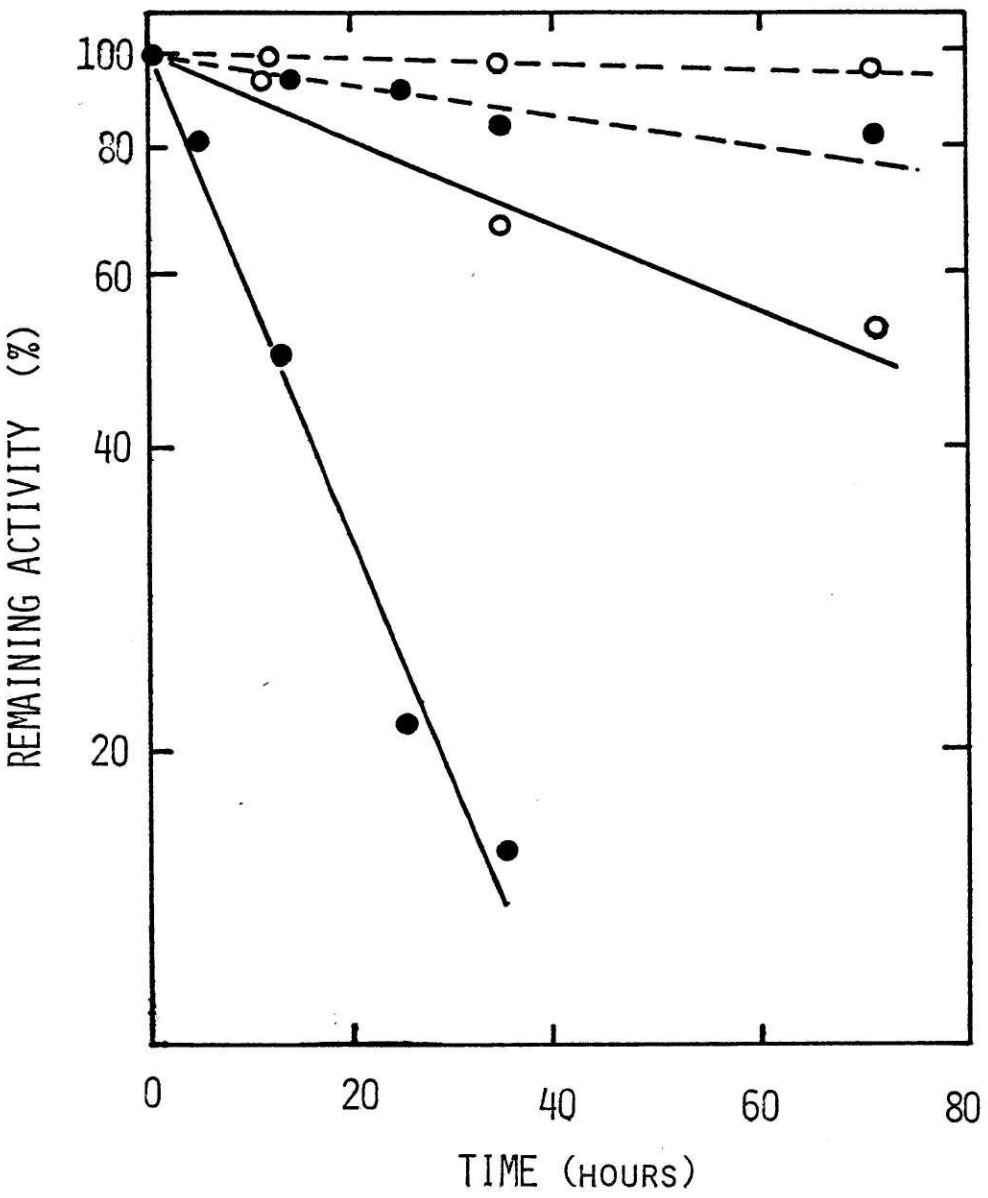


FIGURE 34.

Stability of immobilized (O) and native (●) chromatophores at 3° (dotted line) and 25° (solid line).

Conditions: immobilized chromatophores were stored at the specified conditions in 0.1 M Tricine buffer, pH 7.8 in the dark; samples were taken at various times and then assayed for their photophosphorylating activity.

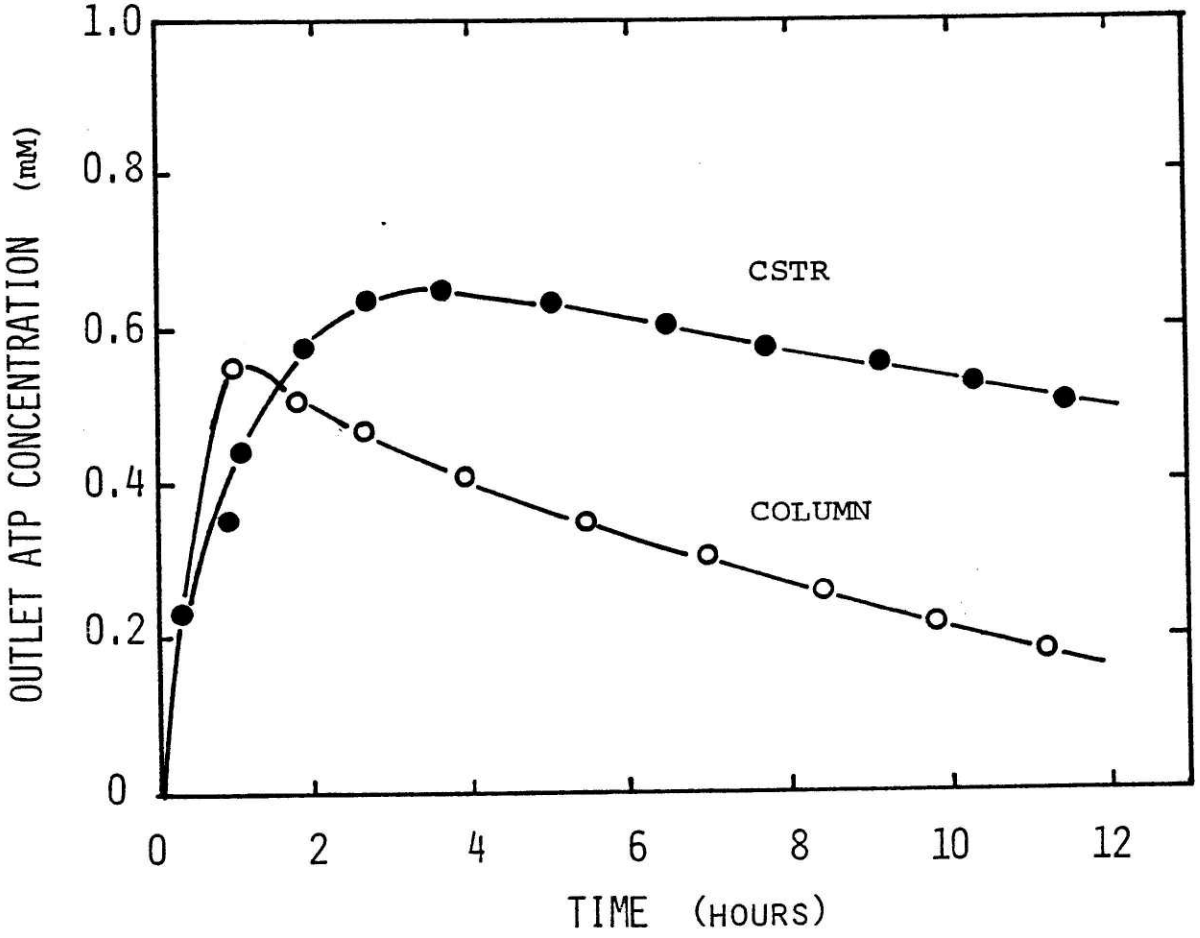


FIGURE 35.

Continuous ATP production by immobilized chromatophores in a Continuous-flow Stirred Tank Reactor (CSTR) and a packed-bed column reactor (Column).

Reactor configuration: CSTR; an ultrafiltration cell with a polypropylene plate(pore size, 10-30 u), 12.5 ml liquid volume : Column; 0.9 cm diameter x 15 cm lenght with a Nylon net (pore size, 10 u), bed volume 3.5 ml.

Conditions for both reactors: total chromatophore activity, 6.0 units(hr); flow rate, 8.9 ml/hr; 1 mM ADP; and other conditions were as described in the text.

total units of immobilized chromatophore activity were the same for both reactors with 1 mM ADP and 8.9 ml/hr flow rate for each. All other conditions including reactor configurations are described in the figure legend. The outlet ATP concentration reached the maximum more rapidly in the column reactor than in the CSTR due to the smaller space volume in the column. Then ATP rpoduction in the column reactor decreased rapidly whereas that in the CSTR remained fairly stable over a period of 12 hours. The rapid decrease in the column reactor is probably a result of a temperature gradient (and possibly light) across the unstirred gel bed while stirring in the CSTR kept the temperature at all points inside the reactor the same as the constant temperature water bath. The rate constant of the decrease in the CSTR ($k = 0.028\ hr^{-1}$) was similar to that determined for thermal inactivation of immobilized chromatophores in a batch reactor. A decrease of stirring speed from 500 r.p.m. to 350 r.p.m., which still allowed enough mixing, gave a only slight improvement in stability. Even though the mechanism of the rapid decrease in the column reactor was not investigated in detail, it is obvious that the CSTR is better in performance for continuous photophosphorylation than the column reactor.

Flow rate

In the early stage of the study of immobilized chromatophores in the CSTR, the flow rates employed were in the same range as those for native chromatophores in the U.F. reactor. At these flow rates (3-4 ml/hr) ATP production appeared to reach to a maximum at 2 hours and this was

followed by a rapid decrease (Figure 36). In fact, the photophosphorylation stopped completely and ATP washed out of the reactor. This indicated that the loss of photophosphorylation activity was enhanced by immobilization at those flow rates. As the flow rate increased to 11 ml/hr, the stability of the photophosphorylation activity of immobilized chromatophores increased.

A possible explanation for the effect of the flow rate is that diffusion of the proposed inhibitor produced during the photophosphorylation is resisted by the gel and the rate of the inhibitor removal is enhanced by the high rate of flow. This effect can be shown mathematically as following.

The flux (J) of the inhibitor(s) through the gel particles is,

$$J = D/X\ (I-I_o) \quad \text{-------- (Eq. a)}$$

where D is the diffusion coefficient of the substrate within the gel, X is the thickness of the particles, and I and I_o are the inhibitor concentrations inside and outside (bulk solution) of the gel particles, respectively.

In the CSTR, the change of inhibitor concentration with time ($\frac{dI}{dt}$) is,

$$\frac{dI}{dt} = -\frac{Q}{V} I_o + \frac{D}{X} (I-I_o) \quad \text{------- (Eq. b)}$$

where Q is the flow rate, V is the reactor volume, and I, I_o, D, and X are as described above. At the steady state, $\frac{dI}{dt} = 0$. Thus,

$$I_o = \frac{I}{\frac{Q/V}{D/X} + 1} \quad \text{----- (Eq. c).}$$

(The inhibitor concentration may not be reached to the steady state at all cases. To explore the hypothesis, we

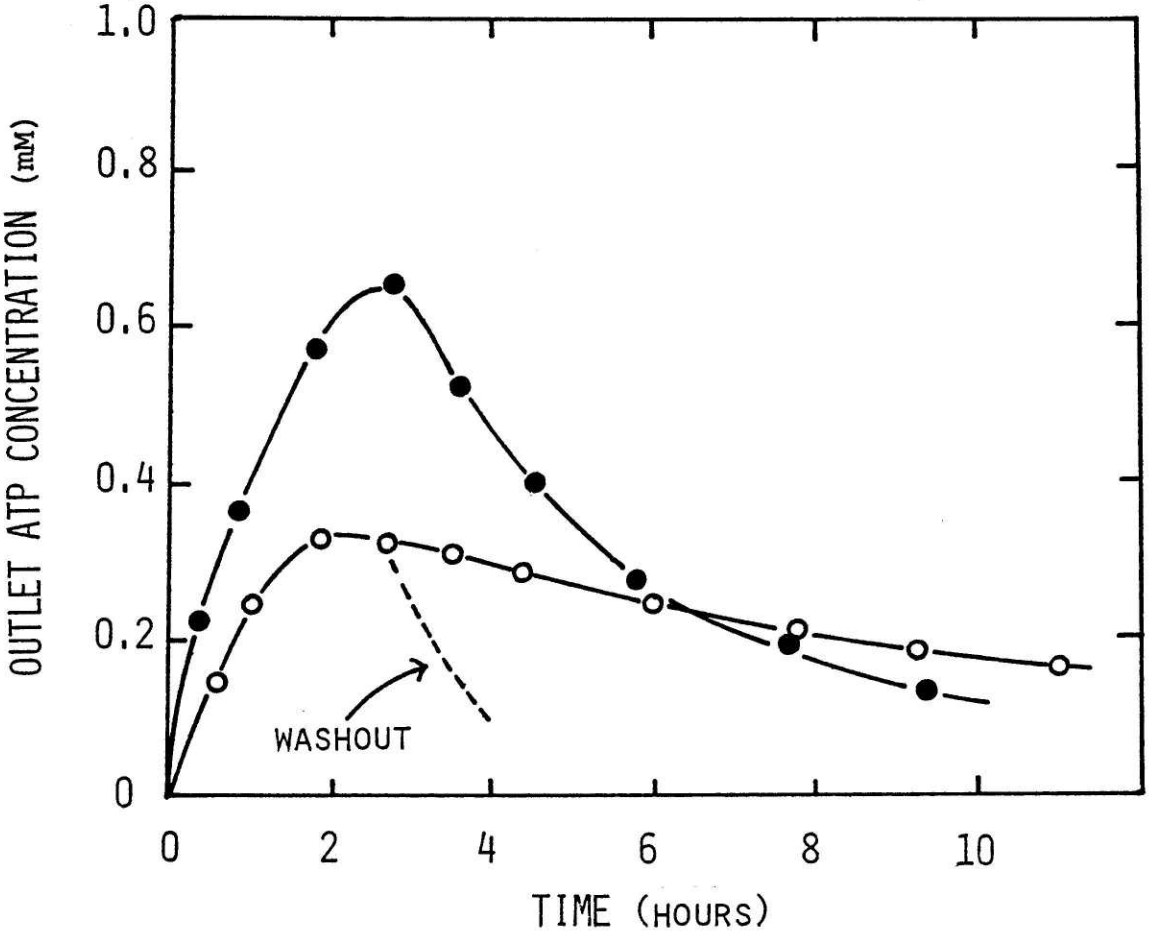


FIGURE 36.

Effect of flow rate on continuous ATP production by immobilized chromatophores in a CSTR.

Flow rate: 3.1 (●),and 11.1 ml/hr (○).

Conditions: 1 mM ADP; total chromatophore activity, 5.2 units(hr); and other conditions were as described in the text.

are making the assumption.)

The experimental values obtained were V, 12.5 ml; and X, 100 u (The gel particle sizes were 100-500 u. Thus the average distance (X) from the chromatophores to the gel particle surface is assumed to be 100 u.). D for the inhibitor is, of course, unknown. Determined D for ADP within the polyacrylamide gel was 3.0×10^{-6}cm sec^{-1}. This value is used for the following calculation. At the flow rate of 11.1 ml/hr, (Eq.C) yields I_o=0.25I; whereas at Q=3.1 ml/hr, I_o= 0.55I. Substitution of these I_o values into(Eq.a) shows that the increase of flow rate from 3.1 to 11.1 ml/hr produces a 2.2-fold increase in the flux (remaval). If D for the inhibitor is greater than that for ADP, the difference in the inhibitor concentration (I_o vs I) will be smaller.

Immobilized chromatophores after reaching the apparent equilibrium of photophosphorylation in batch reactor did not show any significant activity when the activity was measured by adding more substrate to the reaction solution. The reactant solution after removal of chromatophores, however, did not inhibit the activity of fresh chromatophores. This effect was also observed with native chromatophores by Pace(1975). Inhibitor(s) bound to the chromatophores were suggested as an explanation

Washing of native chromatophores after reaction can not be achieved since repeated washing and centrifuging resulted in a reduction (more than 50%) of activity. However, when immobilized chromatophores after photophosphorylation were washed twice with 0.1M Tricine buffer,pH 7.8 and then their activity was measured, 60-80 % of activity was recovered. It appears that immobilization protects the integrity of chromatophores during the recovery procedures. According to the above results, the inhibitor appeared to be bound loosly to the chromato-

phores and thus can be removed by high flow rates in the CSTR or thorough washing.

Concentration of substrates and other reactants

To obtain high conversions of substrates to product at high concentrations, long residence times and/or high chromatophore activities are necessary. An increased residence time can be obtained in a constant-volume reactor by decreasing the rate of flow, which, however, is not desirable in this case for reasons discussed previously. The maximum concentration of immobilized chromatophore particles for good mixing in the CSTR was 0.2 gram gel/ml. Thus, the highest activity possible in the reactor was 0.3 - 0.6 u moles ATP/hr/ml. This low activity yielded product concentration of less than 1 mM at the desirable flow rates (above 8 ml/hr). Thus with these restictions on the reactor parameters, conversions at high concentrations of ADP would be too low to be of any value.

Increasing the concentration of fumarate from 1 mM to 10 mM resulted in a slight increase in ATP production (Figure 37). Anaerobic conditions were necessary for optimum photophosphorylation activity of native chromatophores. There was no difference, however, between aerobic and anaerobic conditions for ATP production by immobilized chromatophores in the CSTR (Figure 37). This suggests that either the immobilized chromatophores are less sensitive to the red-ox potential change or the differneces in the microenviroments of the native and the immobilized chromatophores can produce different red-ox potentials.

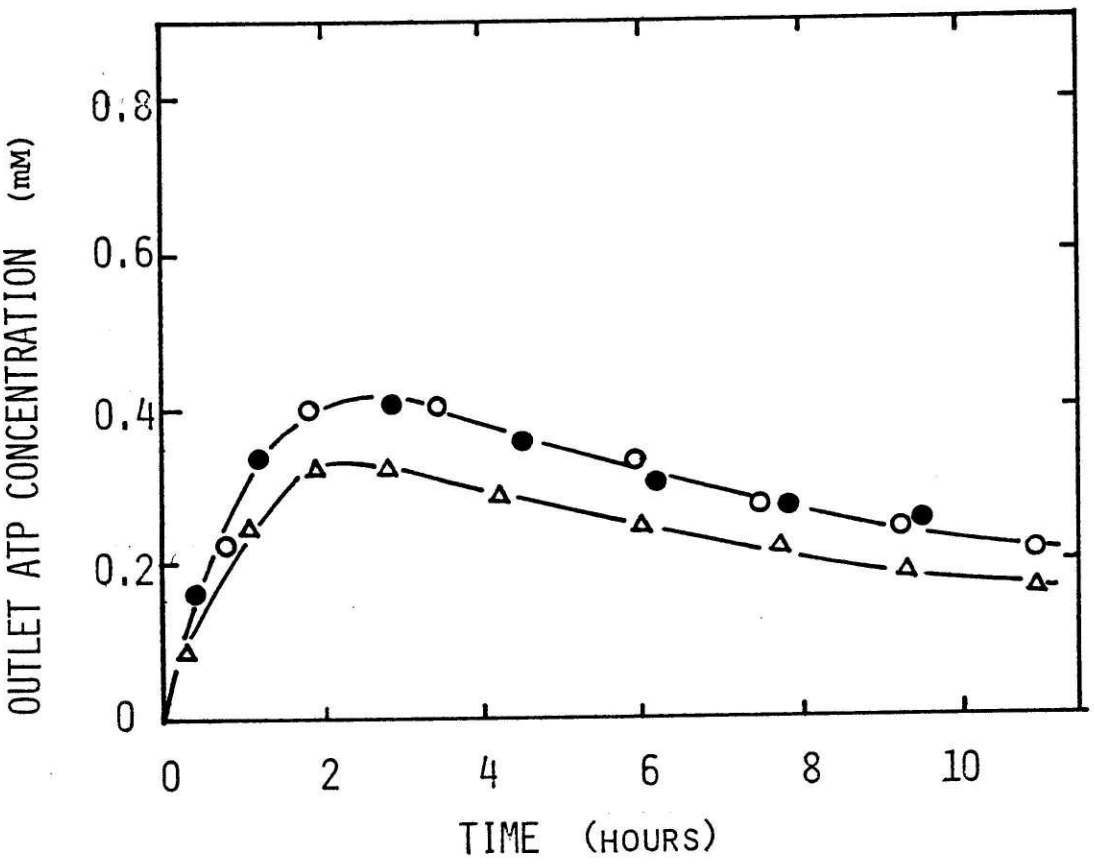


FIGURE 37.

Effect of fumarate concentration and anaerobic conditions of reactants on continuous ATP production by immobilized chromatophores in a CSTR.

Fumarate: 1 mM, aerobic (Δ); and 10 mM, aerobic (O) or anaerobic (●).

Conditions: flow rate 11 ml/hr; other conditions were the same as in Figure 36.

4.3.4. Co-immobilization of Adenylate Kinase and Chromatophores for Regeneration of ATP from AMP.

An attempt has been made to co-immobilize adenylate kinase and chromatophores together in a single matrix to produce ATP from AMP. In addition to providing a system in which chromatophores and enzyme activity are retained, if the two activities are in close proximity, the intermediate substrate, ADP, may be transformed by the chromatophores before it has time to diffuse into the surrounding medium. The net effect will be a higher yield of product per unit time as compared to native enzymes. This type of effect has been demonstrated with a two enzyme sequence consisting of hexokinase and glucose-6-phosphate dehydrogenase (Mosbach and Mattiason, 1970).

When adenylate kinase was entrapped in polyacrylamidee gel by the procedures yielding a high chromatophore activity 10 - 20 % of the enzyme activity was retained. This low yield of activity can be possible improved by optimizing the preparation procedures as for the chromatophores.

Co-immobilized Baker's yeast adenylate kinase and R. rubrum chromatophores in a polyacrylamide gel have produced ATP from AMP and inorganic phosphate under illumination. The specific activity of the immobilized particles was 0.5 u moles of ATP/hr/gram of wet gel.

Due to lack of time, the kinetics of the co-immobilized coupled system were not investigated further.

5. DISCUSSION

5.1. Possible Mechanisms for the Decrease in Photophosphorylation Activity of Chromatophores.

Bacterial chromatophores, which were confined in a U.F. reactor, catalyzed continuous photophosphorylation for the regeneration of ATP. A decrease in ATP production with time was observed resulting from chromatophore inactivation. An understanding of the mechanism for the inactivation would faciliate a more$_{efficient}$ use of the chromatophores in photosynthetic regeneration of ATP. Studies which have appeared in the literature on chromatophores have been mainly concerned with the mechanism and kinetics of photosynthesis. The mechanism of photophosphorylation is not well understood and is under extensive study by several investigators. Chromatophores in most of these studies are used for relatively short periods of time and no reports are available on stability of the photophosphorylating activity.

The rate of activity loss for the chromatophores during continuous photophosphorylation was much greater than for those incubated without reaction under similar conditions. This suggests that different mechanisms are involved in the chromatophore inactivation and the photophosphorylation process itself influences the activity loss. Chromatophores in the U.F. reactor lost a large portion of their initial activity in the early phase of continuous photophosphorylation. This loss in activity took place more slowly in the latter phase. Thus chromatophores appear to be inactivated by two different mechanisms.

a) Early phase inactivation.

The outlet ATP concentration of the U.F. reactor reached a maximum level in the early phase (3-4 hours). The maximum concentration of ATP increased when the concentration of ADP, the limiting substrate, was increased. The increased ATP concentration was a result of the increase in photophosphorylation in the reactor. However, increased amount of photophosphorylation in the early phase appeared to cause a rapid decrease in chromatophore activity, reducing the outlet ATP concentration with time. This rapid decrease in chromatophore activity was also observed in the batch reactors at the latter stage of reaction, lowering the apparent equilibrium conversion, when high concentrations of ADP (above 1 mM) were employed.

Above results indicate that chromatophore inactivation is associated with photophosphorylation and that the rapid inactivation is a consequence of a increased amount of photophosphorylation. A possible explanation for the photophosphorylation-associated inactivation is an inhibition by some factor(s) produced during the photophosphorylation process. Pace (1975) reported an inhibition which lowered the apparent equilibrium conversion. This inhibition was completely relieved by gas bubbling or addition of fumarate. Formation of a volatile inhibitor during photophosphorylation, the synthesis or activity of which is affected by the addition of fumarate, has been suggested. All of the reaction solutions used in this thesis contained 1 mM fumarate. Fumarate, however, relieved the inhibition completely only when ADP concentrations were less than 1 mM and the fumarate effect diminished at high ADP concentrations.An initial increase in fumarate concnetration

or addition during the latter stage of the reaction did not raise the apparent equilibrium conversion. These findings together with those by Pace suggest that the fumarate effect is only partial at increased photophosphorylation with high ADP concentration.

In the U.F. reactor dilution of the reaction solution containing native chromatophores with incoming reactant solution did not wash out the inhibitor(s). Pace(1975) reported that the supernatant from the batch reaction solution after photophosphorylation did not inhibit the chromatophore activity. These studies suggest that the inhibitor is not released into the medium but is bound to the chromatophores.

The immobilized chromatophores in the continuous reactors lost their activity very rapidly after reaching maximal production of ATP. This occured in the early phase and when flow rates were the same as those for the U.F. reactor with native chromatophores. It appears that immobilization enhanced the inhibition. Removal of the inhibitor(s) produced during photophosphorylation from the chromatophores seemed to be hindered by the acrylamide gel layer surrounding the chromatophores. This case is similar to the effect of product inhibition on immobilized enzymes (Engasser and Horvath, 1974). Diffusion resistance can give rise to both substrate depletion and product accumulation in the micro-enviroment of the enzyme. No quantitative determination of the inhibition was attempted in the present study since the identy of the inhibitor(s) is not known and the kinetic analysis of product inhibition in immobilized enzymes has been reported to be very complex (Goldman et al., 1968)

Identification of the inhibitor(s) is desirable in order to control the inhibition. No attempt was made to investigate the inhibitor since it was beyond the scope of the present study. The results lead to a hypothesis that some factor(s) produced during photophosphorylation is responsible for the inhibition. By studying initial rates and final equilibrium conversions of the photophosphorylation in the presence of various ATP concentrations it was found that the product(ATP) is not the inhibitor.Chromatophores contain multienzyme systems for electron transport and phosphorylation as well as photoreactive centers such as bacteriochlorophylls. All these are known to be involved in photophosphorylation (Batscheffsky and Batscheffsky, 1971). Considering the series of reaction which occur during photophosphorylation it would not be surprising if there were by-products along the reaction pathway, and if any of these could inhibit the reaction(s) leading to the final phosphorylation.

b) Latter phase inactivation.

Inactivation of chromatophores in the latter phase (after 3 hours) is much slower giving only 10 - 20 % decrease in ATP production in 10 hours. The rate of this inactivation was comparable to that for the chromatophores incubated in the reaction medium under illumination but without photophosphorylation.

The hydrolysis of ATP to ADP by ATPase is a reversible reaction of the same enzymes which catalyze ATP synthesis in chromatophores (Hochman and Cameli, 1973). The reversal of phosphorylation by ATPase activity of chromatophores,

which was suggested by Pace(1975) as a possible mechanism for inhibition of ATP production, was not a significant cause of the decrease under illuminated conditions. The activity of ATPase in the chromatophores during active photophosphorylation can be determined by using radioactivity-labelled ATP such as (γ-^{32}P)ATP employed by Hochman and Cameli (1973). Appearance of radioactive Pi indicates the active ATPase activity.

Chromatophores, whose activity were not inhibited in the early phase, could lose their activity through the following changes, depending on the enviromental factors:

1. Formation of aggregates as observed by development of a heavy fraction on a sucrose density gradient in chromatophores from the U.F. reactor.

2. Disintegration and separation of soluble factors from the chromatophores. The presence of soluble factors such as phosphodoxin (Black et al., 1963) and coupling factors involved in phosphorylation (Green, 1963) whose separation from the chromatophores destoryed their activity has been reported.

3. Denaturation of chromatophore components i.g. enzymes and pigments.

The value of activation energy for the chromatophore inactivation was between the protein denaturation and the simple chemical reactions. This suggests that the chromatophore inactivation is not simply a result of the denaturation of protein material. Chromatophores have a complex composition including protein, lipid, and pigments. The intimate structual arrangement of these components within the membrane of the photosynthetic cell is not understood. We may assume, however, that the relative configur-

ation of components necessary for photophosphorylation is critical for activity. It seems likely that the inactivation energy reported here is a reflection of dissociation of internal structure possibly involving a number of components.

The effect of light intensity on chromatophore inactivation is probably a result of an increase in internal temperature of the chromatophores due to light absorption by pigments. Light might also cause a photochemical denaturation of the chromatophore components such as pigments.

The activity of the chromatophores during continuous use was affected by enviromental factors - composition of the medium, flow rate, stirring, light, pH, and temperature. Chromatophores, like cell organelles, posses various components capable of biochemical reactions. The enviromental factors then could cause changes not only in the biochemistry of the photophosphorylation process but also in the structure of chromatophores.

5.2. Immobilization of Chromatophores.

Recently there have been numerous reports on immobilization of enzymes and their applications. The reusability of immobilized enzymes allows various modes of the continuous reactor. Immobilization often increases the stability of enzymes. Chromatophores have been successfully immobilized by entrapment in polyacrylamide gel for the first time in this study. The immobilized chromatophores were fully capable of photophosphorylation.

Selection of immobilizing material for the chromatophores has a limitation: it should be transparent so that light can be transferred to the chromatophores. Polyacrylamide, which forms a gel by cross-linking, was chosen as material for the entrapment of chromatophores. Polyacrylamide gel is hydrophilic and neutrally charged as well as transparent if prepared under certain conditions. The gel is also chemically stable and has a fairly good mechanical stability for use in reactors. These characteristics of the polyacrylamide gel allow a favorable microenviroment for chromatophore photophosphorylation. Whole cells (Franks, 1972 and Yamamoto et al., 1974) and macromolecules such as necleic acids (Cavalieri and Carroll, 1970), antigens and antibodies (Goodfriend et al., 1969), and enzymes (Zaborsky, 1973) have been successfully immobilized by entrapment in the interstitial space of crosslinked water insoluble polymers.

Conditions employed in the preparation of immobilized chromatophores affected the characteristics of the gel as well as the retention of photophosphorylating activity. The transparency of the polyacrylamide gel depended on the the degree of cross-linking and the polymerization temperature.

Monomer concentration is also known to influence the transparency (Lamotte, 1974). The gel prepared giving the optimal conditions for high activity was, however, visibly opaque. This was explained by the fact that the gel had a high transmittance in the red region of the spectrum even though it had low transmittance overall in the visible region. Photophosphorylation by chromatophores from R. rubrum has an absorption maximum at 890 nm at which wavelength the gel was quite transparent.

The total acrylamide monomer concentration and cross-linking degree influenced the mechanical stability of the gel. A high concentration of chromatophores in the gel or increased cross linking caused a leak of chromatophores out of the gel matrices. This suggests that there is a saturation concentration of chromatophores for the entrappong sites in the gel matrices.

There was an optimal concentration of total monomers and cross linking for retention of high photophosphorylating activity. Immobilized chromatophores lost a large portion of their activity at high shear when the gel was broken into small particles. By optimizing the conditions immobilized chromatophores retained 40 % of their activity in the native state. The reduction in activity is probably a result of changes not only in the microenviroment but also in the chromatophores themselves. Activity retention of gel entrapped enzymes is dependent on the preparations but usually less than 30 % (Zaborsky, 1973). Thus the immobilization procedure developed gives better retention of activity than most other similar studies with enzymes.

Immobilization often alters the properties of enzymes.

Effects of pH, temperature, substrate concentrations, light intensity, and stability on photophosphorylation by immobilized chromatophores have been investigated in this study. The pH profile of the immobilized chromatophores was the same as that of native chromatophores. This is an expected behavior since the polyacrylamide gel has an electrically neutral character and hence the hydrogen ion concentration in the microenviroment would be the same as in the bulk solution. The slight shift in temperature profile toward lower temperatures probably derived from a slight elevation of the internal temperature of the gel particles as a result of illumination.

In most immobilized enzymes reaction rates are controlled by electrical effects and diffusion of the substrates (Hornby et al., 1968, Bunting and Laidler, 1972, and Hinberg at al., 1974). The Km for ADP was higher in photophosphorylation by immobilized chromatophores than by native ones. For immobilized chromatophores entrapped in polyacrylamide gel, which is neutral in charge, the main cause of change in Km is the diffusion of substrate. Substrate molecules have to be transferred from bulk solution to the external surface of the immobilized chromatophore particles (external diffusion) and then into the interior of particles where the chromatophores are and where the reaction occurs(internal diffusion). External diffusion effects depend on fluid velocy near the particle surface and can be eliminated by stirring or a high flow rate (Hinberg et al., 1974). The rate of ADP transfer for the chromatophore reaction is dependent on the diffusion coefficient of ADP within the gel, the distance between the surface and the inside where the reaction occurs,

and the concentration gradient of ADP over this distance. The determined diffusion coefficient of ADP (3.0×10^{-6} $cm^2 sec^{-1}$) is close to the one reported for a molecule of similar size (Hinberg et al., 1974) where a 10 fold increase in Km was observed with particles of 231 um size. As the particle size of immobilized chromatophores increased, the rate of photphosphorylation decreased. It is evident from these results that internal diffusion becomes increasingly important with increasing particle size. There is also a possibility that diffusion affects the removal from the chromatophores in the gel of by-products which is hypothesized to be produced during photophosphorylation and to inhibit the chromatophore activity.

Another problem which needs to be considered in the kinetics of the immobilized chromatophore reaction is the transfer of light. Light has to penetrate to the chromatophores inside the matrix. This is analogous to the mass transfer in immobilized chromatophores as dicussed previously or in mycelial pellets during fermentation. The light transfer property of the polyacrylamide gel and its effect on chromatophore reactor are discussed elsewhere in this thesis.

By immobilization the chromatophores were enhanced in their stability during storage in buffer (a 6-fold increase in half-life at 25°). Immobilization probably protects the integrity of chromatophores. The increased activity loss during photophosphorylation by immobilized chromatophores and the effect of high flow rate on reduction of the loss are discussed in Section 5.1.

Chromatophores were co-immobilized in a polyacrylamide

gel matrix with adenylate kinase. The co-immobilized two-enzyme system was capable of regeneration of ATP from AMP and inorganic phosphate utilizing light energy. This kind of multienzyme system immobilized in a single matrix can mimic in vivo biological systems (Mosbach, 1972).

Arkles and Bringar (1975) have recently reported the immobilization of rat liver mitochondria by adsorption to the surface of alkylsilylated glass beads. The immobilized mitochondria exhibited normal respiratory behavior and remained viable at 27° for periods up to 4 hours. The immobilized bacterial chromatophores displayed biochemical characteristics similar to the native photosynthetic apparatus. Immobilized organelles of this type may be useful for studying the biochemistry of membrane particles in model systems that simulate natural enviroments within the cell, as well as for commercial production of complex biological materials.

5.3. Chromatophore Reactors in Photosynthetic Regeneration of ATP.

Many different types of reactor have been used with enzymes either in their native or immobilized forms. Lilly and Dunnil (1971 and 1972) have discussed the factors affecting the choice of various modes of enzyme reactor as well as general biochemical reactors. The continuous reactors employed for photosynthetic ATP regeneration in this study were the ultrafiltration cell (U.F.) reactor for native chromatophores and the packed-bed column reactor and the Continuous-flow Stirred Tank Reactor (CSTR). Only the unique

problems to be solved in a reactor utilizing chromatophores are considered here.

One of the unique problems is an efficient transfer of light. The chromatophore reactor must be designed in such a way that utilization of light energy is most efficient. For example, materials for the reactor wall facing the light source should be transparent, in particular in the red region of the light spectrum where maximal photophosphorylation by the chromatophores from purple bacteria occurs.

In the immobilized chromatophore reactor, the absorption of light by the gel and the chromatophores results in the decreased light intensity. This decrease is more important as the reactor becomes large. Thus the light transferred to the chromatophores depends on the size and geometry of the reactor and the concentration of the immobilized chromatophores, as well as the intensity of the light on the surface of the reactor. Since no report was found for this problem, the following equation has been derived to describe light transfer ,using the Beer-Lambert law;

$$I = I_o / e^{kcl}$$

where I is the light intensity at a specific point on the light path in the reactor, I_o is the light intensity on the surface of the reactor, c is the concentration of the immobilized chromatophores, I is the distance from the surface to a specific point on the light path, and k is a constant. I_o is measured at a specific wavelength i.g. 890 nm and is a function of the intensity of light source and the distance from the source to the reactor surface. The constant, k, depends on the light transmittance (or absorption) of the wall material, gel particles, and

chromatophores. By integrating the above equation and then deviding it by the total length of light path in the reactor (L), the average light intensity (I_{ave}) for the reactor can be obtained i.e.

$$I_{ave} = \int_{o}^{L} (I_{o}e^{-kcl})dl \; / \; L$$

This will represent the true light intensity for the photophosphorylation by chromatophores in a reactor.

Another problem, which would not occur in general with enzyme reactors, is a temperature increase caused by heat generated upon illumination. Not only the radiant heat transmitted by the light source but the heat as a result of light absorption by chromatophores should be considered in mataining the reactor temperature at the optimum.

Successful operation of a U.F. reactor is dependent on the permeability characteristics of the membrane employed Ideally the memebrane should retain the chromatophores co completely but allow free passage of products. Chromatophores. were well retained by the membrane employed, PM10 (Amicon). Pace (1975) reported that PM10 is permeable to ATP but UM10 partially rejects ATP. However, the results in this study showed that a substantial amount of ATP accumulates on the PM10 membrane until a stedy-state was reached. ATP rejection was enhanced by reapeated use of the membrane.

Another limitation in the use of the ultrafiltration cell for the chromatophore reactor was that it allowed only low flow rates which possibly limited the inhibitor removal. The formation of a chromatophore gel layer on the membrane, particularly at high pressure, made a small fraction of chromatophores not available for the photophosphorylation and also increased the pressure drop which lowered the flow rate even further.

It was possible to obtain a wide range of desirable flow rates in the immobilized chromatophore reactors in which much larger gel-entrapped chromatophore particles were retained by a Nylon mesh scrren or plastic plate with pore size of 10-30 u. The outlet concentration of ATP during continuous photophosphorylation by immobilized chromatophores decreased with time more rapidly in the column reactor than in the CSTR. The poor performance of the column reactor may be a result of a temperature gradient across the gel bed and/or a resistance in the inhibitor removal due to lack of stirring. The high flow rates reduced the inhibition of photophosphorylation activity in the CSTR and thus the productivity of the reactor was comparable to the initial catalytic activity of the immobilized chromatophores.

One of the drawbacks to using immobilized chromatophores for continuous ATP regeneration is a low catalytic activity per unit weight (4 - 7 u moles ATP/hr/gram of immobilized particles) because of the limit in chromatophore loading in the gel. To achieve good mixing the concentration of immobilized chromatophores in the reactor also has to be limited. Thus, a high volumetric productivity of the reactor can not be achieved. To increase the specific activity of the chromatophores culture conditions of R. rubrum can be modified (Cohen-Bazier and Kunisawa, 1960). It wil also desirable to look for different photosynthetic organisms which give photosynthetic apparatus with high specific activity.

By coupling the chromatophores and the adenylate kinase in a single reactor a complete conversion of AMP to ATP was achieved. The steady-state rate of ATP production by the

coupled system was the same as that predicted by the rate equations for the batch reactor (Appendix II). It appears that the limiting step in the coupled reaction is photophosphorylation by the chromatophores, which is affected by both initial activity and stability.

6. SUMMARY AND CONCLUSIONS

Chromatophores from R. rubrum catalyze the photophosphorylation of ADP using light energy. The conditions for photophosphorylation were optimized and the feasibility of using chromatophores in the regeneration of ATP were demonstrated by Pace (1975). The sujects concerned in this thesis are: first, the stability of chromatophores during continuous photophosphorylation in a U.F. reactor; secondly, regeneration of ATP from AMP by the coupled reactions of adenylate kinase and chromatophores; and thirdly, immobilization of bacterial chromatophores and their use in photosynthetic regeneration of ATP.

1) The stability of chromatophores is a most important factor in their continuous use. The decrease in outlet concentration of ATP in the U.F. reactor during continuous photophosphorylation with native chromatophores followed first-order kinetics and resulted from inactivation of chromatophores. The ATP production in the continuous reactor was expressed in an equation in terms of ATP formation by chromatophores and inactivation of chromatophore activity with time. The rapid inactivation of chromatophore activity in the early phase, in particular at high ADP concentrations, appeared to be a consequence of increased amount of photophosphorylation. The inactivation of chromatophores following photophosphorylation was also observed to lower the apparent equilibrium conversion in a batch reactor where the activity decreased with time at high concentrations of ADP. Fumarate relieved the inhibition completely at low concentrations of ADP (less than 1 mM) but only partially at higher concentrations.

The mechanism of the rapid inactivation which occured in the early phase was hypothesized to be inhibition by some factor(s) produced during photophosphorylation. The product, ATP, was not the inhibitor.

A high concentration of inorganic phosphate increased the inactivation rate probably via aggregation of chromatophores. Other reactants in the inlet stream such as $MgCl_2$, ascorbic acid, and Tricine , which were added for optimal reaction conditions, did not significantly affect the stability of chromatophores. Increased light intensity caused a rapid inactivation. Thermal effect on chromatophore inactivation was substantial and thus the temperature of operation is a important factor for the ATP production.

2) By coupling the chromatophore reaction to the adenylate kinase reaction in a batch reactor, complete conversion of AMP to ATP was achieved. The rate equations derived predicted the kinetics of the coupled reaction. The optimal ratio of the activity of chromatophores to the adenylate kinase was 1:1 as expected. Chromatophore inactivation as a result of increased photophosphorylation appeared to be a cause for the low apparent equilibrium conversion at high substrate concentrations. Adenylate kinase was stable during use in the reaction. Continuous regeneration of ATP from AMP was demonstrated in a U.F. reactor with the coupled enzymes. The rate of decrease in the ATP production by the coupled system in the U.F. reactor was similar to that with chromatophores alone. Thus it appears that the limitation in the use of the coupled system for ATP regeneration is the activity of the chromatophores, both initial activity and stability.

3) Chromatophores were successfully immobilized by entrapment in polyacrylamide gel. The conditions of preparation of immobilized chromatophores affected the retention of activity and the kinetic properties of chromatophores. By optimizing conditions such as total acrylamide monomer concentration, cross-linking degree, chromatophore concentration in the gel, and gel breaking method, 40 % retention of the activity was achieved.

The properties of immobilized chromatophores with this high activity was studied. The profiles for pH and temperature were similar to those of native chromatophores. The Km for ADP was increased as a result of the effect of substrate diffusion. As the size of immobilized chromatophores particles was increased the initial rate of the reaction decreased. The change in Km for light by immobilization was not detected. The immobilization which yielded high activity produced an opaque gel but the the transmittance for light was high in the red region of the spectrum where the maximal photophosphorylation occurs. Light transfer in the immobilized chromatophore reactor will, however, be influenced by the size and geometry of the reactor as well as light absorption by the immobilizing materials and the chromatophores themselves. Immobilization enhanced the stability of chromatophores during storage. The half-life of immobilized chromatophores at 25° was 72 hours which is a 6-fold increase compared to the native chromatophores.

The immobilized chromatophores were used for photosynthetic regeneration of ATP. It was observed that the stability of the immobilized chromatophores during continuous photophosphorylation was better in the CSTR than in the

column reactor. Immobilization appeared to enhance the loss of chromatophore activity. A thorough washing with buffer or high flow rates in the CSTR, however, decreased the activity loss. These results suggest that the removal of an inhibitor(s) produced during photphosphorylation was hindered by the gel surrounding the chromatophores but was enhanced by the high flow rates. The productivity of the chromatophore reactor was comparable to the catalytic activity of the immobilized chromatophores. One of the restrictions in the use of the immobilized chromatophores was their low specific activity per unit weight which, in turn, resulted in a low maximum volumetric productivity of the reactor.

Immobilized cell organelles of the type described here may be useful for studying the biochemistry of membrane particles in natural systems as well as for productions of biological materials.

7. SUGGESTIONS FOR FUTURE RESEARCH

This study has revealed that a better understanding of the biochemical events occuring in the chromatophores is required for efficient utilization of their photosynthetic activity. It will, however, take much time and effort even though the principles of photosynthesis and general energy coupling in living system are currently being studied extensively by many investigators. As our knowledge of bioenergetics accumulates, it will be possible to use the photosynthetic apparatus from organisms, such as bacterial chromatophores, more successfully for conversion of light energy to useful chemical energy.

The following are suggested for future research in photosynthetic regeneration of ATP.

1) Modification of culture conditions of R. rubrum, or screening of other photosynthetic bacteria such as Rhodopseudomonas spheroid and Chromatium, in order to obtain chromatophores with high activity and stability. As shown in my study the stability of chromatophores is a limiting factor in their use for continuous photophosphorylation. Chromatophores with high activity are also desirable to increase the productivity of the immobilized chromatophore reactor. Kinetic properties of R. spheroid and Chromatium have been well studied and their photophosphorylation activity are comparable to R. rubrum. Thus, the stability of chromatophores from those bacteria should be checked for their continuous use.

Another possible approach is use of plant chloroplasts. Chloroplasts have been reported to have higher specific

activity on the basis of chlorophyll content (2,000-2,500 umoles ATP formed/mg chlorophyll/hr for swiss chard chloroplasts - Avron, 1959; Biochim. Biophys. Acta, 40:257) than bacterial chromatophores (60-100). The operational stability of the chloroplasts, however, has not been studied.

2) Study of light transfer in the chromatophore reactor. Success of the photosynthetic regeneration of ATP depends on the efficient use of light. Light intensity will decrease during its path in the reactor due to absorption by immobilizing materials and chromatophores themselves and also scattering by particles. This light reduction will increase in importance as the reactor becomes larger. Extensive studies on mass transfer in immobilized enzymes, such as diffusion of substrates, have appeared in literature. Similar approaches can be applied for the investigation of the light transfer problem and thus theoretical as well as experimental solutions are possible.

3) Optimization of conditions in the preparation and the study of kinetics of co-immobilized chromatophores and adenylate kinase. The co-immobilized chromatophore/adenylate kinase system in this study was capable of photophosphorylation to convert AMP to ATP. The activity of the co-immobilized system can be increased by optimizing the preparation conditions. One of the parameters will be the optimal ratio of enzymes loaded in gel. A higher yield of product per unit time compared to native enzymes, as a result of the proximity of the two enzymes in a single matrix, has been reported. Kinetics of the co-immobilized coupled system should be studied and compared to the native system.

4) Interface of the regeneration system with biosynthetic reactions which utilize ATP. The ultimate goal of the development of a ATP regeneration system is its use to supply energy for biosynthetic reactions such as cell-free synthesis of Gramicidin S or Bacitracin (Hamilton, 1974). The coupling of this ATP regeneration system to the biosynthetic reaction will bring several problems to be solved. One of such examples is inhibition by substrates and products of each reaction to the other. Another problem in a particular concern with the photosynthetic regeneration method will be effects of light on the biosynthetic reaction such as stability of the final products in the light.

5) Scale-up of the regeneration system. The feasibility of the photosynthetic ATP regeneration was demonstrated in a laboratory scale. The data obtained through the laboratory study must be scaled up for use in the industrial production. Problems such as mass and light transfer and effects on the biochemical reaction involved in the scale-up should be investigated.

REFERENCES

Alberty, R. and R. Block, 1953. Alternation of the kinetic properties of an enzyme by the binding of buffer, inhibitor, or substrate. Proc.Nat. Acad.Sci., 39:895.

Arkles, B. and W. Bringar, 1975. Respiratory properties of rat liver mitochondria immobilized on an alkylsilylated glass surface. J. Biol. Chem., 250:8856.

Baltscheffsky, M., 1961. On light induced phosphorylation and oxidation of reduced diphosphoridine nucleotide in R. rubrum. Acta Chem. Scan., 15:215.

Baltscheffsky, H. and M. Baltscheffsky, 1971. Energy conversion reactions in bacterial photosynthesis. Current Topics in Bioenergetics, 4:273.

Bauman, E., L. Goodson, G. Guilbault, and D. Kramer, 1965. preparation of immobilized cholinesterase for use in analytical chemistry. Anal. Chem., 37:1378.

Bernfeld, P. and J. Wan, 1963. Antigen and enzymes made insoluble by entrapping them into lattices of synthetic polymers. Science, 142:678.

Bernfeld, P., R. Bieber, and D. watson, 1969. Kinetics of water-insoluble phosphoglycerate mutase. Biochim. Biophys. Acta, 191:570.

Bernfeld, P. and R. Bieber, 1969. Water-insoluble enzymes: kinetics of rabbit muscle enolase embedded within an insoluble carrier. Arch. Biochem. Biophys., 131:587.

Black, C., A. San Pietro, D. Limbach, and G. Norris, 1963. Photosynthetic phosphorylation catalyzed by factors isolated from photosynthetic organisms. Proc. Nat. Acad. Sci. U.S., 50:37.

Blodin, G. and D. Green, 1975. A unifying model of bio-energetics. C & C. En. News, Nov. 10, page 26.

Brown, H., A. Patel, and S. Chattopadhyay, 1968. Lattice entrapment of glycolytic enzymes. J. Chromatography, 35:151.

Bunting, P. and K. Laidler, 1972. Kinetic studies on solid -supported β-galactosidase. Biochem., 11:4477.

Carrel, S. and S. Barandun, 1971. Protein-containing polyacrylamide gels: their use as immunoadsorbents of high capacity. Immunochemistry, 8:39.

Cavalieri, L. and E. Carroll, 1970. A DNA-acrylamide gel column for analyzing proteins that bind to DNA. I. DNA polymerase. Proc. Nat. Acad. Sci., 67:807.

Charmbach, A. and D. Rodbard, 1971. Polyacrylamide gel electrophoresis, Science, 172:440.

Chiu, C., S. Su, and P. Russell, 1967. Adenylate kinase from Baker's yeast. I. Purification and intracellular location. Biochim. Biophys. Acta, 132:361.

Cohen-Bazire, G. and R. Kunisawa, 1960. Some observation on the synthesis and function of the photosynthetic apparatus in Rhodospirillum rubrum. Proc. Nat. Acad. Sci. U.S., 46:1543.

Cusanovich, M. and M. Kamen, 1968. Light induced electron transport in Chromatium Strain D. I. Isolation and characterization of Chromatium chromatophores. Biochim. Biophys. Acta, 153:376.

Degani, Y. and T. Miron, 1970. Immobilization of cholinesterase in cross-linked polyacrylamide. Biochim. Biophys. Acta, 212:362.

Doherty, M. and J. Morrison, 1963. The conversion of adenosine 5'-phosphoate into adenosine triphosphate as catalyses by adenosine triphosphate-creatine phosphotransferase and adenosine triphosphate-adenosine monophosphate phosphotransferase in the presence of phosphocreatine. Biochem J., 86:344.

Easterby, J., 1973. Coupled enzyme assays: a general expression for the transient. Biochim. Biophys. Acta, 293: 552.

Engasser, J. and C. Horvath, 1974. Inhibition of bound enzymes I. characterization of product inhibition and accumulation. Biochemistry, 13:3849.

Feinstein, G., 1970. Purification of trypsin by affinity chromatography on ovomucoid-sepharose resin. FEBS Letter, 7:353.

Franks, N., 1972. Catabolism of L-arginine by polyacrylamide entrapped Streptococcus faecalis ATCC 8043. in: L. Wingard, Jr.(editor), Enzyme Engineering. New York, John Wiely and Sons, Inc.

Frenkel, A., 1954. Light induced phosphorylation by cell-free preparations of photosynthetic bacteria. J. Am. Chem. Soc., 76:5568.

Frenkel, A., 1956. Photophosphorylation of adenine nucleotides by cell-free preparation of purp e bacteria. J. Biol. Chem., 222:823.

Geller, D., 1969. The effect of phenazine dyes and N,N,N', N'-tetramethyl-p-phenylenediamine upon light-induced absorbance changes and photophosphorylation in R. rubrum extracts. J. Biol. Chem., 244:971.

Geller, D. and F. Lipmann, 1960. Photophosphorylation in extracts of R. rubrum. J. Biol. Chem., 255:2478.

Gest, H., 1972. Energy conversion and generation of reducing power in bacterial photosynthesis. Adv. in Micro. Physiol., 6:243.

Gibson, K., 1965. Isolation and characterisation of chromatophores from Rhodopseudomonas spheroides. Biochem. 10:2042.

Goldman, R. and E. Katchalski, 1971. Kinetic behavior of a two-enzyme membrane carrying out a consecutive reactions. J. Theor. Biol., 32:243.

Goldman, R. O. Kedem, and E. Katchalski, 1968. Papain-colloidon membranes II. Analysis of the kinetic behavior of enzyme immobilized in artificial membranes, Biochemstry, 7:4518.

Good, N. and S. Izawa, 1972. Hydrogen ion buffers. in: (S. Colowick and N. Kaplan (editors), Methods in Enzymology; vol. 23B, A. San Pietro (editor), Photosynthesis and Nitrogen Fixation, pp 53. New York, Academic Press.

Goodfriend, T., D. Ball, and S. Updike, 1969. Antibody in polyacylamide gel, a solid phase reagent for radioimmunoassay. Immunochemistry, 6:481.

Green, D., R. Beyer, M. Hansen, A. Smith, and G. Webster, 1963. Coupling factors and the mechanism of oxidative phosphorylation. Federation Proc., 22:1460.

Guilbault, G. and E. Hrabankova, 1970. An electrode for determination of amino acids. Anal. Chem., 42:1779.

Hamilton, B., 1974. Progress in preparative-scale, ATP-driven, enzyme-catalyzed synthesis of fine organic chemicals. Ph.D. thesis, M.I.T.

Hamilton, B., C. Gardner, and C. Colton, 1974. Basic concepts in the effects of mass transfer on immobilized enzyme kinetics.in: A. Olson and C. Cooney (editors) Immobilized Enzymes in Food and Microbial Process. Plenum Pub. Co., New York.

Hamilton, B., L. Stockmeyer, and C. Colton, 1973. Comments on diffusive and electrstatic effects with immobilized enzymes. J. Theor. Biol., 4:547.

Hicks, G. and S. Updike, 1966. The preparation and characterization of lyophilized polyacrylamide enzyme gels for chemical analysis. Anal. Chem., 38:726.

Herbert, D., P. Philipps, and E. Strange, 1962. in: J. Morris and D. Ribbons (Editors), Methods in Enzymology, vol 5B, p. 249, New York, Academic Press.

Hjerten, S. and R. Mosbach, 1962. Molecular-sieve chromatography of proteins on columns of cross-linked polyacrylamide. Anal. Biochem., 3:109.

Hjerten, S., 1968. Chromatography on polyacrylamide gels. b. Preparation of polyacrylamide spheres. in: C. Williams and M. Chase (editors), Methods in Immunology and Immunochemistry, vol.II. p 142, New York, Academic Press.

Horio, T. and M. Kamen, 1962. Optimal oxidation-reduction potentials of endogenous co-factors in bacterial photophosphorylation. Biochemistry, 1:144.

Horio, T. J. Yamashita, and K. Nishikawa, 1963. Photosynthetic adenosine triphosphate formation and photoreduction of diphosphoridine nucleotide with chromatophore from R. rubrum. Biochim. Biophys. Acta, 66:37.

Horio, T., L. Von Stedingk, and H. Baltscheffsky, 1966. Photophosphorylation in presence and absence of added adenosine diphosphate in chromatophores from R. rubrum. Acta Chem. Scan., 20:1.

Keister, D. and N. Yike, 1967. Energy linked reactions in photosynthetic bacteria. II. The energy dependent reduction of oxidized nicotinamide-adenine dinucleotide phosphate by reduced nicotinamide-adenine dinucleotide in chromatophores of R. rubrum. Biochem., 6:3847.

Hochman, A. and C. Cameli, 1973. ATPase and ATP-Pi exchange activities in Chromatium strain D chromatophores. Photosynthetica, 7:238.

Hornby, W. and M. Lilly, 1968. Some changes in the reactivity of enzymes resulting from their chemical attachment to water-insoluble derivatives of cellulose. Biochem. J., 107:669.

Khoo, J., 1971. The kinetics and multiple molecular forms of adenylate kinase. Ph.D. thesis, University of Kansas.

Khoo, J. and P. Russell, 1970. Adenylate kinase from baker's yeast. IV. Substrate and inhibitor structural requirements. J. Biol. Chem., 245:4163.

Kobayashi, T. and K. Laidler, 1973. Kinetic analysis for solid-supported enzymes. Biochim. Biophys. Acta, 302:1.

Kondrat'eva, E., 1965. Photosynthetic bacteria. Jerusalem: S. Monson.

Kopp, L. and R. Miech, 1972. Nonlinear enzymatic cycling system: the experimental cycling system.1. Mathematical models. J. Biol. Chem., 247:3558.

Kuchel, P. and D. Roberts, 1974. The behavior of coupled enzyme systems in the transient and steady-state regions of the reaction. Biochim. Biophys.acta,364:181.

Lamotte, A., 1975. Ph.D. thesis, M.I.T.

Langer, R., 1974. Enzymatic regeneration of ATP. Sc.D. thesis, M.I.T.

Lascelles, J., 1962. The chromatophores of photosynthetic bacteria. J. Gen. Microbiol., 29:47.

Lasch, J., 1973. Theoretical and experimental analysis of continuous enzyme reactor kinetics. II. diffusion controlled system.sphere-like enzyme carrying particles. Mol. & Cell. Biochem., 2:79.

Lehninger, A., 1971. In: Bioenergetics, The Molecular Basis of Biological Energy Transformation, chapter 6. W. Benjamin.

Levenspiel, O., 1972. Deactivating catalysts. in: Chemical Reaction Engineering, second ed., p 537. John Wiley & Sons, New York.

Lilly, M. and P. Dunnill, 1971. Biochemical reactors. Process Biochemistry, 6(8):29.

Lilly, M. and P. Dunnill, 1972. Engineering aspect of enzyme reactors. in: L. Wingard (ed.), Enzyme Engineering. John wiley & Sons, New York.

Lilly, M. and W. Hornby, and E. Crook, 1966. The kinetics of carboxymethylcellulose-ficin packed beds. Biochem. J., 100:718.

Lilly, M. and A. Sharp, 1968. The kinetics of enzyme attached to water-insoluble polymers. Chem. Eng.No.215,CE12.

Maeda, H., A. Yamauchi, and H. Suzuki, 1973. Preparation of immobilized enzymes by γ-ray irradiation. Biochim. Biophys. Acta, 315:18.

McClure, W., 1969. A kinetic analysis of coupled enzyme assays. Biochem., 8:2782.

Melorose, G., 1971. Insolubilized enzymes : biochemical applications of synthetic polymers. Review Pure Appl. Chem., 21:83.

Moo-young, M. and T. Kobayashi, 1972. Effectiveness factor for immobilized-enzyme reactions. Can. J. Chem. Eng. 50:162.

Mori, T., T. Sato, T. Tosa, and I. Chibata, 1972. Studies on immobilized enzymes. X. preparation and properties of aminoacylase entrapped into acrylamide gel lattice. Enzymologia, 43:213.

Morrison, J. and M. Doherty, 1961. The biosynthesis of N-phosphorylcreatine: An investigation of the postulated alternative pathway. Biochem. J.,79:433.

Morrison, J. and E. Hyde, 1972. Enzymic phosphoryal group transfer. Ann. Rev. Biochem., 41:29.

Mosbach, K., 1970. Matrix-bound enzymes, Part I. The use of different acrylic copolymers as matrices. Acta Chem. Scan., 24:2084.

Mosbach, K., 1972. On establishing optimal conditions for immobilized multienzyme system. in: L. Wingard (ed.), Enzyme Engineering. John Wiley & Sons, New York.

Mosbach, K., and P. Larson, 1970. Preparation and application of polymer-entrapped enzymes and microorganisms in microbial transportation process with special reference to steroid 11-β-hydroxylation and 1-dehydrogeneration. Biotech. Bioeng., 12:19.

Mosbach, K. and B. Mattiasson, 1970. Matrix-bound enzymes, Part II. Studies on a matrix bound two enzyme system. Acta Chem. Scan., 24:2039.

Nemet, M., 1976. Ph. D. thesis, M.I.T.

Noda, K., 1958. Adenosine triphosphate-adenosine monophosphate transphosphorylase. III. Kinetic studies. J. Biol. Chem., 232:237.

Noda, L., 1973. Adenylate kinase. in: P. Boyer (ed.) vol. 8, The Enzymes. Academic Press, New York.

Nilsson, H., R. Mosbach, and K. Mosbach, 1972. The use of bead polymerization of acrylic monomers for immobilization of enzymes. Biochim.Biophys.Acta,268:253.

Nishimura, M, 1962. Studies on bacterial photophosphorylation and adenosine 5'-triphosphatase and effect of divalent metal ions. Biochim. Biophys. Acta, 64:345.

Olesen, H., E. Hippe, and E. Haber, 1971. Nature of vitamin B_{12} binding. I. Covalent coupling of hydroxycobalamine to soluble and insoluble carriers. Biochim. Biophys. Acta, 243:66.

Oleze, J. and G. Drew, 1972. Membranes of photosynthetic bacteria. Biochim. Biophys. Acta, 265:209.

O'Neill, S., P. Dunnill, and M. Lilly, 1971. A comparative study of immobilized amyloglucosidase in a packed bed reactor and a continuous feed stirred tank reactor. Biotech, Bioeng., 13:337.

Pace, G., 1975. Photosynthetic regeneration of ATP using bacterial chromatophores. Ph.D. thesis, M.I.T.

Quintero-ramirez, R., 1974. Purification and kinetics of yeast adenylate kinase. M.S. thesis, M.I.T.

Reed, R. and E. Murray, and N. Smith, 1957. Bergey's Manual of Determinative Bacteriology, 7th ed., p59, Williams and Wilkins Press.

Saxinger, C., C. Ponnamperuma, and C. Woese, 1971. Evidence for the interaction of nucleotides with immobilized amino acids and its significance for the orgin of the genetic role. Nature, New Biol., 234:172.

Selinger, R. and M. Civin, 1971. ACTH diazotized to agarose: effect on isolated adrenal cells. Biochim. Biophys. Res. Commun., 43:793.

Silman, I. and E. Katchalski, 1966. Water-insoluble derivatives of enzymes, antigen, and antibodies. Ann. Rev. Biochem., 35:873.

Su, S., 1966. Kinetic analysis of yeast adenylate kinase. Ph.D. thesis, University of Kansas.

Su, S. and P. Russell, 1968. Adenylate kinase from baker's yeast. III. Equilibria: Equilibrium exchange and mechanism. J. Biol. Chem., 243:3826.

Su, S. and P. Russell, 1967. Adenylate kinase from baker's yeast. II. Substrate specificity. Biochim. Biophys. Acta, 132:370.

Wagner, A., R. Bugianesi, and T. Shen, 1971. Preparation of sepharose bound poly(rI:rC). Biochim. Biophys. Res. Commun., 45:184.

Warburg, O. and W. Christian, 1941. Isolierung and Kristallization des Garungsterments Enolase. Biochem. Z., 310:384.

Woody, B. and E. Lindstrom, 1955. The succinic dehydrogenase of Rhodospirillum rubrum. J. Bacteriol., 69: 353.

Yamamoto, K, T. Sato, T. Tosa, and I. Chibata, 1974. Continuous production of K-citrulline by immobilized Pseudomonas putida cells. Biotech.Bioeng., 16:1589.

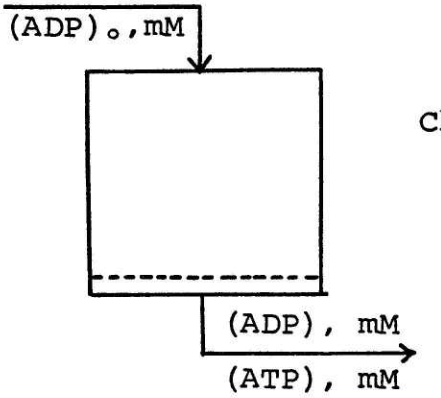
Yamamoto, N., S. Yoshimura, T. Higuti, K. Nishikawa, and T. Horio, 1972. Role of bound ADP in photosynthetic ATP formation by chromatophores from R. rubrum. J. Biol. Chem., 72:1397.

Yamashita, J., S. Yoshimura, Y. Matuo, and T. Horio, 1967. Relationships from light grown cells of R. rubrum. Biochim. Biophys. Acta, 143:154.

Zaborsky, O., 1973. Immobilized Enzymes.CRC press, Cleveland, Ohio.

Appendix I

Continuous Production of ATP in an Ultrafiltration Cell Reactor (a CSTR) with Inactivating Chromatophores.



Chromatophores ; A_o, units/ml*

Reactor volume ; V , ml

Flow rate ; Q, ml/hr

* defined as u moles ATP/hr/ml

(Inorganic phosphate and light are saturating).

a) ATP production in a CSTR without catalyst inactivation.

$$\frac{d(ATP)}{dt} = -D(ATP) + R \qquad \text{---------- (1)}$$

where D (dilution rate) = Q/V

and $R = \dfrac{k_2 (E)(ADP)}{Km + (ADP)}$

(k_2 is the rate constant for reaction and (E) is chromatophore concentration, thus $k_2(E)$ represents A_o in this reaction, and km is the Michaelis constant for ADP of chromatophores.)

b) Chromatophore inactivation (first order).

$\dfrac{d(A)}{dt} = -k(A)$, where k is the inactivation rate constant.

Thus, $(A) = (A)_o e^{-kt}$ ---------- (2)

Combining (1) and (2),

$$\frac{d(ATP)}{dt} = -D(ATP) + \frac{k_2 (E)_o e^{-kt} (ADP)}{Km + (ADP)} \qquad \text{------ (3)}$$

The equation (3) represents the ATP production rate in a U.F. reactor with inactivating chromatophores.

When substrate concentrations in the reactor are excess i.e. (ADP) >> Km,

$R = k_2 (E) = Vm$ (maximum rate of reaction)

and Vm is equivalent to the chromatophore activity per unit reactor volume (or (A));u moles/hr/ml.

Thus, Eq.(3) is $\frac{d(ATP)}{dt} = -D(ATP) + A_o e^{-kt}$.

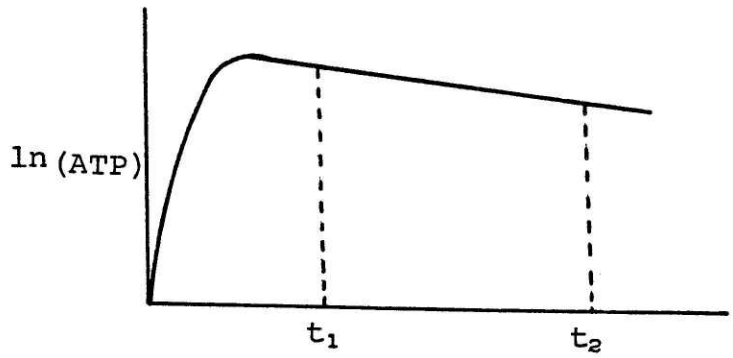
For steady-state operation (Q, V, (E), and pH and temperature are constant) and when the inactivation rate, a "pseudosteady-state", where d(ATP)/dt is close to zero, is assumed (O'Neill, 1972 and Levenspiel, 1972).
Thus,

$$(ATP) = A_o e^{-kt/D} \quad \text{or} \quad A_o e^{-kt \cdot Tr}$$

since Tr (residence time) is 1/D.

Under these conditions, if $(ATP)_1$ is the outlet ATP concentration at t_1 and $(ATP)_2$ is at t_2, then

$$\ln \frac{(ATP)_2}{(ATP)_1} = -k\ (t_2 - t_1).$$



References:

O'Neill, S., 1972. Thermal inactivation of immobilized enzymes in ideal continuous reactors. Biotech. Bioeng., 14:473

Levenspiel, O., 1972. Deactivating catalysts, p537,In "Chemical Reaction Engineering", second ed., John Wiely & Sons, Inc, New York.

Appendix II

Expression of the Reaction Rate and Concentration for Each Adenine Nucleotide during the Time-course of the Coupled Reaction of Adenylate kinase and Chromatophores in Regeneration of ATP from AMP.

The reactions involved are;

$$\text{AMP} + \text{ATP} \underset{\longleftarrow}{\xrightarrow{\text{adenylate kinase}}} 2\ \text{ADP}$$

$$2\ \text{ADP} + 2\ \text{Pi} \xrightarrow[\text{light}]{\text{chromatophores}} 2\ \text{ATP}$$

$$\text{net, AMP} + 2\ \text{Pi} \longrightarrow \text{ATP}$$

Assumptions:
1. pH, temp., (Mg), and red-ox are held constant.
2. Light and Pi are saturating.
3. Inhibitions by substrate and product are not significant.
4. There is no change in enzyme activity during reaction.

For adenylate kinase reaction;

Ka : Km for AMP, mM.
Kt : Km for ATP, mM.

ka : rate constant for the forward reaction (for disappearence of AMP under ATP saturating)
Ea : concentration of enzyme.
Va : maximum rate of reaction, kaEa, u moles/min/ml.

A : activity of adenylat kinase for ADP formation (by denifition), u moles ADP/min/ml; equivalent to 2Va.

For chromatophore reaction;

Kc : Km for ADP, mM.

kc : rate constant of the photophosphorylation.
Ec : concentration of chromatophores.
Vc : maximum rate of reaction, kcEc, u moles/min/ml.

C : activity of chromatophores for ATP formation, u moles ATP/min/ml; equivalent to Vc.

A. Rate expression for the change in the concentration of AMP, ADP, and ATP in the reactors.

Reactions of adenylate kinase and chromatophores follow the Michaelis-Menten kinetics: Thus the rate of reaction of adenylate kinase (Ra) and of chromatophores (Rc) is,

$$Ra = \frac{kaEa\ (AMP)}{Ka + (AMP)}$$ when (ATP) is much higher than Kt (saturating),

and $$Rc = \frac{kcEc\ (ADP)}{Kc + (ADP)}$$ under the saturating Pi and light.

a. Batch reactor.

$$-\frac{d(AMP)}{dt} = Ra \quad \text{---------- (1)}$$

$$\frac{d(ADP)}{dt} = 2Ra - Rc \quad \text{---------- (2)}$$

and $$\frac{d(ATP)}{dt} = Rc - Ra \quad \text{---------- (3)}$$

where (AMP), (ADP), and (ATP) are concentrations of the respective components in the reactor, mM.

b. Continuous-flow Stirred Tank Reactor (U.F. reactor).

Material balance;
Accumulated = In - Out + Formed -Consumed.

Thus,

$$\frac{d(AMP)}{dt} = D((AMP)_o - (AMP)) - Ra$$

$$\frac{d(ADP)}{dt} = -D(ADP) + 2Ra - Rc$$

$$\frac{d(ATP)}{dt} = -D(ATP) + Rc - Ra$$

where (AMP), (ADP), and (ATP) are concentrations of AMP, ADP, and ATP in outlet, respectively, $(AMP)_o$ is concentration of AMP in feed stream, and D is the dilution rate which is the flow rate(Q) over the reactor volume(V) in the CSTR.

B. Expression for the concentration changes of AMP, ADP, and ATP in the batch reactor under the steady-state reaction rate.

The reaction rates do not change and are maximal during the steady-state of production and the concentration of ADP, the intermediate, remains small and constant. (see Section 4.2.1. in text).

a. Steady-state concentration of ADP, $(ADP)_{ss}$

When adenylate kinase activity is rate limiting in the coupled reaction ($C/A \geq 1$, refer to text), (ADP) in reactor remains small and is less then Kc. Then

$$Ra = Va = A/2 \quad \text{---------- (4)}$$

and $$Rc = \frac{kcEc}{Kc}(ADP) = \frac{C}{Kc}(ADP) \quad \text{--- (5)}$$

Substitute Ra (Eq.4) and Rc (Eq.5) into Eq.(2),

$$\frac{d(ADP)}{dt} = A - \frac{C}{Kc}(ADP) \quad \text{--------- (6)}$$

The transient time (tr) to reach the steady-state is dependent on initial ATP concentration added and C/A. At the optimal C/A and $(ATP)_o > 0.1$ mM, Tr is close to zero.

Using the boundary conditions, (ADP)=0 at t=0, Integrate the Eq. (6),

$$(ADP) = \frac{AKc}{C}(1 - e^{-(C/Kc)t}) \quad \text{----- (7)}$$

As $t \rightarrow \infty$ i.e. at the steady state,

$$(ADP)_{ss} = \frac{AKc}{C} \quad \text{---------- (8)}$$

b. Concentration of AMP, (AMP).

Since (AMP) is much greater than Ka,

$$-\frac{d(AMP)}{dt} = kaEa = Va = A/2$$

Integration with the boundary condition, $(AMP) = (AMP)_o$ at t = 0, give

$$(AMP) = (AMP)_o - A/2(t)$$

c. Concentration of ATP, (ATP).

The steady-state rate of ATP production is obtained by substituting Eq.(4) and (5) into Eq.(3), then Eq.(8),

$$\frac{d(ATP)}{dt} = Rc - Ra = \frac{C}{Kc}(ADP)_{ss} - \frac{A}{2} = \frac{C}{Kc}\frac{AKc}{C} - \frac{A}{2} = \frac{A}{2}$$

Integrating the equation with the boundary condition, $(ATP) = (ATP)_o$, which is added initially to "prime" the adenylate kinase reaction, at $t = 0$,

$$(ATP) = \frac{A}{2}(t) + (ATP)_o$$

References:

McClure, W., 1969. A kinetic Analysis of coupled enzyme assays. Biochem., 8:2782.

Easterby, J., 1973. Coupled enzyme assays : A general expression for the transient. Biochim. Biophys. Acta, 293: 552.

APPENDIX III

Development of Procedures for Determination of Adenylate Kinase Activity by Continuous Assay.

The results described here utilize enzyme prepared from Baker's yeast and carried through the first 5 steps of purification (Chiu, et al., 1967.B.B.A., 132:361). In the continuous assay for the activity of adenylate kinase (and Myokinase discussed below), the reaction is run in the direction of the disproportionation of ADP to ATP and AMP,

$$2ADP \xrightarrow[Mg^{++}]{\text{adenylate kinase}} ATP + AMP \ .$$

ATP production is assayed by the sequential enzymatic reaction scheme described in Section 3.5.3. Under the conditions when adenylate kinase reaction is rate limiting, the rate of formation of NADPH measured at 340 nm is proportional to the concentration of adenylate kinase in the system.

The following concentrations of reactants were used: for assay solution (I), 10 mM glucose, 1 mM NADP, 10 units hexokinase (Sigma),and 2 units glucose-6 phosphate dehydrogenase(Sigma); and 5 mM ADP and ~0.2 units/ml adenylate kinase.

All of the above were prepared in 0.2 M Tris buffer, pH 7.4 containing 10 mM $MgCl_2$. Components of the ATP assay solution (I) were initially incubated at 25° for about 1 hour (until a steady absorbance at 340 nm was achieved). At this stage ADP solution was added and the solution was again incubated for about 10 minutes at 25° (this incubation

was necessary due to a small(about 1 %) contamination of ADP with ATP). Then the adenylate kinase preparation was added to the reaction cuvette and a corresponding volume of Tris buffer added to the blank cuvette. The initial velocity of the reaction was determined spectrophotometrically at 340 nm.

Variation of glucose-6-phosphate dehydrogenase concentration from 1 to 10 units/ml and variation of hexokinase from 5 to 10 units/ml had no effect on the rate of reaction using a constant amount of adenylate kinase. Figure 1 shows a linear relationship between enzyme activity and adenylate kinase concentration. One unit of activity is defined as that required to produce 1 uM ATP from ADP in 1 minute; the enzyme in this preparation had a specific activity of 48 units/mg protein.

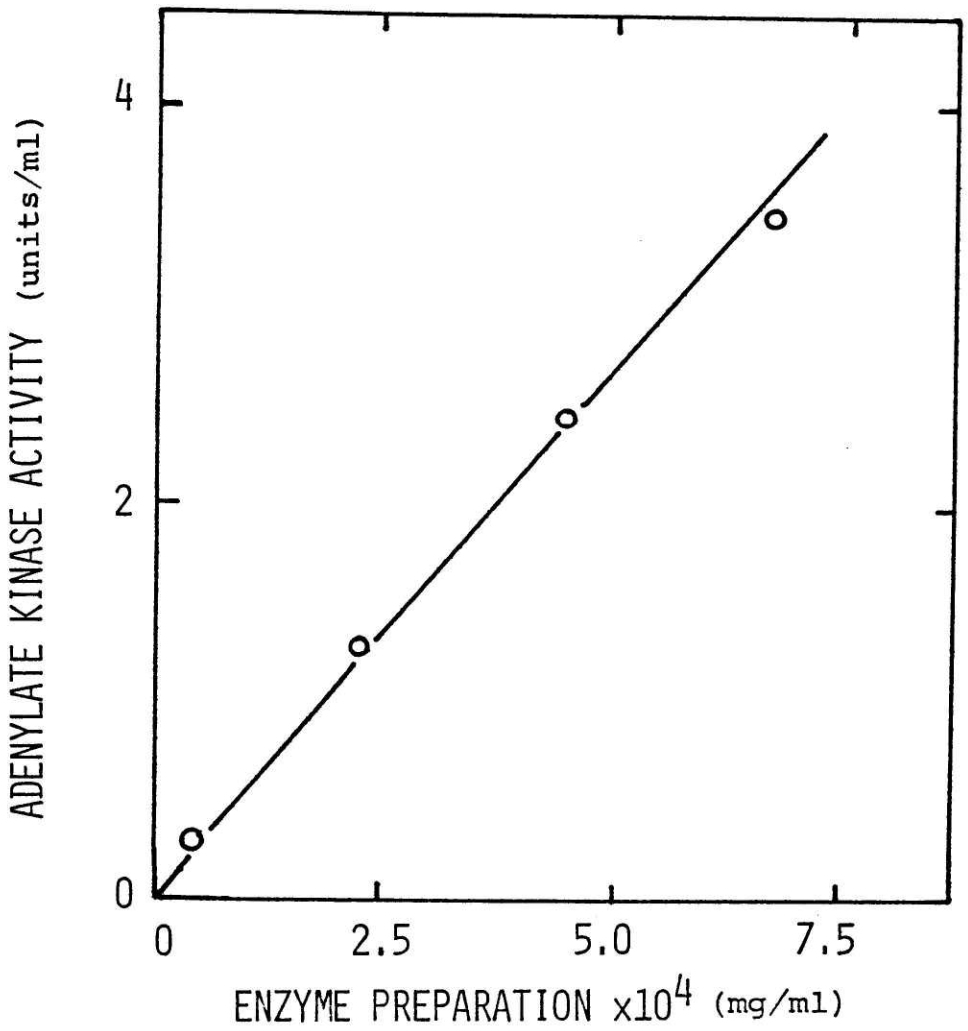


FIGURE 1.

Determined enzyme activity by the continuous assay at various concentrations of adenylate kinase.

APPENDIX IV

Purification and Properties of Adenylate Kinase from Rhodospirillum rubrum.

INTRODUCTION

Adenylate kinase (E.C. 2.7.4.3.) catalyzes a phosphoryl transfer reaction,

$$AMP + ATP \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} 2ADP$$

This reaction can be coupled to photophosphorylation by bacterial chromatophores for regeneration of ATP from AMP with inorganic phosphate using light energy (Pace et al., 1976).

Since Colowick and Kalcar (1943) first reported the enzyme from rabbit muscle (myokinase), adenylate kinases have been found in cells where the turnover of energy from adenine nucleotides is great. Adenylate kinases from rabbit(Noda and Kuby, 1957) and human(Thuma et al., 1972) muscle and rat(Criss, 1970), bovine(Markland and Wadskin, 1965) and swine(Chiga and Plaut, 1960) liver were purified and their properties were studied. Adenylate kinases were also isolated from various microorganisms such as Saccharomyces cerevisiae (Chiu et al., 1967), Escherichia coli (Holmes and Singer, 1972), Thiobacillus neapolitanus (Mazzotta and Johnson, 1973), and Pseudomonas denitrificans (Terai, 1974). Noda(1973) and Terai(1974) have presented summaries of properties of the various adenylate kinases.

Baker's yeast adenylate kinase was isolated and partially purified in large quantity (Quintero-ramirez, 1974) and used for the enzymatic regeneration of ATP (Nemet,

1976). Chromatophores, particulate membrane fragments of photosynthetic bacteria, have been isolated from Rhodospirillum rubrum for their use in photosynthetic ATP regeneration (Pace et al., 1976). Isolation of adenylate kinase, which is present in the soluble fraction of the cell extract, and chromatophores from the same organism is ideal for the economic use of cells. Thus we have investigated the isolation and partial purification of adenylate kinase from R. rubrum.

MATERIALS AND METHODS

R. rubrum (ATCC 11170) was grown, harvested, and stored as described in Pace(1975).

Cell disruption methods:

a. Grinding with sand: frozen cell solid was ground with sand by mortar and pestle.

b. Sonication: A cell suspension of 1.2 g/5 ml was placed in sonicator (Branson). The cell suspension was cooled with salt/ice bath in a surrounding beaker.

c. Braun Homogenizer: A cell suspension of 1.2 g/5 ml was shaken in a pyrex glass bottle (40 ml) together with glass beads (diameter, 0.25-0.30 mm). The whole system was cooled by liquid CO_2.

d. Manton-Gaulin Homogenizer: A cell suspension of 20 g/740 ml was passed through the orfice of Manton-Gaulin homogenizer.

e. French Press: A cell suspension of 1.2 g/5 ml was put in a cold cylinder and pressed in a French press with a pressure of 10,000 psi. The disintegrated cell suspension

was collected through the orfice in the cylinder.
f. Hugher press: 5 g of frozen cell solid between the cold metal block was pressed with a pressure of 10,000 psi.

Extraction of soluble fraction:

Thirty grams of frozen cells were suspended and thawed in 60 ml of 0.05 M Tris buffer, pH 7.8, and disrupted routinely in a Braun homogenizer for 3 min. The extract was centrifuged at 18,800 g for 3 minutes. The resultant supernatant was centrifuged at 10,000 g for 60 min. The supernatant, which was separated from the chromatophore pellet, was designated the crude extract.

Purification of adenylate kinase:

After the addition of 1 mM dithiothreitol(Sigma, St. Louis, MO), the pH of the crude extract was slowly lowered to 3.5 with 1 M citric acid at ice bath temperatures. The solution was allowed to stir for 5 min, then the pH was raised to 7.5 with 1 N NaOH. The precipitate was centrifuged at 12,000 g for 15 min.

Solid ammonium sulfate was added to the supernatant fraction to yield 30 % solution. The precipitate was centrifuged and the supernatant was brought to 75 % saturation and recentrifuged. All centrifugations were at 12,000 g for 15 min.

The precipitate after ammonium sulfate fractionation was redissolved in 1-2 ml buffer and applied to a column of Sephadex G-75 (Pharmacia Fine Chemicals, Upsala, Sweden). The fractions with high activity were pooled and then applied to a column of DEAE-cellulose (Bio-Rad Laboratories, Richmond, CA). Specific conditions of the chromatography

are described in each figure legend.

Isoelectrofocusing:

An isoelectrofocusing column LKB 8100 (LKB Produkter, Bromma, Sweden), 110 ml capacity, containing ampholytes (Ampholine from LKB Produkter) with a pH range of 3-10 was prepared. The current was maintained at 5 mA for 24 hours and the column was cooled to 3° by a circulating refrigerated water bath. After equilibration, 3 ml fractions were collected from the bottom of the column. Each fraction was assayed for adenylate kinase activity and protein content.

Determination of protein content:

Protein concentration was determined by the method of Lowry et al.,(1951). The protein content in the fractions of the eluent from the columns were estimated by the spectrophotometric method of Warburg and Christian (1941).

Enzyme assay:

Adenylate kinase activity was determined by measuring the rate of ATP production using a coupled enzymatic system in continuous assay as described in the preceeding Appendix III.

RESULTS AND DISCUSSION

1. Cell disruption method.

In an attempt to optimize the yield of adenylate kinase

the effect of various methods of cell disruption have been studied and the results are shown in Table 1.

Although the Manton-Gaulin homogenizer gave the overall best yield of adenylate kinase, the method requires a minimum volume of about 500 ml per run. Thus for routine preparation of small batches of cell extract, the Braun homogenizer run for 3 minutes, is used. Routine extraction with the Braun homogenizer yielded about 12 units of adenylate kinase activity per gram of wet cells. with 44 mg of protein per gram of wet cells. This yields a specific activity of 0.25 units of adenylate kinase/ mg of protein. Adenylate kinase activity in an extract of R. rubrum with a specific activity of about 3 units/g wet cells (estimated) was reported by Oliver and Peel (1956).

2. Purification of adenylate kinase.

Soluble fractions of cell extracts were purified for adenylate kinase following the procedures of acid precipitation, ammonium sulfate fractionation, Sephadex G-75 gel chromatography, and DEAE-ion-exchange chromatography as described in materials and methods.

Little loss of activity was detected during acid precipitation at pH 3.5, whereas at pH 3.0 more proteins were denaturated but there was a concurrent loss of enzyme activity. Although there is only a small increase in specific activity following acid precipitation, the step

is included because it eliminates any red pigment in the extract which would interfere with the operation of the fraction collector.

An 80 % recovery of enzyme activity was observed during ammonium sulfate precipitation; no activity was found in the precipitate of the 30 % saturated fraction, and only a trace amount in the supernatant of the 75 % fraction.

Typical volumes of enzyme fraction applied to the Srphadex column were of the order of 2-3 ml. This produces a good separation.

Separation by Sephadex G-75 is shown in Figure 1. A large portion of protein was eluted with red colored pigments just after the void volume. Approximately 85 % of the protein applied to the column was eliminated during pooling of the peak fractions resulting in about a 3.5-fold increase in purity of the enzyme. The gel filtration also removed ammonium sulfate present in the applied fraction from proteins and thus eliminated the need of dialysis of the sample prior to ion-exchange column.

Following gel filtration, about 60 % of the activity applied was recovered; the peak fractions were pooled and then applied to DEAE column. The results of the separation are shown in Figure 2.

12-20 % of the proteins applied to the DEAE column was not adsorbed and washed out with the 0.05 M Tris pH7.8 at

the solvent front; no enzyme activity was found in these fractions. Volumes of solution applied to the column ranged from 10 to 15 ml. The recovery and protein profile depended upon the elution conditions.

A linear salt gradient of 0 to 0.5 M NaCl in 0.05 M Tris pH 6.0 yielded only about 30 % recovery and no sharp enzyme peak. However, a linear salt gradient of 0 to 0.4 M $MgCl_2$ in 0.05 M Tris pH 6.0 yielded a 66 % recovery with a sharp enzyme peak, but much of the other proteins were eluted with the peak fractions. A Tris gradient of 0.05 to 0.50 M pH 6.0 yielded a 40 % recovery and a sharp peak as shown in Figure 2.

A summary of the purification is given in Table 2. The resulting enzyme fraction from the DEAE-cellulose column was subject to polyacrylamide gel electrophoresis to check purity. Two minor bands were observed along with a major band which was estimated to represent approximately 90 % of total protein. The specific activity of the final fraction was 6.39 unit/mg protein. This value is low compared to those from other microorganisms, 627 for E. coli (Holmes and Singer, 1974) and 50 for Ps. nitrificans (Terai, 1974), both of which were claimed to be pure. It is not known in present study whether the low specific activity is just a result of incomplete purification or R. rubrum adenylate kinase simply has a low specific activity.

3. Enzyme stability.

The effect of temperature and addition of dithiothreitol (DTT), a reducing agent, on the stability of adenylate kinase activity was investigated. The study was done on an ammonium sulfate fraction (specific activity, 0.244). The results are shown in Figure 3.

In the frozen state, with 20 mM DTT 85 % of the activity remained after 72 hours and after 84 days, 44 % of activity still remained. At 3°, the normal temperature during purification, the half-life of the enzyme is only about 55 hours with DTT and 35 hours without DTT. It appears that the time spent during purification is critical for a high yield of activity of the enzyme. At 30° half the activity disappears after about 3 hours regardless of the presence of DTT.

The results indicate that the reducing agent is necessary to maintain the activity probably by protecting sulfuhydryl group of the enzyme. It has been reported that adenylate kinases from yeast (Chiu et al., 1967) and T. neapolitanus (Mazzotta and Johnson, 1973) have no free-SH group whereas myokinase (Noda, 1973), adenylate kinase from rabbit muscle, requires two-SH groups for activity.

4. Isoelectric point of enzyme.

The isoelectric point(pI) of enzyme was determined as shown in Figure 4. pI of the R. rubrum adenylate kinase

from the Sephadex G-75 fraction was determined to be around 5.0. pIs of adenylate kinases from various sources have been reported (Russell et al., 1973 and Sapico et al., 1971). Values vary between 4 and 10 . Criss(1970) observed isoenzymes of rat liver adenylate kinase with different pIs.

REFERENCES

Chiga, M. and G. Plaut, 1960. J. Biol. Chem., 235:3260.

Chiu, C., S. Su, and P. Russell, 1967. Biochim. Biophys. Acta, 132:361.

Colowick, S. a d H. Kalcar, 1943. J. Biol. Chem., 148:117.

Criss, W. 1970. J. Biol. Chem., 245:6352.

Holmes, R. and M. Singer, 1973. J. Biol. Chem., 248:2041.

Lowry, O., N. Rosenbrough, A. Farr, and R. Randall, 1951. J. Biol. Chem., 193:265.

Markland, F. and C. Wadskin, 1966. J. Biol. Chem.,241:4124.

Mazzotta, M. and E. Johnson, 1973. Biochim. Biophys. Acta, 321:512.

Nemet, M., 1976. Ph.D. thesis, M.I.T.

Noda, L., 1973. In:P. Boyer(ed.), The Enzymes, Vol. 8, Academic Press, New York.

Noda, L. and S. Kuby, 1957. J. Biol. Chem., 226:541.

Oliver, I. and J. Peel, 1956. Biochim. Biophys. Acta,20:390.

Pace, G, 1975. Ph.D. thesis, M.I.T.

Pace, G., H. Yang, M. Archer, and S. Tannenbaum, 1976. Biotech. Bioeng. (in press).

Quintero-ramirez, R. 1974. M.S. thesis, M.I.T.

Russell, P., J. Horenstein, L. Goins, D. Jones, and M. Laver, 1974. J. Biol. Chem., 249:1847.

Sapico, V., G. Litwack, and W. Criss, 1972. Biochim. Biophys. Acta, 258:436.

Terai, H., 1974. J. Biochem., 75:1027.

Thuma, E. R. Schirmer, and I. Schirmer, 1972. Biochim. Biophys. Acta, 268:81.

Warburg, O. and W. Christian, 1941. Biochem. Z., 310:384.

TABLE 1

Cell Disrution for Rhodospirillum rubrum

Disruption Method	Protein Released	Ad. Kinase Activity	Specific Activity
	mg / g wet cell	unit / g wet cell	unit / mg
Grinding with sand	27	7.3	0.27
Sonication			
0.5 min	20	8.9	0.46
1 min	32	9.7	0.30
2 min	29	9.7	0.33
3 min	39	0.5	0.01
Braun Homogenizer			
0.5 min	32	0.5	0.16
1 min	22	6.8	0.31
3 min	33	10.8	0.33
Manton-Gaulin Homogenizer			
1 pass	18	10.0	0.55
3 pass	54	14.0	0.25
French Press (10,000 psi)	28	9.7	0.34
Hughes Press (10,000 psi)	18	5.7	0.32

TABLE 2

Purification of adenylate kinase from Rhodospirillum rubrum*

Fraction	Total activity	Total protein	Specific activity	Yield	Purification
	unit	mg	$\frac{\text{unit}}{\text{mg}}$	%	
Crude extract	61.7	560.4	0.110	100	1.0
Acid	69.3	533.5	0.130	100	1.18
Ammonium sulfate	48.9	200.0	0.244	79.2	2.22
G-75 gel	23.5	38.0	0.618	38.1	5.62
DEAE-cellulose	7.3	1.14	6.39	11.8	58.05

* started with 30 gram of wet cells.

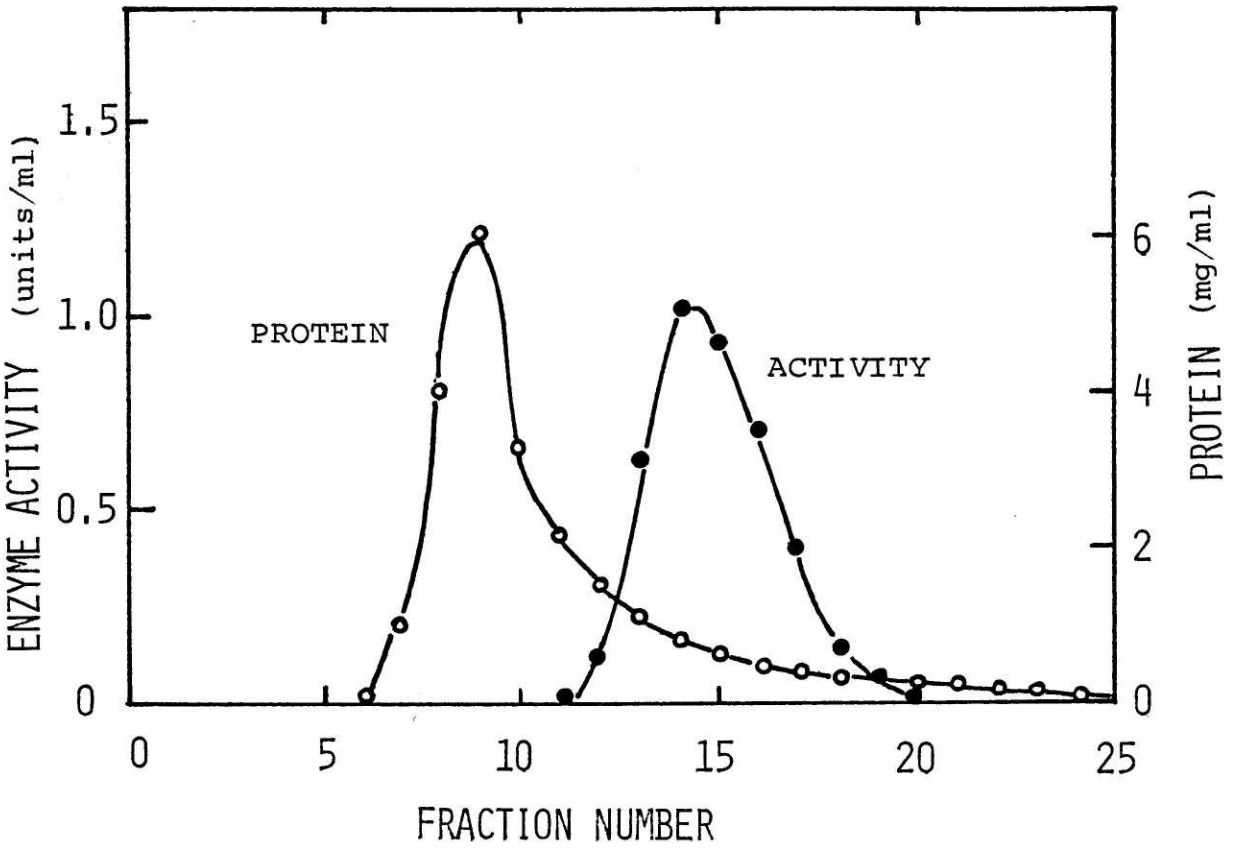


FIGURE 1.

Chromatography on Sephadex G-75. The enzyme fraction from ammonium sulfate fractionation was placed on a column of Sephadex G-75, 1.5 x 95 cm, equilibrated with 0.1 M Tris, pH 7.8 and eluted with same buffer. Fractions of 4.5 ml were collected at a flow rate of 20 ml/hr. Then the fractions were assayed for protein and adenylate kinase activity.

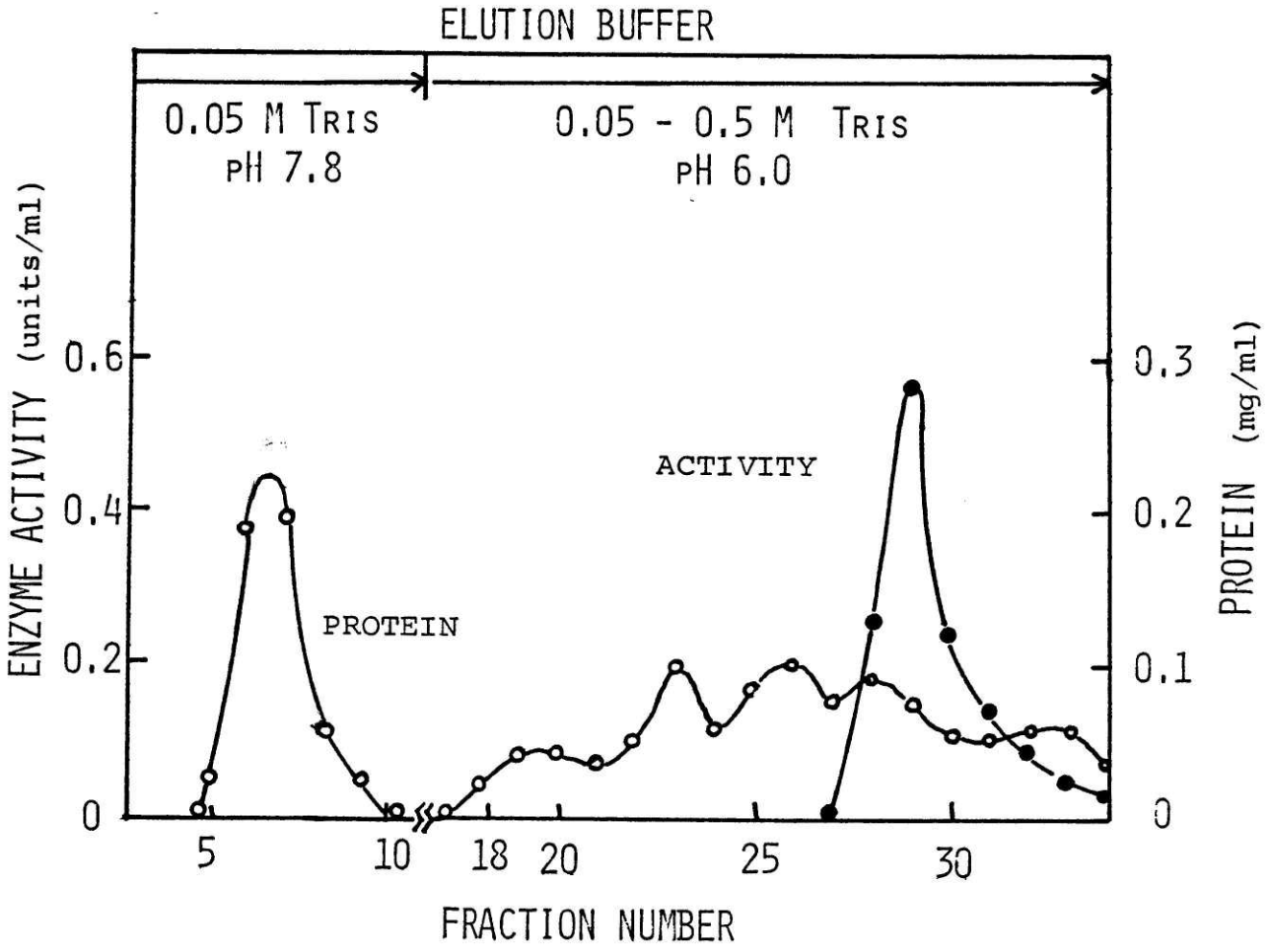


FIGURE 2.

Purification of adenylate kinase by DEAE-cellulose chromatography. The peak fractions of G-75 chromatography were pooled and then applied to a column of DEAE-cellulose, 1.5 x 18 cm, equilibrated with 0.05 M Tris buffer, pH 7.8. After non-adsorbed proteins were washed out with the same buffer, the enzyme was eluted with a linear gradient of 0.05 to 0.5 M Tris, pH 6.0, generated bt mixing 150 ml of each. Fractions of 4.5 ml were collected at a flow rate of 9 ml per hour. The fractions were assayed for protein and adenylate kinase activity.

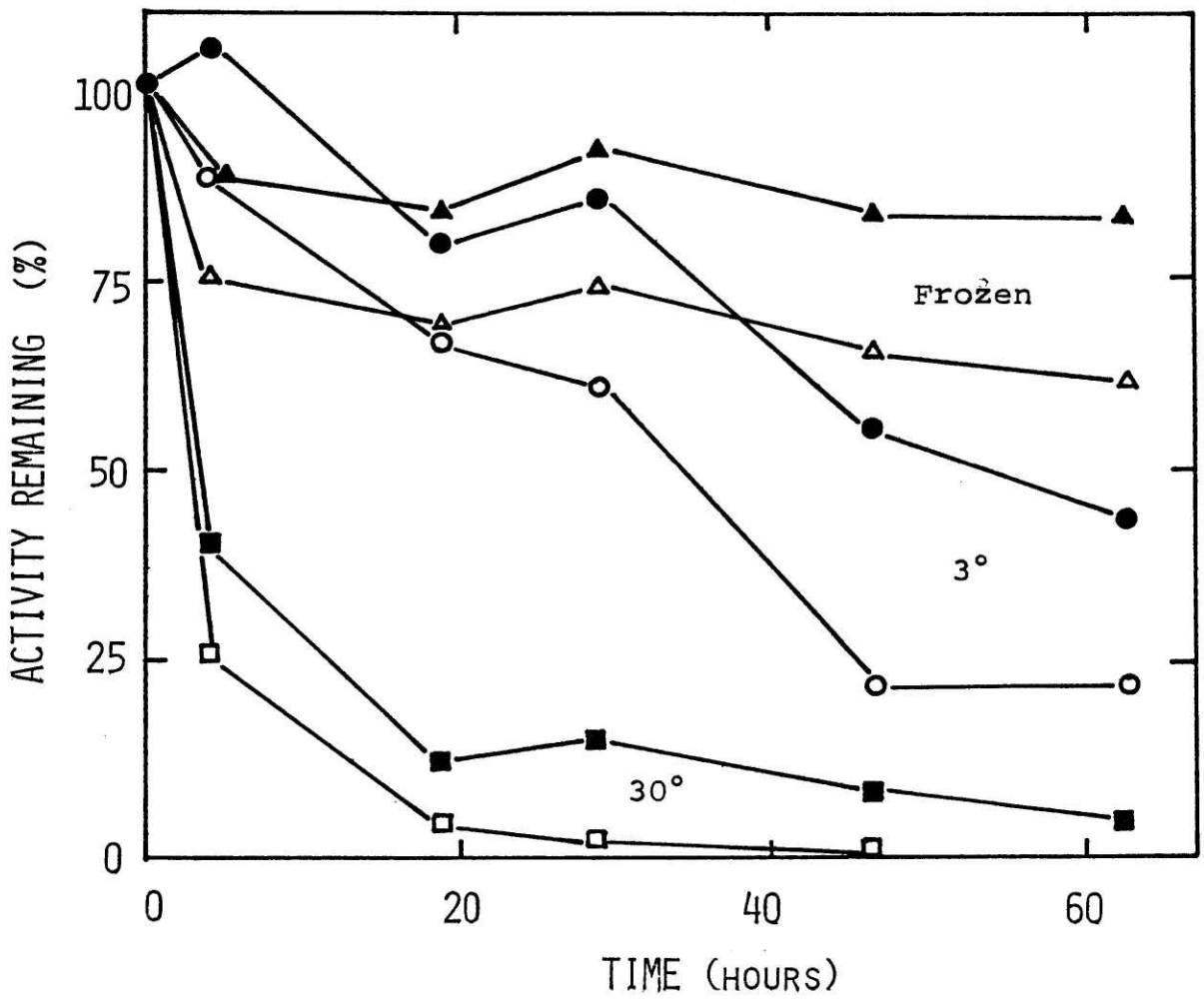


FIGURE 3.

Stability of R. rubrum adenylate kinase at various storage temperatures with (open dots) and without (closed dots) addition of 20 mM dithiothreitol (DTT).

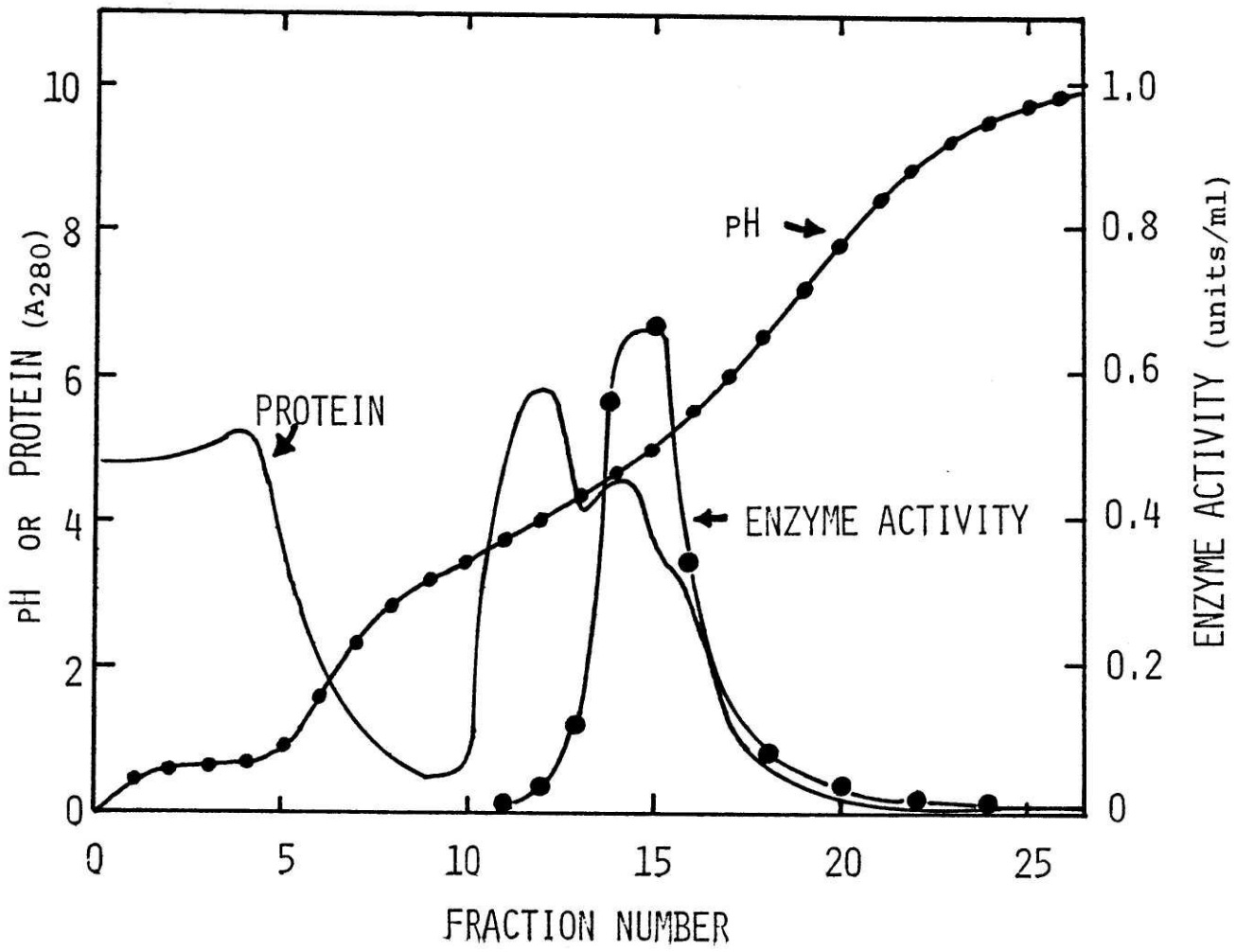


FIGURE 4.

Determination of isoelectric point of R. rubrum adenylate kinase by isoelectrofocusing in ampholytes, pH 3-10. see text for detailed procedures.

BIOGRAPHICAL NOTE

The author was born on December 13, 1947 and was raised in Kwangju, Korea. Later he moved with his family to Seoul where he spent most of his young life. In February, 1969 he was granted a Bachelor of Science degree in the Department of Animal Science, Seoul National University (Korea), and received the Presidential Award as one of top graduates. In the same year, after a long journey across the Pacific Ocean, he entered the University of Minnesota, where he obtained his Master of Science degree in Nutrition with a minor in Biochemistry in December, 1971.

After beginning work towards a Ph.D. at Minnesota, the author decided instead to switch to M.I.T., where he pursued a Ph.D. in Food Science and Technology, with a minor in Biochemical Engineering, from July, 1972 to May, 1976. His major field of interest and research at M.I.T. was Biochemical Engineering, particularly as applied to food. He was a teaching assistant in the biochemical analysis laboratory courses for two terms.

He has been supported throughout his graduate studies by research assistantships.

He is a member of Institute of Food Technologists.

His publications are;

Yang, H.S., P.E. Waibel, and J. Brenes. Evaluation of vitamin D3 supplements by biological assay using the turkeys. J. Nutrition, 103:1187 (1973).

Pace, G.W., H.S. Yang, S.R. Tannenbaum, and M.C. Archer. Photosynthetic regeneration of ATP using bacterial

chromatophores. Biotech. Bioeng., (in press, 1976).

Yang, H.S., K.H. Leung, and M.C. Archer. Preparation and properties of bacterial chromatophores entrapped in polyacrylamide. Biotech. Bioeng., (in press, 1976).