STUDIES ON THE SUBUNIT INTERACTIONS

IN ASPARTATE TRANSCARBAMYLASE

by

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SUBUNIT INTERACTIONS IN

ASPARTATE TRANSCARBAMYLASE

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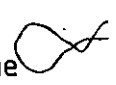SUPERVISOR:　Professor W.W.-C. Chan.

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ii

ABSTRACT

The effects of subunit interactions on the function of aspartate transcarbamylase from _Escherichia coli_ have been investigated by the characterization of an intermediate formed during the reassembly of the native enzyme (c_6r_6) from its constituent catalytic (c_3) and regulatory (r_2) subunits. The intermediary complex was formed by mixing a low concentration of c_3 with excess r_2 and under these conditions was stabilized against reassociation to the native c_6r_6 structure. Sucrose density gradient analysis indicated that the complex sedimented at a position intermediate between the native enzyme and the catalytic subunit. These and other results indicated that the complex has the structure c_3r_6. Similar sedimentation experiments showed the reversibility of c_3r_6 formation and its conversion to c_6r_6 at low r_2 concentrations.

While the native enzyme gives markedly sigmoid initial velocity kinetics with respect to the substrate aspartate the c_3r_6 complex gives a hyperbolic dependence similar to c_3 but with a much reduced K_m. The high affinity of c_3r_6 for aspartate suggests that the complex may represent the relaxed state of aspartate transcarbamylase. Further evidence for this was provided from studies of the pH activity profiles of the various enzyme forms.

Although both regulatory and catalytic subunits are present in the c_3r_6 complex the allosteric inhibitor CTP and activator ATP were found to have no effect on c_3r_6.

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATCase	aspartate transcarbamylase EC 2.1.3.2
ATP	adenosine-5'-triphosphate
BrCTP	5-bromocytidine-5'-triphosphate
BSA	bovine serum albumin
c	catalytic polypeptide of ATCase
c_3	catalytic trimer of ATCase
cpm	counts per minute
c_6r_6	native ATCase containing 6 catalytic and 6 regulatory polypeptides
CTP	cytosine-5'-triphosphate
EDTA	ethylene diamine tetra-acetate
GTP	guanosine-5'-triphosphate
I	inhibitor concentration
K_i or K_{is}	dissociation constant of an enzyme-inhibitor complex
K_{ii}	dissociation constant of an enzyme-inhibitor-substrate complex
K_m	the substrate concentration which gives rise to half maximal velocity
M	Molar
mM	millimolar
M.W.	molecular weight
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of NAD
nm	nanometer = 10^{-9} meter

LIST OF CHEMICAL STRUCTURES

SUBSTRATES

Carbamyl Phosphate

$$H_2N-\overset{\overset{\displaystyle O}{\|}}{C}-O-\overset{\overset{\displaystyle O^-}{|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}=O$$

$$\underset{\displaystyle \underset{CO_2^-}{|}}{CH_2}-\overset{\overset{\displaystyle NH_3^+}{|}}{\underset{\displaystyle CO_2^-}{CH}}$$

Aspartate

PRODUCTS

$$\overset{\overset{\displaystyle O}{\|}}{NH_2-C}$$
$$\overset{\displaystyle NH}{|}$$
$$\underset{\displaystyle \underset{CO_2^-}{|}}{CH_2}-\underset{\displaystyle CO_2^-}{CH}$$

Carbamyl-L-Aspartate

Phosphate

$$HO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O^-$$

TRANSITION STATE ANALOGUE

$$\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-\overset{\overset{\displaystyle O^-}{|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}=O$$
$$\overset{\displaystyle NH}{|}$$
$$\underset{\displaystyle \underset{CO_2^-}{|}}{CH_2}-\underset{\displaystyle CO_2^-}{CH}$$

N-(Phosphonacetyl)-L-Aspartate

ASPARTATE ANALOGUES

$$\underset{\displaystyle \underset{CO_2^-}{|}}{CH_2}-\underset{\displaystyle \underset{CO_2^-}{|}}{CH_2}$$

Succinate

$$\overset{\displaystyle \overset{H}{|}}{\underset{\displaystyle \underset{CO_2^-}{|}}{C}}=\overset{\displaystyle \overset{H}{|}}{\underset{\displaystyle \underset{CO_2^-}{|}}{C}}$$

Maleate

ACKNOWLEDGEMENTS

I. INTRODUCTION

"From a glance at a drawing condensing what
is now known of cellular metabolism we can
tell that even if at each step each enzyme
carried out its job perfectly the sum of
their activities would be chaos were they
not somehow interlocked as to form a co-
herent system"

Monod (1971)

Regulation of the synthesis and degradation of biological

molecules takes place at several levels in the living cell. On a coarse

scale the amounts of the enzymes catalyzing the chemical reactions in a

particular metabolic pathway can be varied by induction or repression

of the genes for these proteins in response to cellular conditions

(Jacob & Monod, 1961). Similarly the degradation rate of enzymes is a

regulating factor. A more immediate form of control, however, is exer-

cised by certain enzymes themselves.

1. Subunit Proteins and Allosteric Interactions

A large number of enzymes have been found to consist of more than

one polypeptide chain (see Darnall & Klotz, 1972). Some of these

oligomeric enzymes catalyze reactions occurring at the branch points

between various metabolic pathways, and their activities are thought to

be modulated by interactions between the subunits of the protein in

response to the binding of compounds unrelated to the substrates or

products of the reaction under catalysis. For example, in biosynthetic

1

pathways the final product of a whole sequence of reactions often inhibits
the first enzyme. Accordingly a large amount of unused final product
can retard its own further synthesis. This form of control is known
as feedback inhibition (Umbarger, 1956).

The modulation of enzymic activity is not limited to inhibition
by the end products of the same pathway but can be effected by products
of unrelated pathways. The resulting interactions may cause either
inhibition or activation.

Such effector compounds usually do not bind at the active site
of the subunit enzyme but to an alternate site, or in some cases to a
special regulatory subunit. This form of control has been designated as
"allosteric" (Monod & Jacob, 1961; Monod et al., 1963; from the Greek
"allo" meaning other and "steric" meaning space or site).

In many cases binding of the substrate to the subunit enzyme
introduces another form of control. Here interactions between the poly-
peptide subunits on substrate binding cause the substrate saturation
profile to become a sigmoid curve rather than the normal hyperbola. A
sigmoid relationship allows a much greater activity response to small
changes in substrate concentration.

The subunit interactions introduced by the binding of substrates
are termed homotropic and those introduced by non-substrate effector
molecules are termed heterotropic interactions (Monod et al.,1965).

The mechanisms by which these two forms of interaction actually
influence the catalytic power of an enzyme are still uncertain, however
two main theories have been proposed (Monod et al., 1965; Koshland et al.,
1966). Both of these hypotheses start from the assumption that the

protein subunits can exist in two conformational forms, one with a high (the R or "relaxed" state) the other with a low (the T or "taut" state) affinity for the substrate. Homotropic interactions are explained by the stabilization of the high affinity form of the remaining empty sub-units on the binding of the first substrate molecule. Heterotropic inhibitors stabilize the low affinity form and activators promote the high affinity form. Evidence from the study of hemoglobin shows that these assumptions are justified (Perutz, 1970). The difference between the two theories arises from the proposed mode of interaction between the protein subunits. According to the scheme of Monod et al. symmetry con-strains the enzyme so that all the subunits exist in one of the two con-formations. It follows then that the binding of the first ligand will convert the whole enzyme to one conformation in a concerted fashion. In contrast Koshland et al. (1966) suggest that change of conformation of the individual subunits follows a sequential pattern, the binding of the first ligand changing the affinity for the next ligand by influencing neighbouring subunits according to the induced fit hypothesis (Koshland, 1959). The binding of a second ligand then produces an even higher af-finity for a third. The above case has been termed positive cooperativity in that the binding of one ligand promotes the binding of the next. Koshland et al. have pointed out that this model also allows negative cooperativity, that is the binding of the first ligand decreases the affinity of the enzyme for the next ligand. An example of this was found in the case of CTP synthetase (Levitzky & Koshland, 1969). The symmetry model of Monod et al. does not predict this behaviour.

Aspartate transcarbamylase (ATCase) from Escherichia coli is
a multisubunit enzyme which shows both the homotropic and heterotropic
properties described above. Available in large quantities it has
become one of the best characterized subunit enzymes (for reviews see:
Jacobson & Stark, 1973; Gerhart, 1970). The mechanism whereby inter-
actions between the various protein subunits modulate the rate of reaction
is still not understood. The experiments to be described have allowed
an attempt at a mechanism to be made.

2. ATCase and Control of Pyrimidine Biosynthesis in Escherichia coli:

Pyrimidine metabolism in E. coli is regulated by both of the
control mechanisms previously described. ATCase has been found to be
a major regulatory enzyme in this pathway.

(a) Control of Enzyme Activity-Feedback Inhibition

Early in the 1950's the biosynthesis of pyrimidines in
cultures of E. coli was observed to be greatly suppressed by addition of
small amounts of uracil or cytosine to the growth medium (Bolton et al.,
1952; Bolton & Reynard, 1954). Yates and Pardee (1956) then showed
in vitro that cytidine and especially cytidine-5'-phosphate were inhibitors
of carbamyl aspartate formation. The production of this compound from
aspartate and carbamyl phosphate (Jones et al., 1955) is catalysed by
aspartate transcarbamylase (Reichard & Hanshoff, 1956) and is the first
committed step in pyrimidine biosynthesis (see Fig. 1).

Later, after Shepherdson and Pardee (1960) had purified ATCase
to near homogeneity, Gerhart and Pardee (1962) showed that the trinucleo-

BIOTIN — CO₂ + ATP

CARBAMYL - P
SYNTHETASE
Mg⁺⁺

NH₃
(FROM
GLUTAMINE)

H₂N
C
O   O - Ⓟ
Carbamyl
phosphate

+   +

CH₂NH₂
CH₂
CH₂
CHNH₂
COOH
ORNITHINE

HOOC
CH₂
H
H₂N   COOH
Asparate

ASPARTATE
TRANSCARB-
AMYLASE

Pi

HO - C
O
H₂N   CH₂
H
O   N   COOH
H
Carbamyl asparate
(ureidosuccinate)

DIHYDRO
OROTASE

H₂O

O
HN   CH₂
H
O   N   COOH
H
Dihydro-orotate

NAD⁺

DIHYDRO-OROTATE
DEHYDROGENASE

NADH + H⁺

Pi

ORNITHINE
TRANSCARB-
AMYLASE

NH₂
C=O
NH
CH₂
CH₂
CH₂
CHNH₂
COOH
CITRULLINE
(CARBAMYL
ORNITHINE)

CO₂

OROTIDYLIC ACID
DECARBOXYLASE

PPi   PRPP

OROTIDYLIC PYRO-
PHOSPHORYLASE

O
HN
O   N
R-5-Ⓟ
Uridylate (UMP)

O
HN
O   N   COOH
R-5-Ⓟ
Orotidine-5'-phosphate
(orotidylate)

O
HN
O   N   COOH
H
Orotate

UDP   REDUCTASE   dUDP (DEOXYURIDINE DIPHOSPHATE)

UTP

NH₃

dUMP

CH₃
(FORMATE)

THYMIDYLIC
SYNTHETASE

NH₂
C
N
O   N
R-5-Ⓟ-Ⓟ-Ⓟ
Cytidylate-triphosphate
(CTP)

O
HN   CH₃
O   N
dR-5-Ⓟ
Thymidylate
(TMP)

dR = deoxyribose
R = ribose

NH₂
C=NH
CH₂-NH
CH₂
CH₂
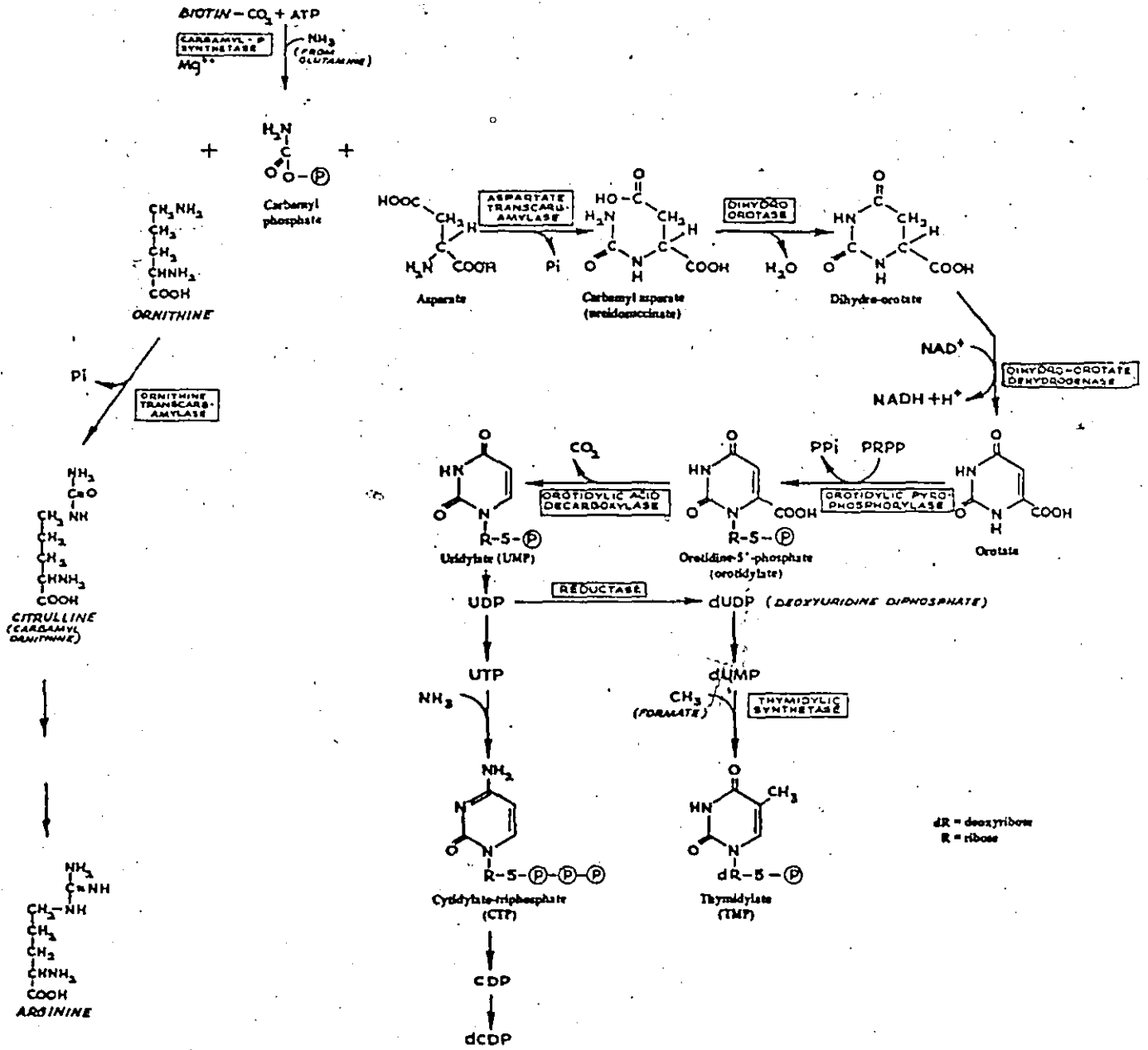CHNH₂
COOH
ARGININE

CDP

dCDP

Fig. 1.   Metabolic Fates of Carbamyl Phosphate

(Extensively modified from Harper, 1969)
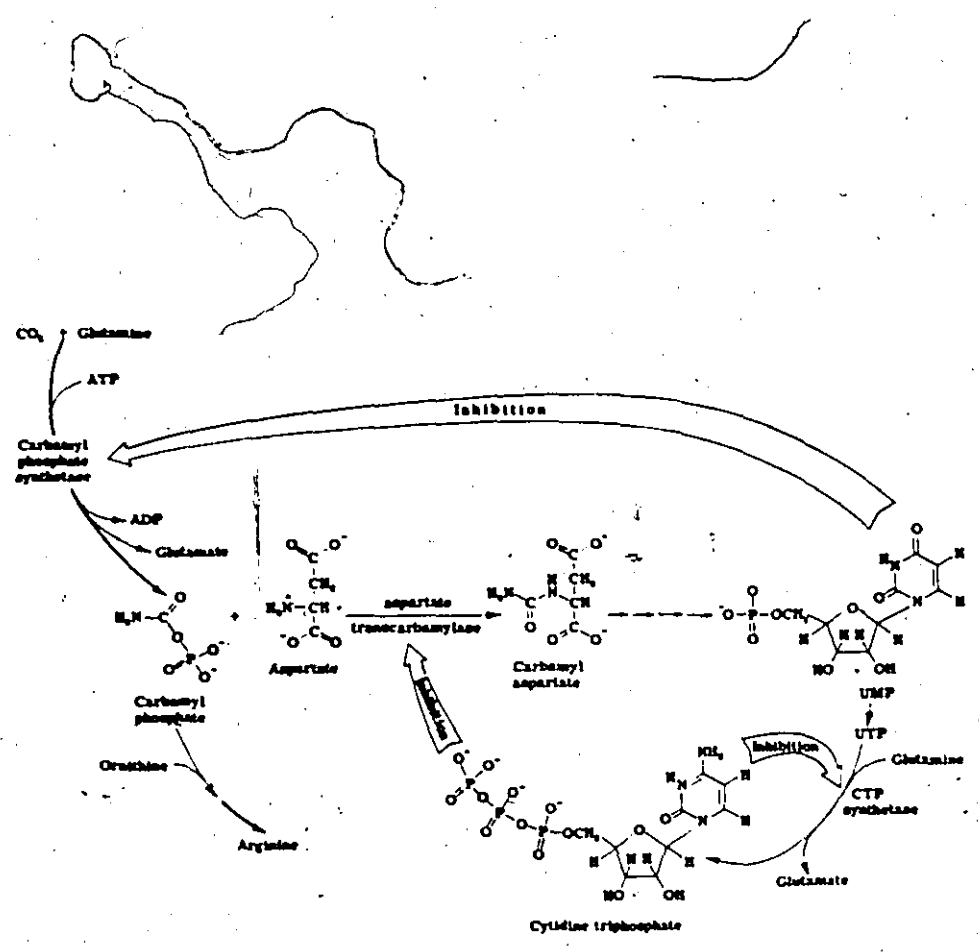
Fig. 2 Regulatory Circuits Controlling Pyrimidine Biosynthesis at
the Enzyme Level in E. coli.
      The heavy open arrows signify the inhibitory action of a
specific metabolite on one of the enzymes of the pathway.
From Gerhart (1970), redrawn by Jacobson and Stark (1973a).

(b) Control of Enzyme Levels

In addition to the modulation of enzyme activity discussed above Yates and Pardee (1957) found that the synthesis of ATCase was repressed by addition of uracil to the growth medium. This discovery was later used by Gerhart and Holoubek (1967) to isolate a strain of E. coli which produces extremely large quantities of ATCase. The strain is diploid in the region containing the cistrons for ATCase and therefore produces twice the normal amount of enzyme under derepressed conditions. In addition the bacterium is defective in the gene for orotidylate decarboxylase (see Fig. 1) and thus grows only slowly in the absences of pyrimidines. For production of large amounts of ATCase the bacteria are grown to a certain titer in the presence of uracil. Under these conditions ATCase synthesis is repressed but cell growth is rapid. Upon depletion of the supply of uracil however, ATCase synthesis becomes derepressed. Extremely large amounts of this enzyme then accumulate as reinstitution of repression is not possible due to the mutant form of orotidylate decarboxylase blocking the remainder of the pyrimidine biosynthesis pathway.

The large amounts of ATCase that can be isolated from this mutant of E. coli have allowed detailed study of the enzyme by the various techniques of enzymology and protein chemistry.

3. Kinetic and Physical Properties of ATCase

    (a) Kinetic Properties of ATCase

         Working with the pure enzyme, Gerhart and Pardee (1962) found that the rate of reaction in the presence of saturating carbamyl phosphate showed a sigmoidal dependence on the aspartate concentration. Such an initial velocity versus aspartate concentration profile is shown in Fig. 3.

         The heterotropic inhibition by the feedback inhibitor is also illustrated as is the activation of the enzyme by ATP. As may be expected the inhibition by CTP can be reversed by ATP (Gerhart & Pardee, 1962).

         The sigmoidicity of the initial velocity profile and the susceptibility to CTP and ATP are characteristic properties of the native form of ATcase

    (b) Desensitization of ATCase

         Gerhart and Pardee (1962) also found that treating ATCase with various mercurials or heating it to 60° at low ionic strength rendered the enzyme insensitive to CTP inhibition. The initial velocity profile of the treated enzyme with respect to aspartate showed a hyperbolic rather than sigmoid dependence.

         Dissociation of the enzyme was suggested as the cause of desensitization (Gerhart & Pardee, 1964) and thus focused attention on the subunit structure of ATCase.

    (c) Elucidation of the Subunit Structure of ATCase

         Compared to most oligomeric enzymes the subunit structure of ATCase is complex and therefore remained uncertain for some time.
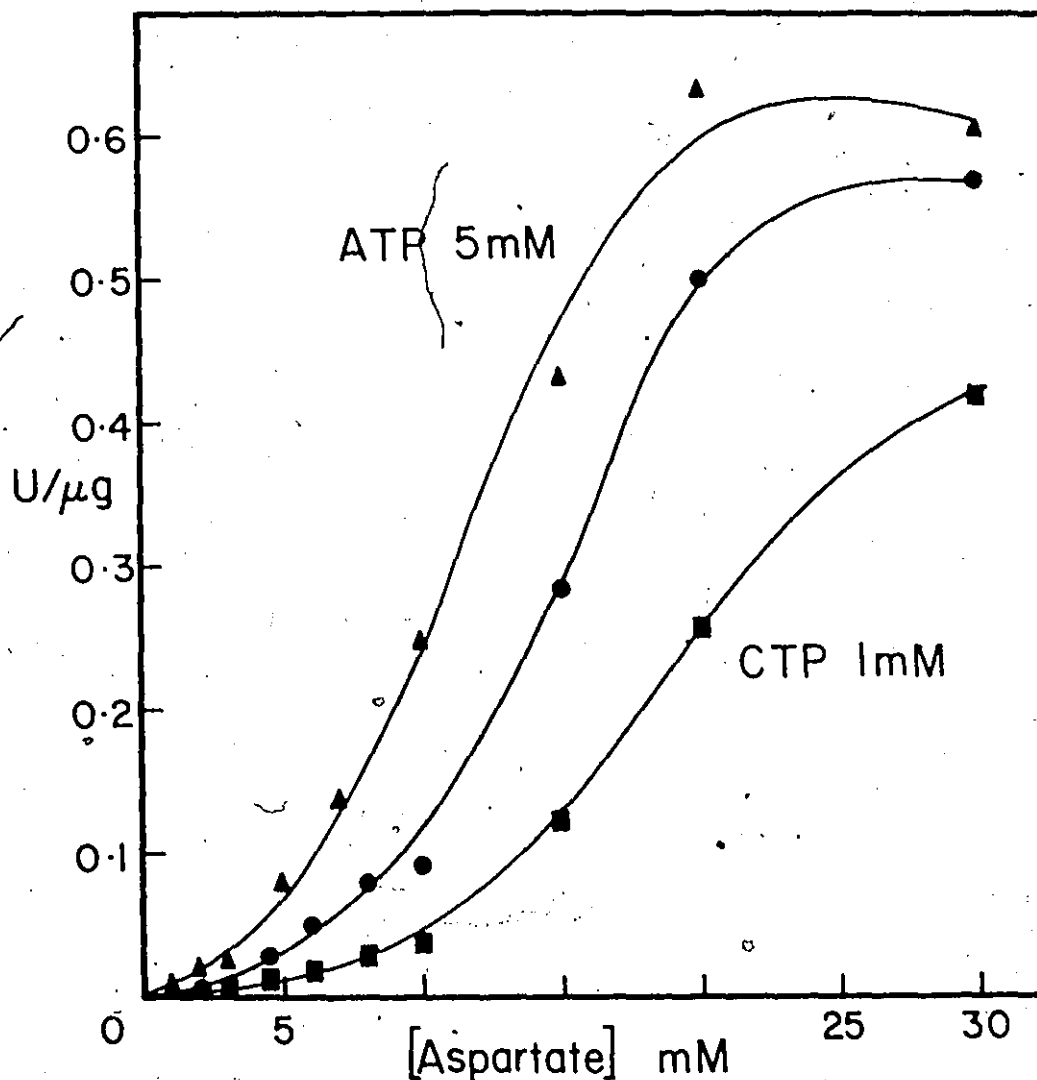
Fig. 3 <u>Initial Velocity Profile of ATCase and Effect of CTP and ATP</u>.
ATCase activity was measured at pH 8.5 by titration assay
(See Methods and Materials) at varied aspartate concentrations
and saturating carbamyl phosphate (5 mM). As indicated squares
show activity in the presence of CTP (1 mM) and triangles in
presence of ATP (5 mM) circles indicate assays with no nucleo-
tides.

These results are similar to the well-known profiles obtained
at pH 7.0 by Gerhart and Pardee in 1962 and agree with the recent
data of Evans et al. (1975) at pH 8.3.

A major breakthrough in the solution of this problem was the discovery that the densensitization of ATCase by treatment with para-hydroxymercuribenzoate (pHMB) gave rise to two different subunit species (Gerhart & Schachman , 1965). The larger of the two was found to retain the catalytic activity but gave simple hyperbolic initial velocity kinetics. Also its catalytic activity was insensitive to the hetero-tropic inhibitor CTP. This species was termed the catalytic subunit. The smaller product of pHMB dissociation of ATCase showed no catalytic activity but was found to bind the inhibitor CTP. (Changeux et al., 1968). This species has been termed the regulatory subunit.

Upon removal of the mercurial, the catalytic and regulatory subunits were found to reassociate spontaneously to form an aggregate having all the properties of native ATCase. (Gerhart & Schachman , 1965).

For several reasons values of the molecular weights of ATCase, the constituent subunits and their individual polypeptide chains and consequently the number of each chain in the active enzyme, were un-certain. In 1968 however, the complete sequence of the regulatory sub-unit was published (Weber, 1968b), and a molecular weight of 17,000 was calculated for the polypeptide. Polyacrylamide gel electrophoresis in sodium dodecylsulphate (SDS) gave a similar result for the regulatory polypeptide and a value of 33,000 for the catalytic chain. From these data it had to be concluded that the native enzyme was an aggregate of six catalytic and six regulatory chains. Further X-ray crystallographic analysis by Wiley and Lipscomb (1968) revealed that the enzyme molecule had a three fold symmetry axis in addition to the previously discovered two fold axis (Steitz et al., 1967). Such symmetry characteristics are

in agreement with the twelve chain finding of Weber.

Work from many sources (Table I) has borne out the above model and the arrangement of the individual subunits is now known (Fig. 4). The catalytic subunit is composed of three 33,000 M.W. chains and will be abbreviated as c_3. The regulatory subunit is a dimer (r_2) of 17,000 M.W. chains. Native ATCase ($c_6 r_6$, M.W. 300,000) is constructed of two catalytic subunits interposed by three regulatory subunits (Fig. 4). This model was first proposed by Markus et al. (1971) and Rosenbusch and Weber (1971a) based on indirect evidence. Electron microscopy (see Fig. 5) (Richards & Williams, 1972) and X-ray crystallography (Wiley et al., 1971; Warren et al., 1973) later provided strong evidence for this arrangement.

(d) Zinc and the Regulatory Subunit

Some of the difficulties experienced in the elucidation of the subunit structure of ATCase were due to the formation of a mercury derivative of the regulatory subunit when the native enzyme was dissociated with para-hydroxymercuribenzoate. The native enzyme was found to contain six zinc ions (Gerhart, 1970; Rosenbusch & Weber, 1971a) which are located in the regulatory subunits, one per polypeptide chain (Rosenbusch & Weber, 1971b). Upon dissociation of ATCase by the above mercurial it was found that mercury replaced the zinc ions of the free regulatory subunit (Nelback et al., 1972). This form of the regulatory subunit participates in a monomer-dimer equilibrium (Cohlberg et al., 1972). A procedure for displacing these mercuric ions by zinc has been described by Nelbach et al. (1972). The zinc form of r_2 shows much less tendency to dissociate and recombines much more effectively with catalytic subunit

13

Fig. 4. Recent Models for Arrangement of Subunits in ATCase.

(i) Models Proposed from Indirect Evidence.

(a) Model of Marcus et al. (1971) showing the regulatory dimer units between catalytic trimers.

(b) Model of Rosenbusch and Weber (1971a) showing the twofold and threefold axes of symmetry. Regulatory polypeptides are represented by the smaller spheres.

(ii) X-ray Cystallographic Results at 5.5Å (Warren et al., 1973).

(c) A schematic view of the molecule expanded along the threefold axis showing a central cavity bounded at the sides by regulatory chains and at the top and bottom by catalytic trimers. The dots within the catalytic subunits represent the position of mercury atoms which were introduced to aid the study and are thought to be close to the active sites.

(iii) Model from Chemical and Hydrodynamic Studies and Electron Microscopy.

(d) Model of Cohlberg et al. (1972) who demonstrated by chemical crosslinking studies that the regulatory chains exist as dimers in the native enzyme. On the basis of hydrodynamic data for c_6r_6, c_3 and r_2 and the electron micrographs of c_6r_6 and c_3 of Richards and Williams (1972) (see Fig. 5) this detailed model was proposed. The regulatory dimers are seen as V-shaped rods. As indicated from X-ray data the catalytic trimers of c_6r_6 are believed to be separated in native ATCase.

a

b

c

d
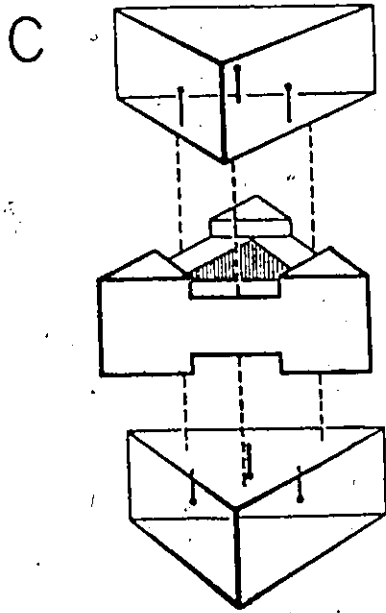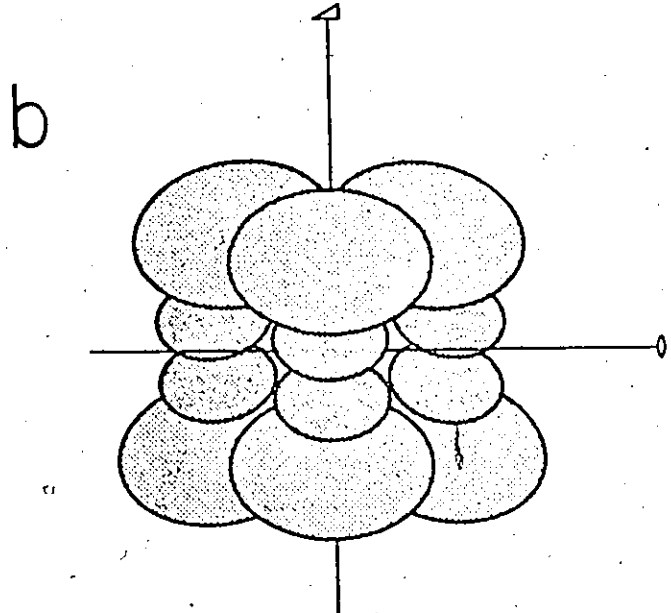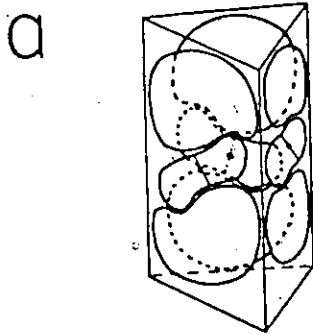
Fig. 5. <u>Electron Micrographs of ATCase and its Catalytic Subunit.</u>

Preparations of c_6r_6 (upper) and c_3 (lower),1 mg/ml
were negatively stained with potassium phosphotungstate
(previously adjusted to pH 7) essentially as described
by Richards and Williams (1972).

Grids were viewed on a Philips 301 transmission microscope
using the standard anticontamination device. Photographs
were taken at a magnification of 110,000x enlarged photo-
graphically, to give a final magnification of 440,000x.

The results are similar to those obtained by Richards
and Williams (1972).

Beside each micrograph is a schematic drawing of the
proposed structure.

$C_6 r_6$

C_3
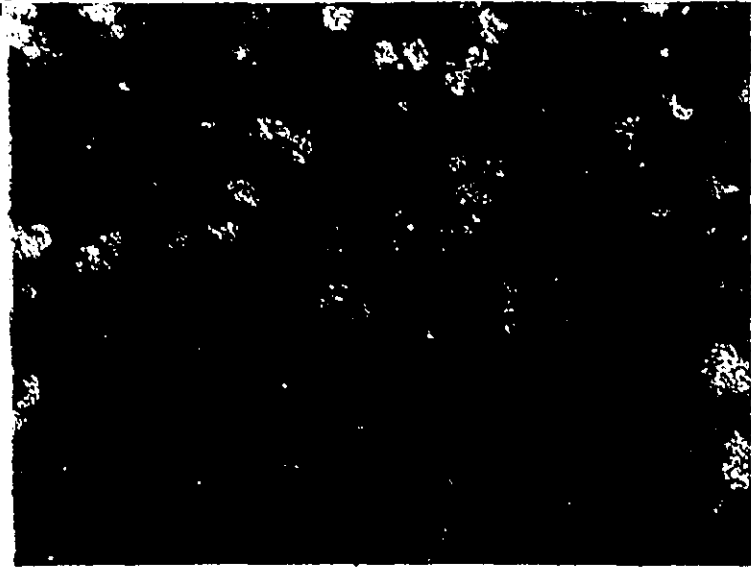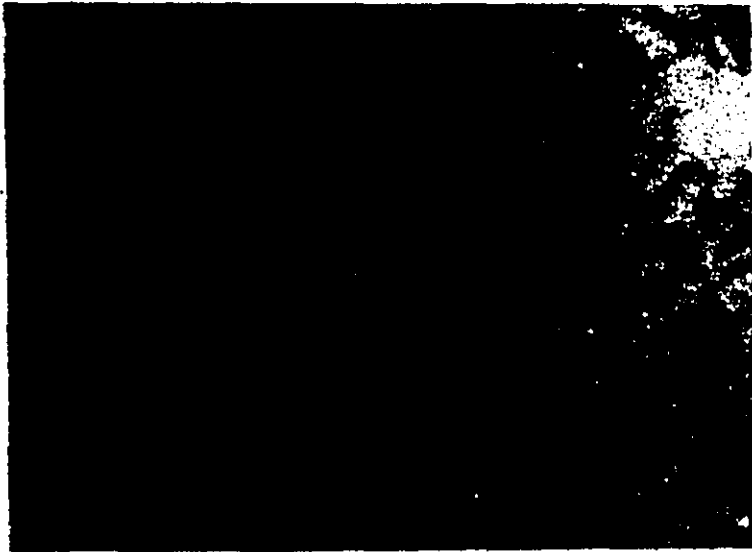
Equilbrium dialysis Ultracentrifugation	6 binding sites for CTP in ATCase	Winland & Chamberlin, 1970; Gray & Chamberlin, 1971; Buckman, 1970
X-ray crystallography	Preliminary 5.5 $\overset{\circ}{A}$ structure showing arrangement of subunits	Wiley et al., 1971
M.W. determination of polypeptide chains by various methods correlated with M.W. determination of subunits and native enzyme	M.W. regulatory chain 17,000 6 chains/native enzyme 2 chains/r_2 M.W. catalytic chain 33,500 6 chains/native enzyme 3 chain/c_3	Rosenbusch & Weber, 1971a
Metal content	6 g atoms of zinc per mole of ATCase No zinc associates with c_3 but found associated with r_2	Rosenbusch & Weber, 1971b
Covalent crosslinking of r_2 and reassociation with c_3	r_2 is incorporated into ATCase as a dimer	Cohlberg et al., 1972
Electron Microscopy	c_3 is visualized as a trimer ATCase visualized see (Fig. 5)	Richards & Williams, 1972
X-ray crystallography	5.5 $\overset{\circ}{A}$ structure showing arrangement of subunits	Warren et al., 1973
Equilibrium dialysis	6 sites for ATP binding in ATCase	Matsumoto & Hammes, 1973 Gray et al., 1973

than the mercury containing regulatory subunit. The discovery of the formation of the mercury containing form of r_2 and its conversion to the native zinc form were of utmost importance in the work to be presented.

Recent elegant work using several physical chemical techniques has demonstrated that in $c_6 r_6$ the zinc binding site on the regulatory subunit is close to the catalytic subunit whereas the CTP binding site is far removed from both the catalytic chains and the zinc atom. (Griffin et al., 1973; Fan et al., 1975; Matsumato & Hammes, 1975). It seems therefore that the zinc ions are involved in the association of c_3 with r_2 and have little to do with the binding of the effector molecules CTP and ATP. In fact the above studies illustrate the true allosteric nature of the action of the heterotropic effector.

(e) Kinetic Mechanism of ATCase

During the course of the reaction catalysed by ATCase the two substrates carbamyl phosphate and aspartate are bound by the enzyme and yield (under physiological conditions irreversibly) the two products carbamyl aspartate and phosphate. According to the nomenclature of Cleland (1963a) this is a Bi Bi reaction and it could proceed by one of three possible kinetic mechanisms. In fact some controversy has arisen as to which mechanism should be assigned to the ATCase reaction.

The possibility of a Ping Pong mechanism was ruled out by the early work of Reichard and Hanshoft (1956) and the case against this mechanism was further strengthened by Schaffer and Stark (1972).

A detailed kinetic study of the catalytic subunit lead Porter et al. (1969) to propose an ordered mechanism for ATCase —

$$\begin{array}{ccccc}
\text{A} & \text{B} & \text{P} & \text{Q} \\
\downarrow & \downarrow & \uparrow & \uparrow \\
\hline
\text{E} & \text{EA} & \left(\begin{array}{c}\text{EAB}\\\text{EPQ}\end{array}\right) & \text{EQ} & \text{E}
\end{array}$$
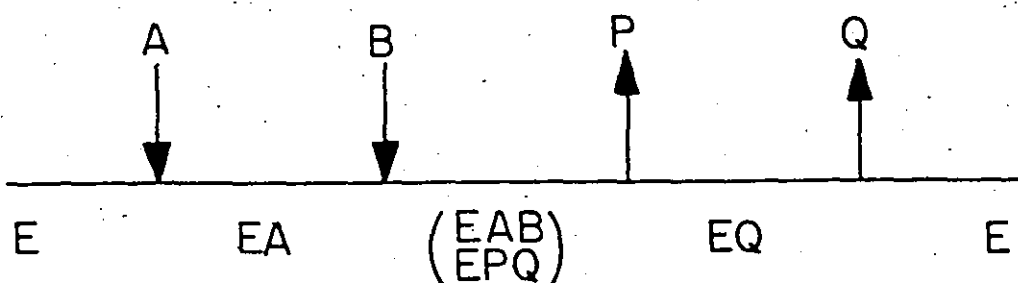
where carbamyl phosphate (A) must bind before aspartate (B) and carbamyl aspartate (P) leaves before phosphate (Q). The absolute assignment of the mechanism was however complicated by the enzyme's thousand fold greater Km for aspartate than for carbamyl phosphate and by the formation of several dead end complexes.

The case for an ordered mechanism was strengthened by studies with N-(phosphonacetyl)-L-aspartate (PALA) an analogue of the transition state between aspartate and carbamyl phosphate synthesized by Collins & Stark (1971). This compound binds very tightly to the enzyme and is a competitive inhibitor with carbamyl phosphate and noncompetitive inhibition with aspartate even when aspartate was present at concentrations many fold that of its Km.

In spite of the evidence outlined above Heyde et al. (1973) have argued convincingly for a Rapid Equilibrium mechanism with several dead end complexes. Here the order of binding would be random and the limiting step in the reaction is the rate of conversion of the ternary complex.

While initial velocity kinetics alone cannot resolve the kinetic mechanism of ATCase, a decision between an ordered and a rapid equilibrium random mechanism is best decided by isotope exchange studies at chemical equilibrium (Boyer 1959).

Although the ATCase reaction is essentially irreversible under physiological conditions Wedler and Gasser (1974) have used this technique in vitro and the results clearly indicate an ordered mechanism.

The elucidation of the kinetic mechanism of ATCase is an important step in the understanding of the allosteric mechanism giving the basic reaction on which the subunit interactions act.

4. Approaches to Studying the Dependence of the Allosteric Properties of ATCase on Subunit Interactions.

As already described, the ATCase activity profile with respect to aspartate concentration is sigmoidal (Fig. 3) and the binding of the substrate analogue succinate shows a similar relationship (Changeux et al., 1968). However, the kinetic behaviour of the isolated catalytic subunit (c_3) and its binding of succinate show a normal hyberbolic concentration dependence. Also while the native enzyme responds to the effector molecules CTP and ATP, the catalytic subunit itself does not. It is known (Changeux et al., 1968) that CTP binds to the regulatory subunit and that this binding is opposed by ATP.

Heterotropic and homotropic interactions are therefore observed only upon association of the different subunits.

It has been shown (Gerhart & Schachman, 1965) that when the isolated c_3 and r_2 subunits of ATCase are mixed in approximately equal proportions under the correct conditions they spontaneously reassociate to form an aggregate that has physical and kinetic characteristics indistinguishable from those of the native enzyme ($c_6 r_6$); as would be ex-

pected this reassociation was found to be irreversible.

The process whereby the ATCase subunits, isolated in vitro, will combine to reform the native structure would be most likely to occur by way of several intermediates formed by the gradual association of c_3 and r_2 units. A study of the properties of any such intermediate would be of great interest as only a portion of the subunit interactions would be present while others would not yet have formed.

(a) Examples of Approaches Previously Used.

In past work on the tetrameric enzyme rabbit muscle, aldolase two approaches were pursued which allowed characterization of a normally transient intermediate, namely the active monomeric subunit.

In the first approach (Chan, 1970; Chan & Mawer, 1972) native aldolase was coupled to an insoluble matrix (Sepharose 4B) under conditions such that the enzyme was attached by only one of its subunits (Fig. 6a). Unbound subunits were then washed off by denaturing the protein with urea and the remaining polypeptide chains were allowed to renature. An active species was found to renature on the matrix but it had properties which differed from those of the native tetramer in several respects particularly its stability to denaturants and proteolytic enzymes.

This difference in stability compared with the native enzyme led to a second approach for characterizing intermediate species. In the assembly of aldolase (Chan et al., 1973), it was expected that during the course of renaturation from the completely unfolded polypeptide chains several intermediates would occur. The renaturation process is envisioned as proceeding by the scheme illustrated in Fig. 6b whereby the unfolded polypeptide chains refold to form active monomers which then aggregate
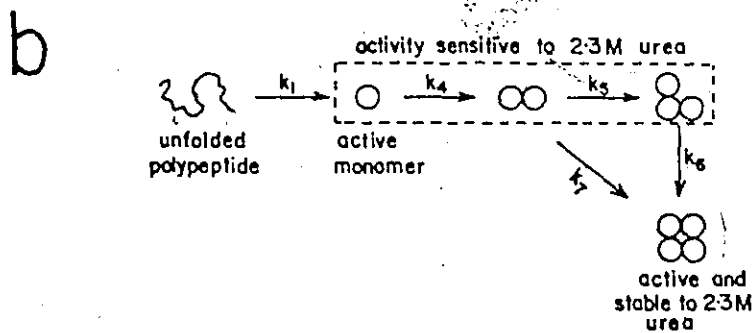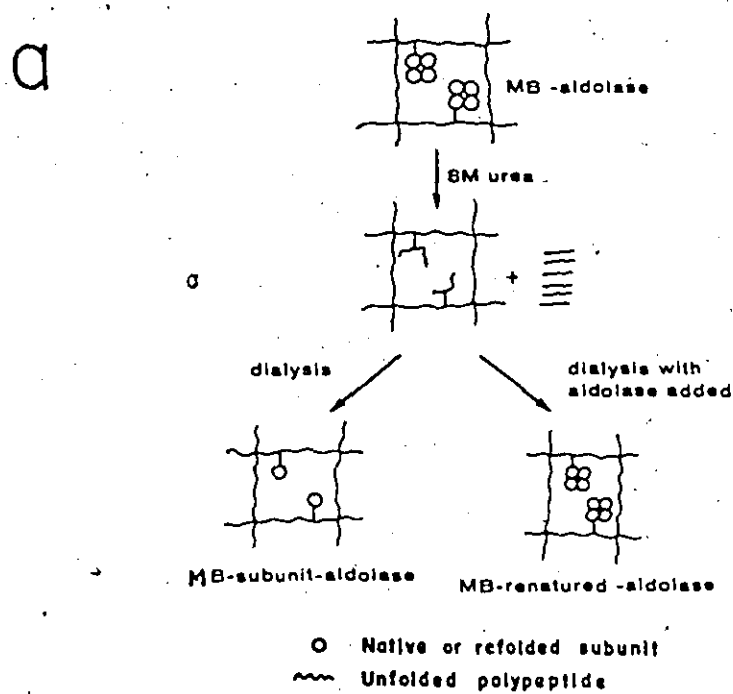
Fig. 6. Examples of the Two Approaches used to Study Subunit Intermediates of Aldolase.

    (a) First approach using matrix bound derivatives (from Chan, 1970)

    (b) Second approach studying intermediate formed during renaturation. (from Chan et al., 1973, slightly modified).

to irreversibly produce the native tetrameric form.

As would be expected from Fig. 6b the relative amount of the intermediate species at any time is very dependent on the total protein concentration while the rate of chain refolding to form the subunit is not. Thus by using very dilute enzyme concentrations the aggregation steps can be retarded. However, the low protein concentrations used severely limit the techniques available to study this process. The most easily measured property under these conditions is enzymic activity.

An assay was developed which registered only the activity of native aldolase. This was achieved by the inclusion in the assay system of a concentration of urea (determined from the above matrix studies) which did not affect the tetrameric form of aldolase but completely inactivated the monomer. In comparison the standard assay measured both the native activity and that of the intermediates. The way was then open to look at the occurence of intermediates in solution.

While matrix-bound enzyme studies have yielded interesting results with ATCase (Chan, 1974) the work presented here is the result of following the second approach.

The search for partially reassembled forms of ATCase was in several ways similar to the above example of aldolase. Here however the starting materials were the isolated catalytic and regulatory subunits rather than denatured polypeptide chains. As the formation of the c_6r_6 structure is known to be irreversible the longevity of intermediates was expected to be enhanced (i) by keeping the total protein concentration as low as possible, (ii) by mixing a large excess of one subunit with the other.

(b) Detection of Intermediates

Because low concentrations of enzyme were to be used
enzyme activity was again chosen as the detection system. The difference
between the initial velocity profiles of the isolated catalytic subunit
and native ATCase and the lack of action by the effectors CTP and ATP
on c_3 allow easy differentiation between these two species. It was
expected that any intermediates formed would have activity profiles
intermediate and perhaps quite different to those of c_3 and c_6r_6 although
it was not possible to predict these properties at the outset.

Using the procedures outlined above it was found that a reasse-
mbly intermediate (c_3r_6) could be detected. This complex showed quite
distinctive kinetic properties and could be stabilized by maintaining a
fairly high ratio of. r_2 to c_3. Under these conditions characterization
of the intermediate was experimentally feasible. Because this complex
contained only part of the subunit contacts present. in the native enzyme,
its properties provided insight into the allosteric mechanism of intact
ATCase. In this thesis evidence will be presented demonstrating that
the intermediate formed under the conditions described is most likely to
have the structure c_3r_6. The nature of the complex and   its formation
and breakdown will also be discussed. Finally the results will be
discussed in relation to the allosteric mechanism of ATCase.

II. METHODS AND MATERIALS

A. Methods

1. Preparation of ATCase

The strain of $\underline{E}$. $\underline{coli}$ specially prepared for the production of ATCase (Gerhart & Holoubek, 1967) was kindly provided by Dr. J.C. Gerhart, University of California. The cells were grown either in a 200 litre batch at the University of Toronto or in a 800 litre batch at the research fermenting facilities of John Labatt Ltd., London, Ontario, following the procedure of Gerhart & Holoubek (1967).

The enzyme was isolated as described by Gerhart and Holoubek (1967). The purification is a seven-step procedure involving ammonium sulphate fractionation of the disrupted cell suspension followed by a 72° heat precipitation step, further ammonium sulphate treatment and column chromatography on DEAE-Sephadex. The peak fractions were then submitted to two low ionic strength precipitations at low pH. The enzyme was stored in 40 mM potassium phosphate at pH 7.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA.

The purified enzyme had high specific activity (see section 3c) and gave a single peak on cellulose acetate electrophoresis (Fig. 7).

As reported by Nelbach et al. (1972) on storage the native enzyme tends to form a small amount of aggregate. Fig. 8 shows the results of Sephadex G200 chromatography of a four month old sample. The small peak eluting in the void volume was found to have the same ultraviolet absorption spectrum and pH ATCase activity profile in 25 mM aspar-

26

preparation were taken and the radioactivity measured to determine the concentration of aspartate which was then adjusted to the required value, usually 50 mM.

This procedure lowered the background to 0.3% of the counts in freshly purified aspartate but the figure tended to increase upon storage. Due to slow decomposition samples were stored at -20°.

(ii) Assay Procedure

Unless specified otherwise, the reaction was conducted in Tris acetate buffer (0.2 M, pH 8.5) containing 2-mercapto-ethanol (20 mM) and BSA (50 µg/ml). The enzyme was preincubated at 25° in this buffer (final volume 0.5 ml) together with labelled aspartate of sufficiently high specific activity to give at least 3500 cpm in the assay. The reaction was initiated by the addition of carbamyl phosphate (4 mM) and terminated with 0.1 ml 1 M phosphoric acid. The reaction mixture (final pH 2.5) was then applied to Pasteur pipette columns containing Amberlite CG-120 (100-200 mesh) in the H^+ form and the columns were washed twice with 1 ml aliquots of 0.2 M acetic acid. The eluant and washings were collected directly in scintillation vials. Without further treatment, scintillation fluid was added to the samples and radioactivity was determined. The measured cpm was corrected by sub-straction of blank values obtained as above but with the omission of carbamyl phosphate. This compensated for background radioactivity and remaining small amounts of impurities in the aspartate preparation.

When the c_3r_6 complex was assayed it was necessary also to correct for the small amounts of enzymically active material present as contaminants in the r_2 preparation since a large excess of r_2 (50 µg/ml) was used.

In radioactive assays, r_2 was added to c_3 30 seconds before the start of the assay to minimize possible conversion to c_6r_6. Control experiments showed that linearity with time was obtained and therefore no significant conversion took place.

(iii) Radioactivity Estimation

Radioactivity was determined by liquid scintillation counting at room temperature in 20 ml of the Triton X114 and xylene mixture (1:3, v/v) described by Anderson and McClure (1973) containing 3 g 2,5-diphenyloxazole and 0.2 g p-bis[2-(5-phenyloxazolyl)]-benzene per liter. The maximal aqueous sample used was 2.6 ml which when mixed with the scintillation fluid, gave a clear solution. Counting efficiency was approximately 36% for tritium. In each series of assays a quenched standard was always included.

(c) Unit of Activity and Specific Activity of ATCase Preparation

A unit of activity is defined here as the amount of enzyme converting 1 μmole of aspartate into carbamyl aspartate per minute at 25°. Using this definition, the specific activity of purified enzyme ranged between 105-130 U/mg at 15 mM aspartate and 4 mM carbamyl phosphate in 40 mM potassium phosphate pH 7.0. This specific activity corresponds to 7300-9200 U/mg using the units defined by Gerhart and Holoubek (1967) and indicates preparations of high purity. Comparison between the titrimetric and radioactive methods of assay was only possible at pH 8.5. Similar activities were obtained at low aspartate concentrations. At higher aspartate concentrations, (e.g. 30 mM) the activity measured with the radioactive assay amounted to only 63% of that

determined by titration probably due to inhibition by buffering
ions (Bethell et al., 1968).

For ease of comparison between the various forms of ATCase,
activity is always expressed in units/µg c_3. Accordingly 1 µg of native
ATCase is considered to contain 0.67 µg c_3, the remainder of the protein
consisting of enzymically inactive regulatory subunits.

4. Protein Estimation

Protein concentrations were generally
determined from the absorbance of the solution at 280 nm. Values of
absorbance for 1 mg/ml solutions (1 cm path length) were taken as 0.59
for ATCase, 0.72 for the catalytic subunit and 0.32 for the zinc
regulatory subunit (Nelbach et al., 1972). Protein measurements were
also made using the methods of Lowry et al. (1951) or Bucher (1947)
and each method was standardized against the absorbance method.

5. pH Profiles

The following buffers were used for assays
at different pH values: pH 6-7, imidazole acetate; pH 7-9, Tris
acetate; pH 9-10, ethanolamine acetate; and pH 10.3-10.7, triethylamine
acetate. The assays were conducted using the radioactive method described
above with 0.2 M of the appropriate buffer. The 2-mercaptoethanol and
unlabelled aspartate were titrated to the required pH before addition
to the assay mixture. When activities were determined in 50 mM
aspartate, 8 mM carbamyl phosphate was used so that an adequate yield of
carbamyl aspartate could be obtained while maintaining carbamyl phosphate
saturation.

6. Sucrose Density Gradients

Linear 5-20% sucrose gradients were made according to Martin and Ames (1961), each gradient (4.6 ml) containing 0.2 M Tris-acetate pH 8.5, 20 mM 2-mercaptoethanol, BSA 50 µg/ml and for certain runs also 50 µg r_2/ml. Enzyme samples (50 or 75 µl) were centrifuged for 9 hours at 47,000 rpm in a Beckman SW 50.1 rotor on a L2-65B ultracentrifuge; temperature was maintained at 3-4°. The tubes were then punctured and 3 drop fractions (∿ 0.17 ml) were collected. The activities of the fractions were determined using the radioactive assay by adding the appropriate amount of substrate and effectors in 10 µl to aliquots from the gradients (50 µl), and incubating at 25°. The reaction was terminated by the addition of 0.5 ml 0.2 M phosphoric acid to make up for the smaller volume in the assay.

The linearity of the gradients was checked by measuring the refractive index of the fractions using a Bausch and Lomb Abbe refractometer.

7. Assay of Marker Enzymes of Known $S_{20,w}$.

Enzymes used in sucrose gradients were estimated by continuous spectrophotomeric assay carried out at 25° using either a Gilford Model 2000 density convertor connected to a Beckman DU monochrometer and a self balancing recorder or a Cary 118 spectrophotometer.

(a) Bovine liver catalase was assayed by the decrease in absorbance at 240 nm due to hydrogen peroxide utilization (Beers & Sizer, 1952).

(b) E. coli alkaline phosphatase
was assayed at 410 nm by following the increase in absorbance due to
the production of o-nitrophenol from o-nitrophenylphosphate (Garen &
Levinthal, 1960).

(c) Yeast alcohol dehydrogenase was
assayed at 340 nm following the production of NADH from the oxidation
of ethanol by NAD (Vallee & Hoch, 1955).

(d) Rabbit muscle aldolase was
assayed at 340 nm following the decrease in absorbance due to depletion
of NADH in an auxiliary reaction. Fructose diphosphate is cleaved
by aldolase to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate,
and the latter is converted to glyceraldelyde 3-phosphate by triose
phosphate isomerase. The reduction of glyceraldehyde 3-phosphate
and concomitant oxidation of NADH is then catalyzed by α-glycerophosphate
dehydrogenase (Racker, 1947).

8. Analysis of Kinetic Data

The original data were fitted to various
equations using the computer programs of Cleland (1963b, 1967) or
modifications of these.
Appendix 1 shows the basic program modified for fitting

$$v = \frac{V_{max}S}{K_m + S + S^2/K_i}$$

the equation for substrate inhibition, and the results obtained from a
sample data set. The programs were run on a Hewlett Packard 3000 computer.

Data was also analyzed using the program KINET (Dye & Nicely, 1971). This non-linear curve fitting program was kindly provided by Dr. J.L. Dye, Michigan State University and was run on a CDC 6400 computer.

Although it has been shown that the double reciprocal plot (Lineweaver & Burk, 1934) is not statistically sound (Hofstee, 1952; Wilkinson, 1961; Eisenthal & Cornish-Bowden, 1974), this representation of kinetic data is the form normally used. In this work the data are most often presented as double reciprocal plots, however, estimation of kinetic constants was carried out by the above numerical procedures on the untransformed data.

B. Materials

1. Chemicals

All chemicals, where possible, were reagent grade. Ultra pure sucrose from Schwartz Mann was used in the sucrose density gradients. Triton X114 was a product of Rohm and Haas.

Dilithium carbamyl phosphate, NAD, NADH, p-nitrophenylphosphate, fructose-1,6-diphosphate, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co.. CTP was obtained from P.L.Biochemicals and ATP from Calbiochem.

L-aspartic acid 2,3 ^{3}H (N) (24 ci/mmole) and ^{14}C L-aspartic acid (U) (208 mci/mmole) were products of New England Nuclear.

2. Enzymes

Bovine liver catalase, E. coli alkaline phosphatase, and a mixture of α-glycerophosphate dehydrogenase with triose phosphate isomerase were obtained from Sigma Chemical Co.. Yeast alcohol dehydrogenase was purchased from Worthington. Rabbit muscle aldolase was prepared by Mr. D.K.K. Chong in this laboratory according to Taylor et al. (1948).

III. RESULTS

The search for possible intermediate species in the assembly of ATCase from c_3 and r_2 was begun by incubating a dilute concentration of c_3 in the presence of excess r_2. Because the detection system used was the measurement of enzyme activity the lack of ATCase activity from the regulatory subunit makes it possible to use arbitrarily high concentrations of r_2 and at the same time allows the detection of very low concentrations of c_3.

1. Initial Experiments

In one of the first experiments reassociation of subunits was initiated by mixing an excess of r_2 (100 μg/ml) with either 50 or 10 μg/ml c_3. At various times aliquots from the reassociation mixtures were withdrawn and their activities measured using the radioactive assay. To detect the appearance of possible intermediates three assay conditions were used.

    (a) pH 7.0

    (b) pH 7.0 plus CTP

    (c) pH 8.5

All assay mixtures contained a nonsaturating concentration of aspartate. The pH of 7 was the normal assay condition (Gerhart & Pardee, 1962). It was felt that the difference between the first two conditions would show whether a quick onset of heterotropic effects took place on mixing of the subunits.

40

The study at pH 8.5 was undertaken because at this pH and non-saturating aspartate levels, the specific activity of c_6r_6 is very much lower than that of c_3 (Gerhart & Pardee, 1964). If any partially re-associated species had properties intermediate between those of c_3 and c_6r_6 it was expected that they would be easier to detect at pH 8.5 than at pH 7. The results (Fig. 9) are plotted as a percentage of the activity of the c_3 initially added. In separate experiments it was found that no further changes took place after 4-5 hr.

At pH 7 both with and without CTP a gradual decrease in activity is seen, this decrease was more rapid at the 1:2 c_3/r_2 ratio; a ratio at which c_6r_6 formation (Gerhart & Schachman, 1965) was known to occur. A lower c_3 level simply seemed to allow slower reassociation. After complete reassociation (5 hr) the activity differences seen by assay with and without CTP show that the heterotropic effects are regained.

At pH 8.5 the result was different. Again at a high c_3 concentration a rapid fall in activity was seen, however at a low c_3 level (10 μg/ml) this fall was preceded by a _rise_ in activity. This increase in activity at pH 8.5 can only be explained by the formation of a new species of ATCase as the activity is higher than that of c_3 under these assay conditions, whereas the final product of reassociation (c_6r_6) has lower activity than c_3.

Although evidence of a new form of the enzyme was indicated by the above experiment at pH 8.5, its existance under the conditions used was only transitory. This was probably due to the relatively high concentration of catalytic subunit (10 μg/ml) used.

Fig. 9. Reassociation of c_3 and r_2. Mixtures of 50 µg/ml
         c_3 and 100 µg/ml r_2 (squares) or 10 µg/ml c_3 and 100
         µg/ml r_2 (circles) were allowed to reassociate in
         40 mM potassium phosphate pH 7 containing 20 mM
         2-mercaptoethanol at 25°C.

         At the time indicated aliquots were withdrawn
         and assayed by the radioactive procedures in

         (a) 40 mM potassium phosphate pH 7 containing
              20 mM 2-mercaptoethanol, 100 mg/ml BSA and
              20 mM aspartate, 4 mM carbamyl phosphate

         (b) the same as (a) but also in the presence of
              0.2 mM CTP.

         (c) the same as (a) except that 40 mM Tris acetate
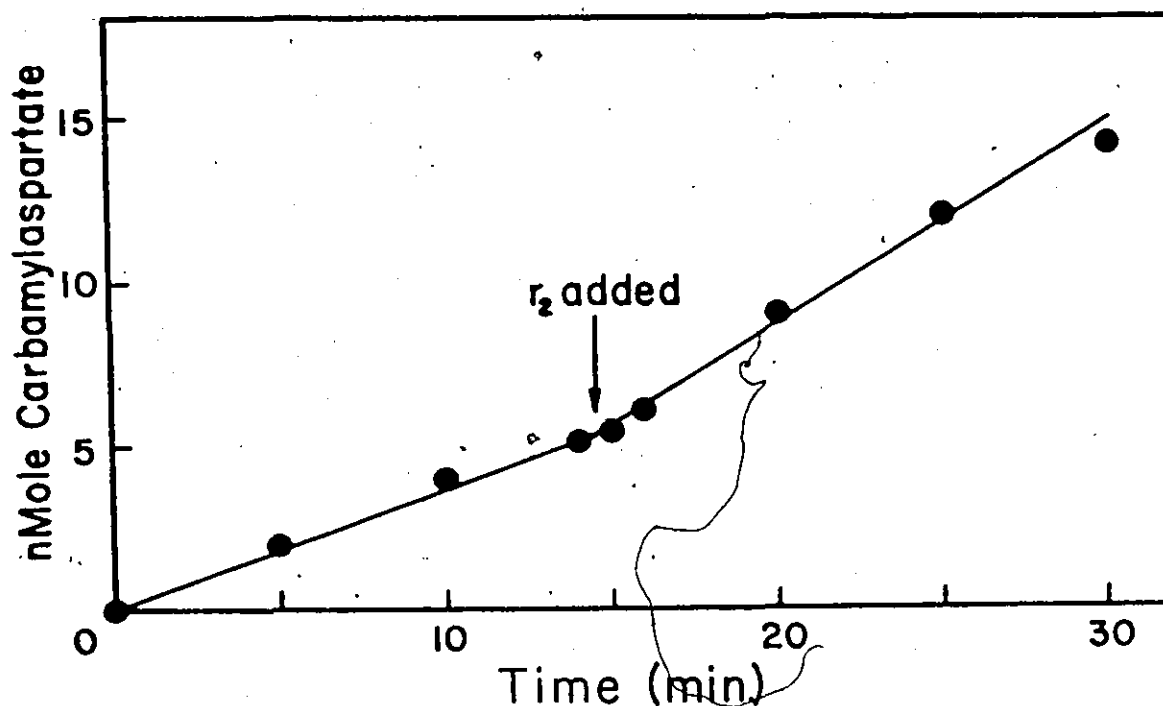   pH 8.5 was used instead of 40 mM potassium
              phosphate pH 7.
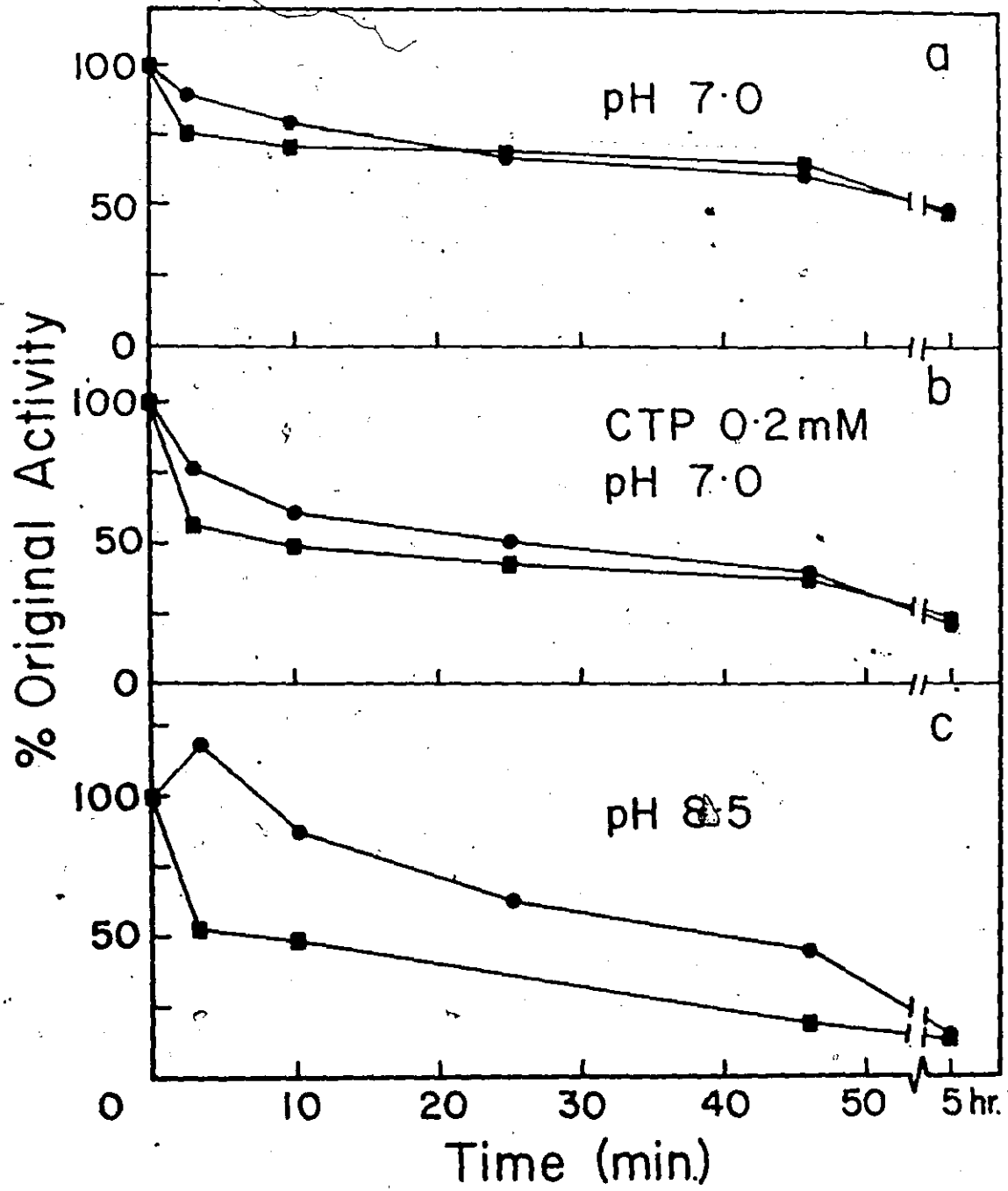
Fig. 10. Reassociation Intermediate Formation Detected During Assay.
c_3 0.05 µg/ml was assayed in 0.2 M Tris acetate pH 8.5,
containing 20 mM mercaptoethanol, 50 µg/ml BSA, 2 mM
potassium aspartate [^{3}H] and 4 mM carbamyl phosphate.
At the times indicated 100 µl aliquots were withdrawn
and treated as described for the radioactive assay
in Methods and Materials section. The arrow represents
the introduction of r_2 to give a final concentration
of 20 µg/ml.

Instead of taking aliquots from a reassociation mixture and assaying these, it was decided to allow the reassociation to take place in the assay mixture itself, take out aliquots from this and separate the products of the reaction by the usual manner. In this way a very low concentration of catalytic subunit (0.05 μg/ml) could be used. After recording the rate of reaction with c_3 alone at pH 8.5 a large excess of r_2 was added (Fig. 10). Directly after this addition a large increase in activity was seen and the rate continued linearily. Under these conditions therefore, the species exhibiting increased activity appeared to be stabilized.

For further work a continuous assay technique seemed to be more convenient than the tedious sampling method. The titrimetric assay was therefore used to further characterize this phenomenon.

2. Initial Characterization of the Intermediates

An increase in activity is seen after addition of r_2 to the c_3 assay (Fig. 10). This increase seemed to be dependent on the excess of r_2 over c_3. It is possible therefore that complexes with the structure c_3r_n where n is 2,4 or 6, are forming and that at high r_2 concentrations a full complement of three r_2 subunits add to each c_3 subunit to produce c_3r_6 (Fig. 11B). This complex, a "truncated" form of the native enzyme (Fig. 11A) would be expected to be stable at high r_2 concentrations as no free c_3 would be available to add to it to complete the c_6r_6 structure.

(A)   (B)
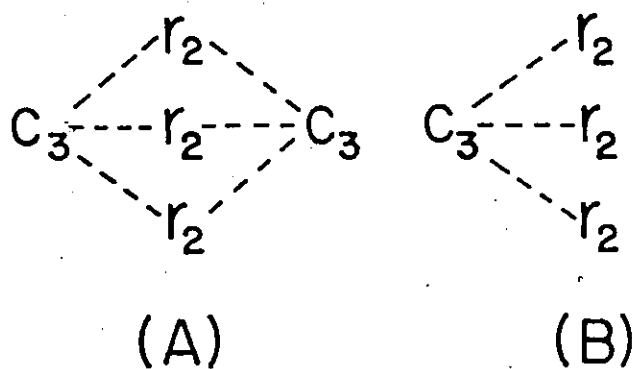
Fig. 11.   Representations of ATCase and the c_3r_6 Complex.
           Schematic representation of the structure of native
           aspartate transcarbamylase (A) and the proposed
           c_3r_6 complex (B).
           Dashed lines represent noncovalent interactions.

           (From Chan & Mort, 1973).

If this line of reasoning is correct then the activation of c_3 by r_2 seen in Fig. 10 should reach a saturating level above which further addition of regulatory subunits has no effect, reflecting the complexing of all r_2 accepting sites on the c_3 subunits. The activity of a mixture of c_3 and a less saturating concentration of r_2 would however, be expected to fall with time due to reassociation of the r_2 and c_3 to form $c_6 r_6$. The native enzyme has been shown to remain associated at very low dilution (Schachman & Edelstein, 1966) so reassociation to $c_6 r_6$ is an irreversible step.

The continuous titrimetric assay of Wu and Hammes (1973) allowed these proposals to be evaluated.

(a) Saturation

The activation caused by several independently tested concentrations of r_2 is shown in Fig. 12. A saturating value was attained at 30-40 µg r_2/ml. When the initial slopes were plotted against the r_2 concentration (Fig. 13) a hyperbolic relationship was found. This suggested that the activation of the catalytic subunit is a simple binding function.

The following results from control experiments show the specificity of the activation effect.

(i) No significant activity was found in the r_2 preparation under these assay conditions.

(ii) The presence of the BSA added to stabilize the c_3 (Porter et al., 1969) could be eliminated in short term experiemnts without changing the results.
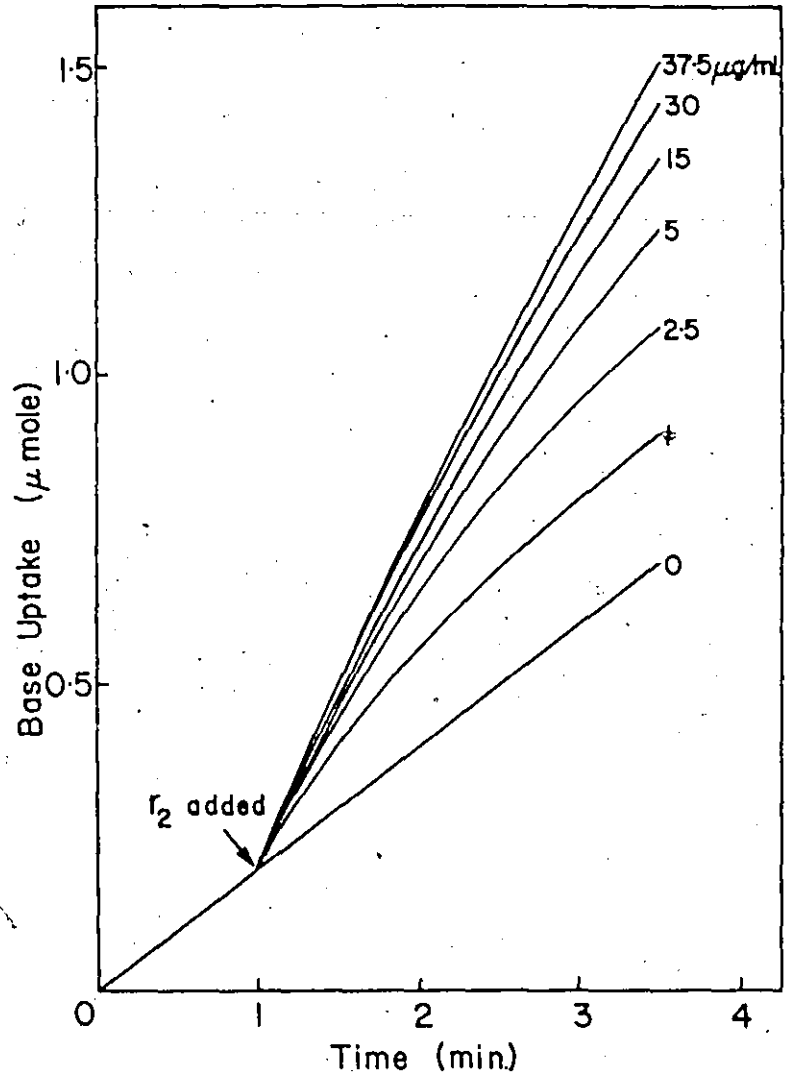
Fig. 12.   Saturation of c_3 by r_2. The first minute shows
the reaction rate of c_3 (1.5 µg/ml) measured by
the standard titration assay at pH 8.5 with 2 mM
aspartate and 5 mM carbamyl phosphate. In separate
experiments different amounts of r_2 were then added.
The curves for the various r_2 concentrations have been
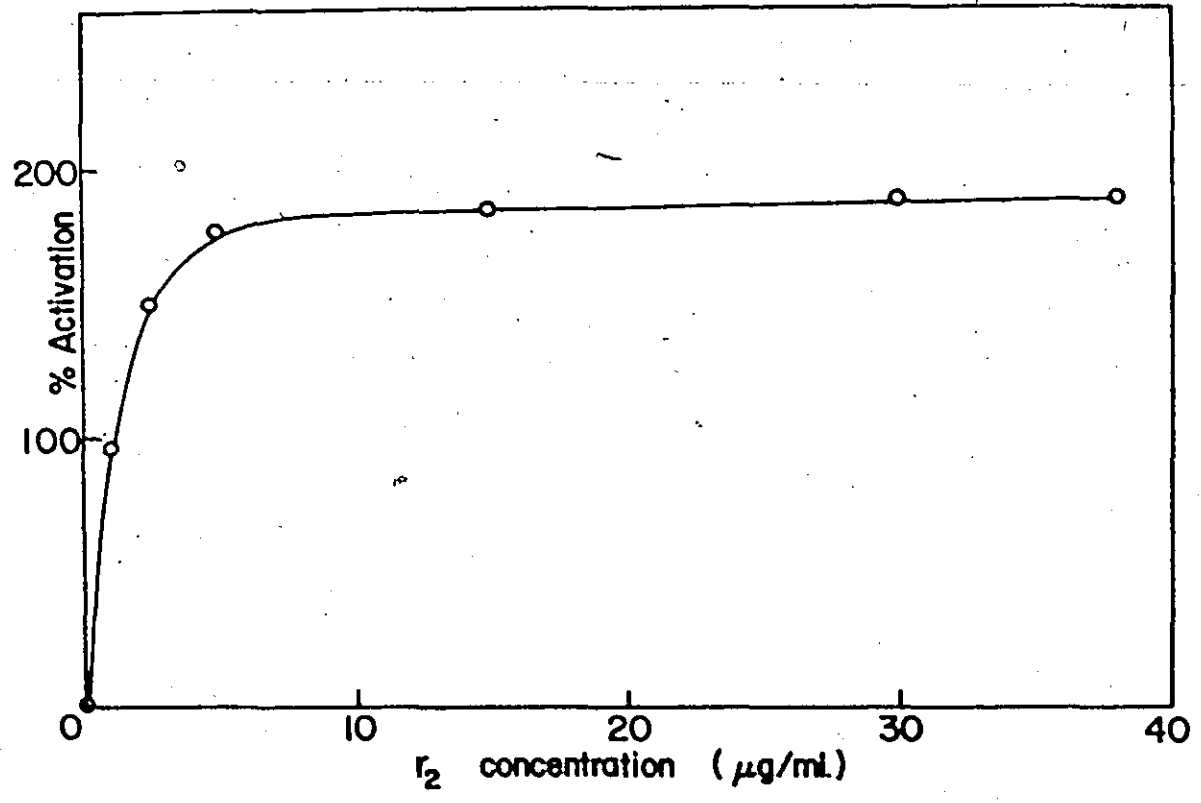superimposed.

(From Chan & Mort, 1973).

Fig. 13. The Initial Activation Relative to r_2 Concentration.
The rates immediately after the addition of r_2
(obtained from Fig. 12) expressed as the percent
increase in activity above the rate due to c_3
alone is plotted against the concentration of r_2
present.

(iii) Addition of r_2 to a preparation of c_6r_6 caused no increase in activity.

Further r_2 saturation studies were carried out in the presence of 6 mM and 15 mM aspartate. Again activation was seen, increasing to maximum value at 30-40 μg/ml r_2. In these cases however the activation at saturation was 2.1 fold and 1.3 fold respectively. These are the characteristics expected if the c_3r_6 complex has a much lower K_m for aspartate than c_3.

(b) Rate of Formation of the Complex

In addition to illustrating a saturation phenomenon the results presented in Fig. 12 also indicate that the association of c_3 and r_2 subunits is so rapid that the rate of complex formation cannot be detected by the continuous assay system. The increase in activity after addition of r_2 was instanteneous even at the lowest concentration of r_2 tested.

(c) Effect of the Allosteric Modifier CTP on r_2 Saturation

As CTP is known to bind to the regulatory subunit (Changeux et al., 1968) conformational changes induced by this nucleotide might be expected to alter both the activity properties of the complex and the affinity of r_2 for c_3 and thus the r_2 saturation profile.

The saturation curve shown in Fig. 13 was obtained using the titration assay (1.5 μg/ml c_3). At low concentrations of r_2 a linear activity response is seen only directly after addition of regulatory subunit; therefore evaluation of small changes in the catalytic activity after r_2 addition is not accurate by this method. The sensitivity of the radioactive assay technique allows the use of extremely low c_3 levels .
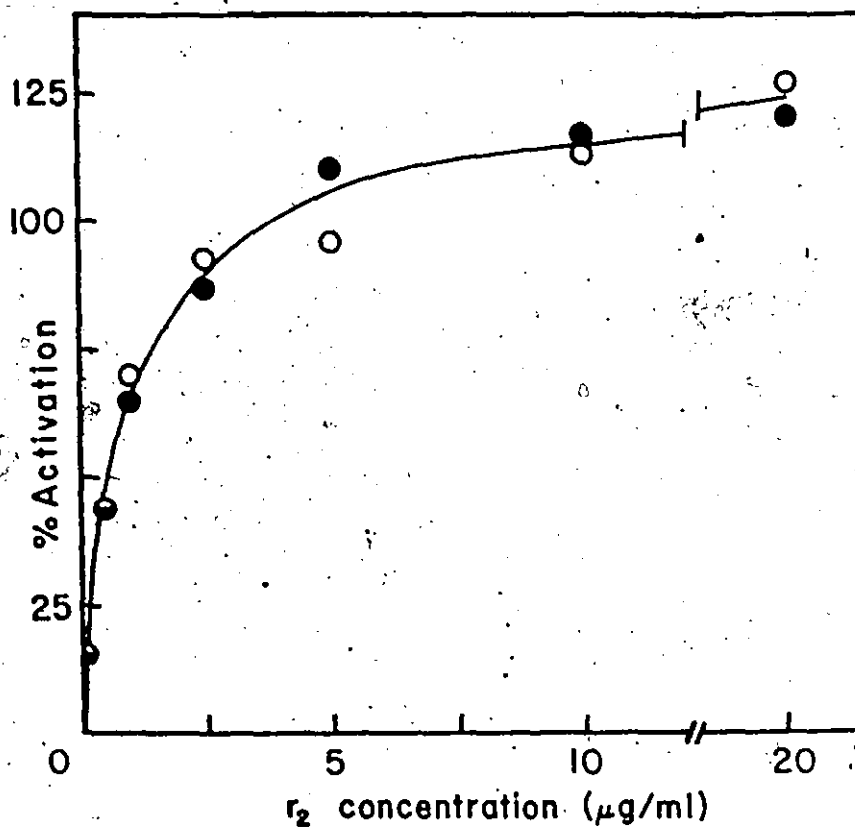
Fig. 14. Effect of CTP on r_2 Saturation. The activities of c_3
(0.075 µg/ml) assayed by the radioactive method in
the presence of various concentrations of r_2 at pH 8.5
with 2 mM aspartate and 4 mM carbamyl phosphate.
Open circles denote no further additions. Closed
circles denote assay in the presence of 1 mM CTP.
Linearity of assay was checked by taking several
time points as in Fig. 11. Percent increase in
activity above the c_3 rate is plotted against the
r_2 concentration present.

(0.075 µg/ml) where the activity after addition of low r_2 concentration remained linear.

Regulatory subunit saturation of c_3 with and without CTP is shown in Fig. 14. Surprisingly CTP was found to have little effect on the activity of the complex, nor did it seem to change the shape of the saturation profile. Chan (1975a) has found that neither ATP nor change in aspartate concentration affects the saturation profile significantly.

(d) <u>Reassociation to c_6r_6 at Non-Saturating r_2 Concentrations</u>

In the presence of high r_2 concentrations an intermediate between the catalytic and regulatory subunits is formed which seems to be stable during the time of the assay as judged by the linear activity response (Fig. 10, and Fig. 12 at saturating levels of r_2). At nonsaturating r_2 levels however, an increase in activity is still observed on addition of the regulatory subunit but with time the assay profiles curve off indicating a gradual lowering of activity. This is to be expected for under these conditions reassociation to c_6r_6 should be possible and at the low aspartate concentration used in these studies c_6r_6 has a much lower specific activity than either c_3 or the c_3r_6 complex. In order to demonstrate that reassociation to c_6r_6 was indeed occurring at non-saturating r_2 levels the activity profiles were studied over a more extended time course (Fig. 15).

A non-saturating concentration of r_2 (2.5 µg/ml) was added to c_3 (0.75 µg/ml) and the activity profile was again measured using the continuous titrimetric assay (Fig. 15, curve A). An increase in activity resulted as before which was less than the maximum produced at saturating r_2 levels (line C). However, as the time of assay in-

53

Fig. 15. Interaction Between c_3 and Non-Saturating r_2 over an Extended Time. The first seven minutes show the rate due to c_3 alone measured by the standard titrator assay at pH 8.5 with 6 mM aspartate.

   Regulatory subunit was added as indicated by the arrow. The results of five experiments have been superimposed. The conditions were:

   A. c_3 0.75 µg/ml, r_2 2.5 µg/ml

   B. c_3 0.75 µg/ml, no r_2

   C. c_3 0.75 µg/ml, r_2 37.5 µg/ml
       or c_3 0.075 µg/ml, r_2 37.5 µg/ml

   D. c_3 0.075 µg/ml, r_2 2.5 µg/ml

When the c_3 concentration was 0.75 µg/ml the carbamyl phosphate concentration was 5 mM and the titrant contained 40 mM NaOH and 40 mM aspartate, while when the c_3 concentration was 0.075 µg/ml the concentration of all these components was reduced 10 fold. Base uptake is therefore expressed as µmoles per µg c_3 to allow comparison between experiments at the different c_3 concentrations.

(From Chan & Mort, 1973).
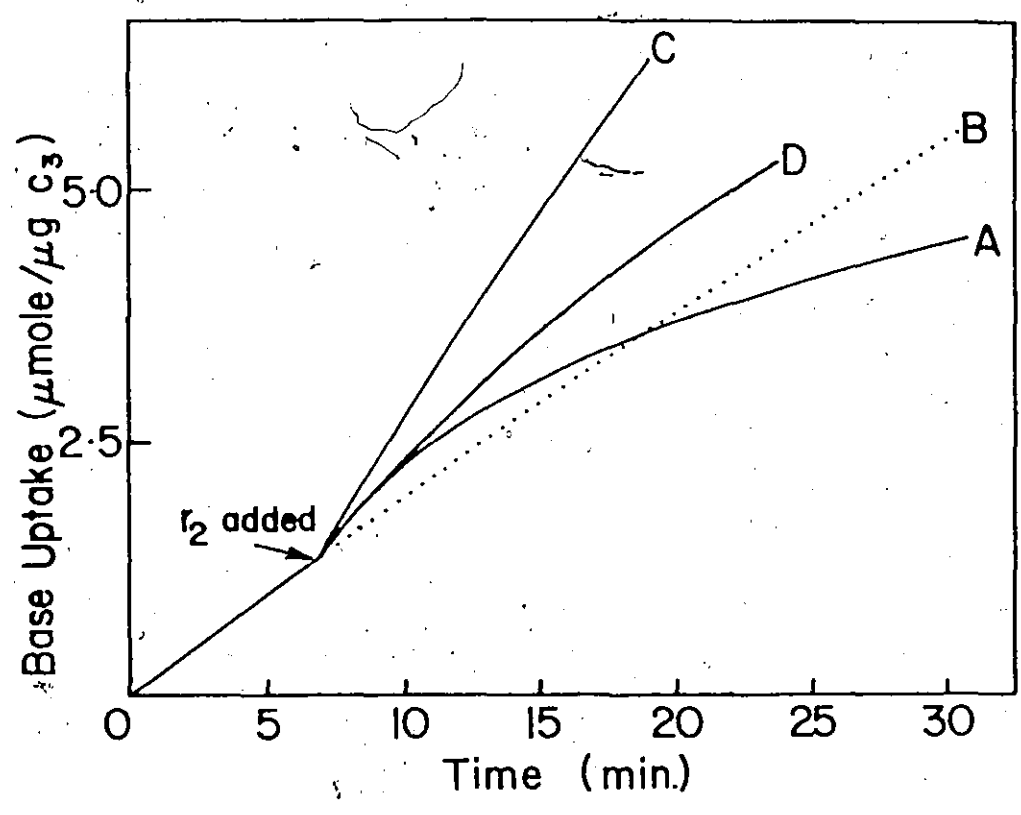
creased the activity at non-saturating r_2 gradually decreased (curve A).
After 30 minutes the rate of reaction had decreased to well below that of
c_3 (line B) the assay of which remained essentially linear over this period.
If at this time the species present are either c_6r_6 or forms with the
activity characteristics of c_3r_6 then it was estimated that after the
thirty minute period illustrated in Fig. 15, curve A, 90% of the c_3 had
been converted to c_6r_6. (From separate experiments it was found that un-
der these assay conditions c_6r_6 had a specific activity equal to one
quarter that of c_3, measured in units/μg c_3 as described in Methods and
Materials, Section 3c). As expected at a saturating concentration of r_2
there was almost no tendency to curve off (line C). This is presumably
due to the complexing of all c_3 subunits into the form c_3r_6 from which it
is difficult to see how reassociation to c_6r_6 woul be possible.

As a further test of the proposal that the gradual lowering of
activity at non-saturating r_2 concentrations is due to reassociation to
c_6r_6, the effect of a much lower c_3 concentration was studied. Formation
of c_6r_6 from c_3 and r_2 would be expected to show a second order de-
pendence on c_3 so lowering the c_3 concentration should greatly slow
the rate of reassociation. When the c_3 level was reduced ten fold
(0.075 μg/ml) a saturating level of r_2 gave an activity profile super-
impossible with that at the higher c_3 concentration (curve C, note that
the Y-axis of Fig. 15 is adjusted to the c_3 concentration used). However,
when the same non-saturating level of r_2 was used (2.5 μg/ml) the
tendency of the activity to curve off was much reduced although still
present (curve D). Thus, as expected, reassociation is possible but
its rate is much reduced.

(e) Effect of CTP on the Reassociated Product

If the reason for the gradual decrease in activity with time upon mixing c_3 with a non-saturating level of r_2 as seen in Fig. 15, curve A, is the gradual formation of c_6r_6 then it would be expected that at later times on curve A the activity should be susceptible to inhibition by CTP. As already described c_3 itself is not significantly inhibited by this nucleotide and the results presented in Fig. 14 show that the c_3r_6 complex is similarly unresponsive.

A mixture of c_3(0.75 μg/ml) and r_2(1.5μg/ml) was allowed to reassociate under similar conditions to those shown in Fig. 15, curve A. After 16 minutes CTP (previously adjusted to pH 8.5) was added to the assay to give a final concentration of 0.4 mM (Fig. 16, lower curve). The experiment was then repeated with the exception that water was added to the reaction after 16 minutes instead of CTP (Fig. 16, upper curve). The assay curves were identical until the additions were made. At this point a significant inhibition of the reaction rate was seen by CTP. Of the catalytic species discussed above only c_6r_6 is known to be responsive to CTP. Additional evidence is therefore provided that after activation of c_3 by nonsaturating r_2 levels there is reassociation of the subunits to form the native structure, c_6r_6.

(f) Effect of CTP on the Reassociation Process

In addition to studying the effect of the allosteric inhibitor CTP on the r_2 saturation profile and consequently on the activity of the c_3r_6 complex it was also of interest to find out whether CTP influences the reassociation process at non-saturating r_2
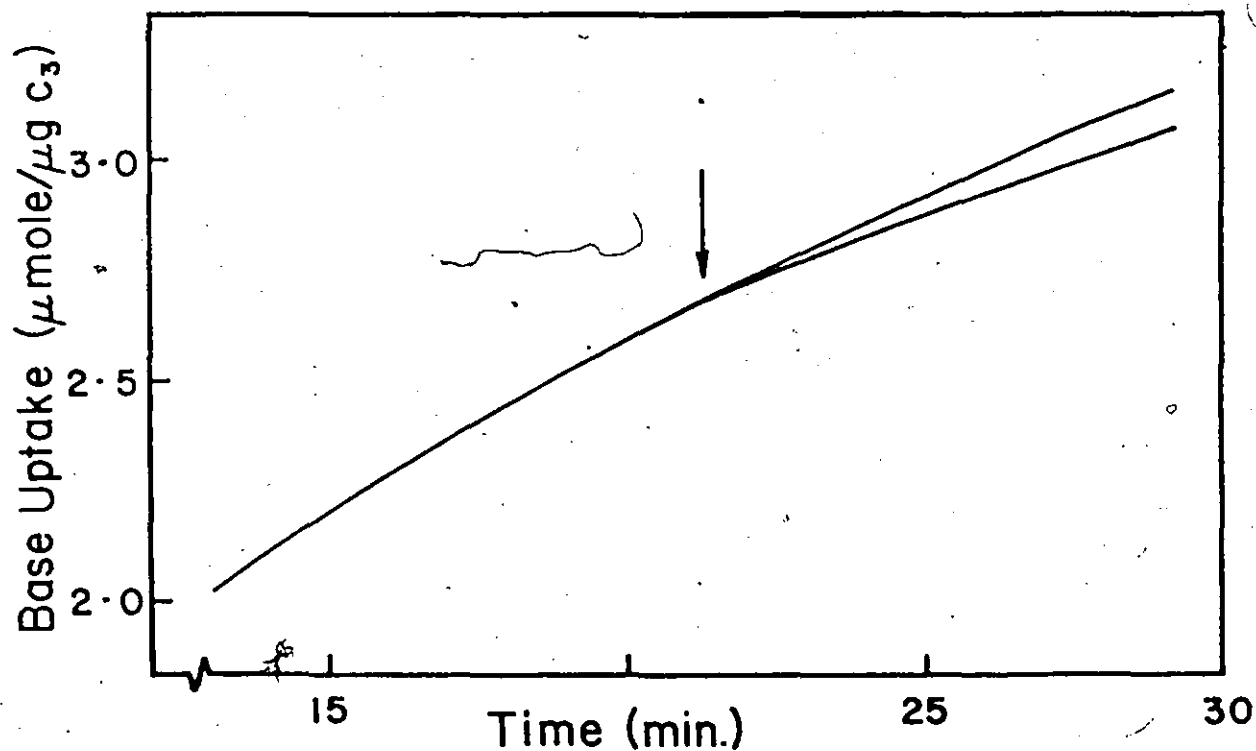
Fig. 16.   Effect of CTP on the Product of Reassociation.   A mixture of
           c₃ (0.75 µg/ml) and r₂ (1.5 µg/ml) was allowed to reassociate
           under the conditions described in fig. 15.   The figure
           represents the superimposition of two experiments of which
           only the final portions are presented.   At the time indicated
           by the arrow CTP (previously adjusted to pH 8.5) was added to
           give a final concentration of 0.3 mM.   This gave rise to the
           lower curve.   In the other experiment at the time indicated
           by the arrow a volume of water similar to that of the CTP
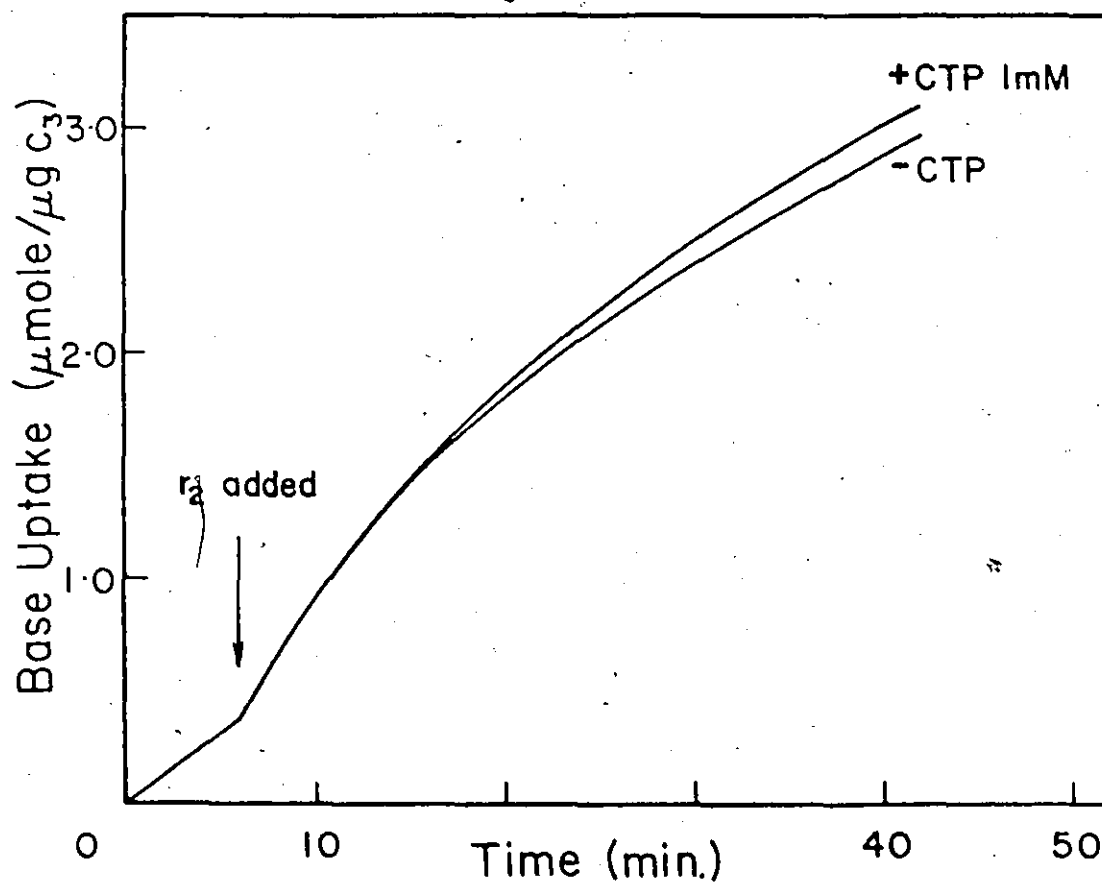   used above was added: This gave the upper curve.

Fig. 17. Effect of CTP on Reassociation. A mixture of c_3 (0.75 µg/ml) and r_2 (3 µg/ml) was allowed to reassociate as described in Fig. 15 except that 2 mM aspartate was used. The figure represents the superimposition of two experiments. As indicated one assay mixture contained CTP (1 mM) the other was a control with no CTP.

levels. The result of the presence of 1 mM CTP in the reassociation
mixture is shown in Fig. 17. After addition of r_2 the catalytic rate
is unaffected by the presence of the nucleotide. Although it was
observed in Fig. 16 that the product of reassociation (c_6r_6) is in-
hibited by CTP, the curve for reassociation with CTP present at the
beginning of the process (Fig. 17, upper curve) shows less of a tendency
to decrease than the control reaction with no CTP (Fig. 17, lower curve).
If CTP had no effect on the reassembly of subunits then a much lower
final activity than the control would be seen. Thus while CTP seems
to have little effect on the formation and the activity of the c_3r_6
complex, this modifier significantly retards the rate of reassociation
of c_3 and r_2 to form c_6r_6.

Because CTP has two effects in the above study (slowing down
the reassociation process and inhibiting the c_6r_6 formed) this finding
is only qualitative. Further and more accurate work (Chan 1975a) in
which samples were withdrawn from the reassociation mixture at various
time intervals and assayed separately has confirmed this finding and
has shown that ATP similarly retards reassociation.

3. Physico-Chemical Characterization of the Complex and the
   Reassociation Products.

The kinetic data already presented indicate that a complex
between c_3 and saturating concentrations of r_2 is stable over an ex-
tended assay period. It was therefore expected that further character-
ization of the complex would be possible by zonal centrifugation in a
sucrose density gradient if the gradient itself contains a saturating

concentration of r_2 to stabilize the complex during centrifugation. As r_2 is a small protein (2.8S) it sediments slowly. A uniform concentration of r_2 throughout the sucrose density gradient would thus ensure that the c_3, whose own sedimentation coefficient (5.8S) was expected to be increased by the formation of the complex, would at all times during the run encounter saturating levels of r_2.

A similar strategy can be used to analyze the products of reassociation of c_3 at nonsaturating concentrations of r_2 in terms of c_3 containing (c_3r_2, c_3r_4 and c_3r_6) and c_6 containing (c_6r_2 (?), c_6r_4 and c_6r_6) species.

(a) <u>Sedimentation Coefficient of the Complex.</u>

The sedimentation pattern obtained when a mixture of 0.037 µg c_3 and 25 µg r_2 is sedimentated in a gradient containing 50 µg/ml of r_2 is shown in Fig. 18a. The only major activity peak is located intermediate between the positions observed for c_3 and c_6r_6 under similar conditions (Fig. 18b). Naturally in order to observe the position of c_3 the gradient shown in Fig. 18b contained no r_2. Throughout these experiments rabbit muscle aldolase was used as an internal marker; control gradients showed that aldolase had no measurable effect on the sedimentation of the species under study. The inclusion of the regulatory subunit in the gradient similarly had no effect on the sedimentation rates of either c_6r_6 or the aldolase marker.

In addition to the peak of activity seen in Fig. 18a, small amounts of activity were seen at the bottom of the gradient. A control experiment (see Section 3d) indicated that this activity was due to small contaminating amounts of enzyme present in the r_2 preparation.

Fig. 18. <u>Sedimentation of Various Forms of ATCase in Sucrose Density</u>
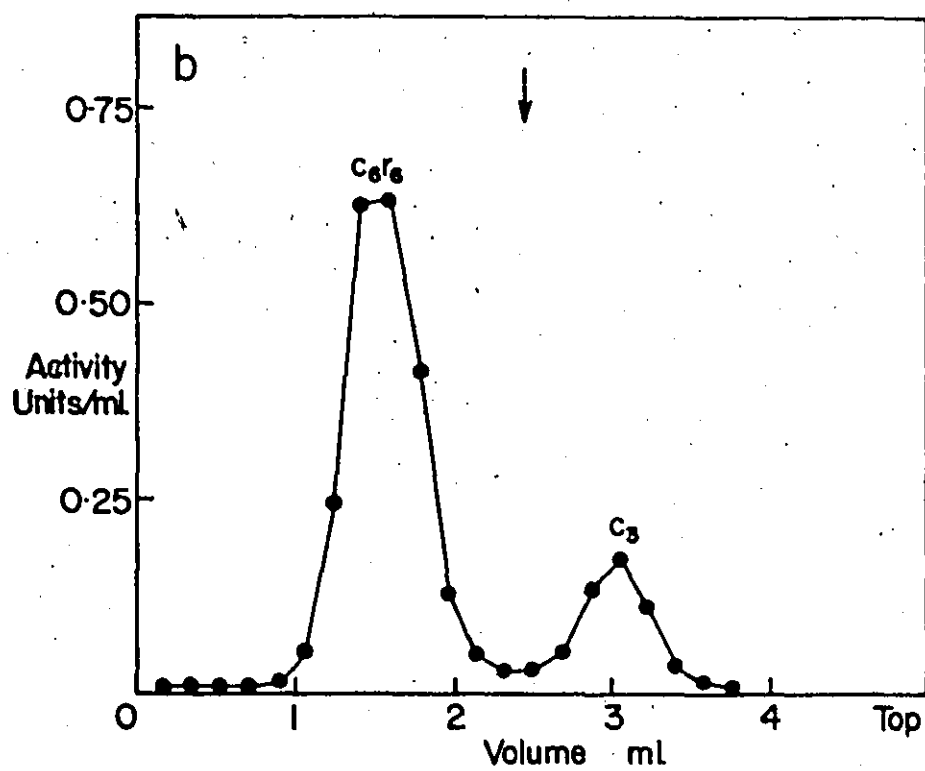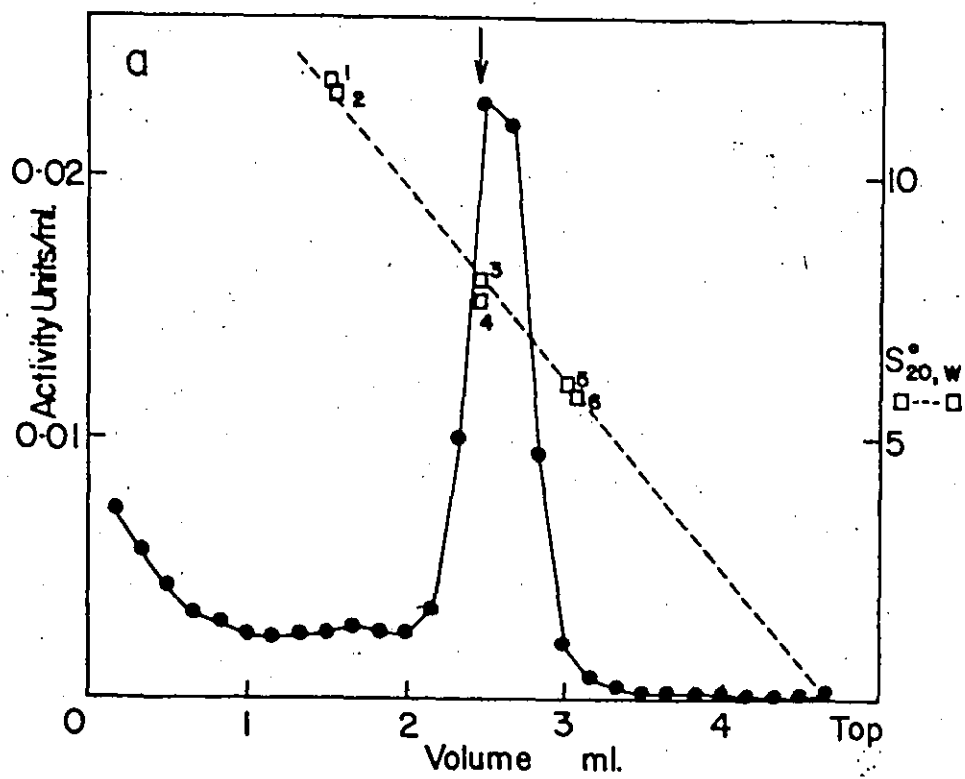<u>Gradients</u>.

(a) A sample of the c_3r_6 complex produced by mixing
0.037 µg c_3 and 25 µg r_2 in 50 µl of buffer, pH 8.5 and
containing 22 µg aldolase (as an internal marker) was
centrifuged in a gradient containing 50 µg/ml r_2. Circles:
ATCase activity assayed in 6 mM aspartate. Squares: the
position of marker proteins determined in separtate
experiments.

    (1)   Native ATCase 11.7 S (Gerhart & Schachman, 1965)
    (2)   Bovine liver catalase 11.3 S (Tanford & Lovrien, 1962)
    (3)   Rabbit muscle aldolase 8.0 S (Kawahara & Tanford, 1966)
    (4)   Yeast alcohol dehydrogenase 7.6 S (Kuff <u>et al</u>., 1955)
    (5)   E. <u>coli</u> alkaline phosphatase 6.0 S (Applebury &
          Coleman, 1969)
    (6)   ATCase catalytic subunit 5.8 S (Gerhart & Schachman,
          1965)

The arrow indicates the position of the internal marker,
aldolase.

(b) Native ATCase (2 µg) and c_3 (0.15 µg) centrifuged under
similar conditions except that no r_2 was present in the
gradient and ATCase assays were conducted at 25 mM as-
partate. As above the arrow indicates the position of the
internal marker aldolase.

(From Mort & Chan, 1975)

To estimate the sedimentation constant of the complex, calibration runs were carried out with enzymes of known $S_{20,W}^{o}$. As expected a linear relationship between S value and the position of the marker in the gradient was found. From this line (Fig. 18a) a sedimentation coefficient of 7.7 S is obtained for the complex. This value is somewhat less than that expected for a complex with a molecular weight of 200,000, however this may be due to the nonspherical shape, and unusual hydration probably associated with the c_3r_6 complex. (see Discussion and Mort & Chan, 1975).

A demonstration that the complex contained the maximal number of regulatory subunits was provided by centrifuging a sample of the complex through a gradient containing 100 µg/ml r_2 rather than 50 µg/ml r_2 as used in Fig. 18a. No change in the position of the activity peak was observed.

(b) Reversibility of the Formation of the Complex

The results presented so far are in agreement with the projections of the kinetic studies. Although the dependence on saturating r_2 levels for the stability of c_3r_6 argues for the reversibility of the formation of the complex this has not yet been tested directly. Sedimentation of a sample of c_3r_6 in a sucrose density gradient containing no r_2 allows direct verification of this proposal. As the sample of c_3r_6 with excess r_2 is centrifuged through a gradient containing no r_2 the free regulatory subunit gradually separates from the c_3r_6 complex due to the large differences in molecular size between these two species. Non-saturating levels of r_2 will then form the environment of the complexed catalytic subunit. If the formation of

c_3r_6 is reversible then these non-saturating r_2 levels will allow re-association to take place.

The results of such an experiment are shown in Fig. 19. The c_3r_6 complex which sedimented as a single peak in an r_2 containing gradient was found to yield two species in a gradient containing no r_2. In order to characterize these species the fractions were assayed at two aspartate concentrations (Fig. 19a). The ratio of activities at 6 mM and 25 mM aspartate are characteristic for c_3, c_3r_6 and native ATCase (Table II). This ratio is very low for c_6r_6 as at 6 mM aspartate the native enzyme is still in the sigmoid range while at 25 mM aspartate maximal activity results (see Fig. 3). As the catalytic subunit shows a simple hyperbolic aspartate saturation profile the 6 mM : 25 mM ratio is much higher for this species. Control experiments showed that the ratio for c_3r_6 was close to unity. The reason for this is explained in Section 4a. By these criteria the faster sedimenting peak (Peak I, Fig. 19) shows activity properties of c_6r_6 and the slower (Peak II) those of c_3. A range of activity ratios is seen throughout the peaks due to slight overlap. To strengthen these assignments the activities of the two peaks were tested in the presence of CTP. Only the activity of the faster sedimenting species was significantly inhibited.

In comparison to the positions expected for c_3 and c_6r_6, the two activity peaks found on sedimentation of c_3r_6 in an r_2-free gradient are both displaced towards the centre of the gradient. This is to be expected if the c_3r_6 [which was observed (Fig. 18a) to sediment to the middle of the gradient in the presence of saturating r_2] sediments down the gradient initially intact. Later in the run the saturating

Fig. 19. <u>Breakdown of the c_3r_6 Complex upon Separation from Excess r_2</u>.

(a) A sample of c_3r_6 was prepared identically to that described in Fig. 18a and was centrifuged as before except that the gradient contained no r_2. ATCase activity was measured in 6 mM aspartate (open circles) and 25 mM aspartate (closed circles).

(b) A similar experiment to above with more c_3 (0.054 µg) and r_2 (37 µg) applied in the sample. ATCase activity was assayed in 20 mM aspartate (closed squares) and 20 mM aspartate plus 1 mM CTP (open squares).

As before the solid arrow indicates the internal marker aldolase. The dotted arrows represent the expected positions of c_3 and c_6r_6.
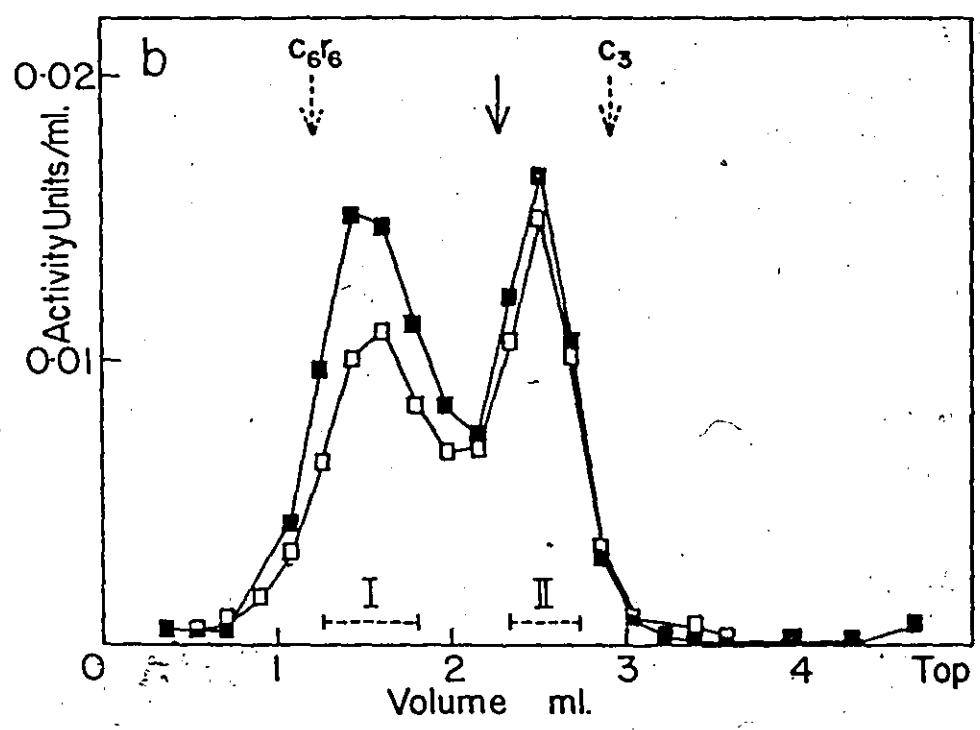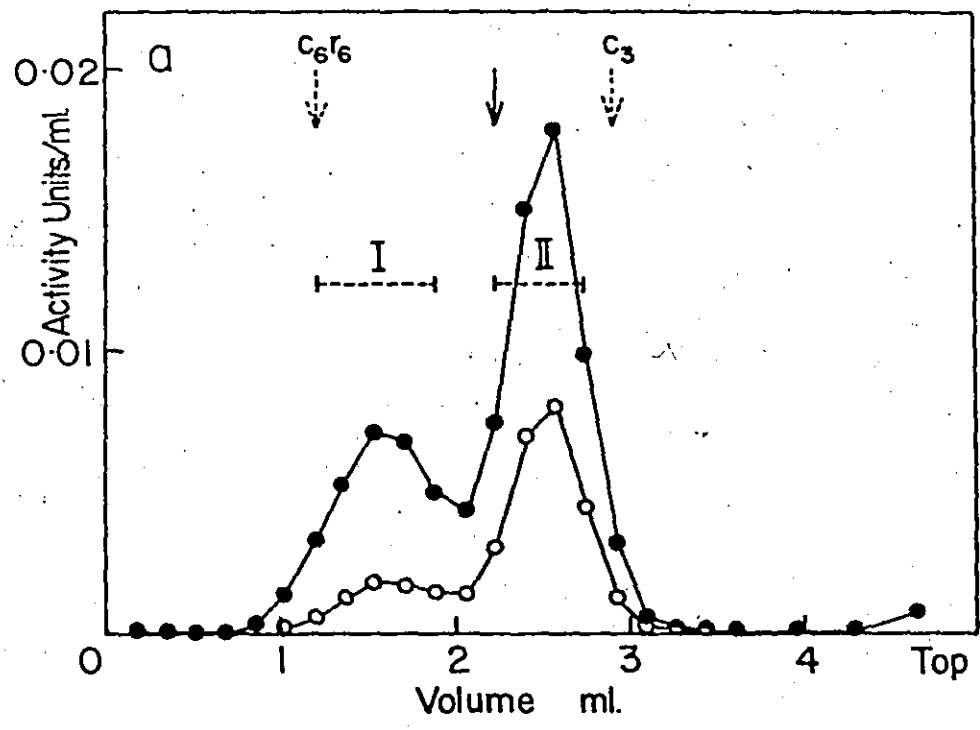
(From Mort & Chan, 1975).

Table II

Properties of the Breakdown Products of c_3r_6

Activity Ratio

Sample	$\dfrac{6\ mM\ Asp}{25\ mM\ Asp}$	$\dfrac{1\ mM\ CTP}{no\ CTP}$
c_3[a]	0.55	1.07
c_3r_6[a]	1.33	.98
c_6r_6[a]	0.18	.67
Peak I[b]	0.18 - 0.30	.67 - .75
Peak II[b]	0.42 - 0.47	.87 - .94

[a] Dilutions of these enzyme forms were made into the same medium as used in the sucrose gradients, containing concentrations of sucrose equal to those present in the region of the gradient at the final position of each particular species.

[b] Peaks I and II refer to Fig. 19.

69

Fig. 20. <u>Conversion of c_3 to c_6r_6 in the Presence of Nonsaturating Concentrations of r_2.</u>
A mixture of c_3 (0.8 μg/ml) and r_2 (2.5 μg/ml) was allowed to reassociate at 25° in 0.1 M potassium acetate, 20 mM 2-mercaptoethanol and 50 μg/ml BSA at pH 8.5. After the times indicated below, samples were withdrawn, r_2 and aldolase (internal marker) were added to give final con-concentrations of 500 μg/ml and 420 μg/ml respectively, and 75 μl aliquots (containing 0.04 μg catalytic chains) were applied to sucrose gradients containing 50 μg/ml r_2.

(a)   0 min.
(b)   4 min.
(c) and (d) 30 min.

Fractions were assayed (a, b and c) in 6 mM aspartate (open circles) and 25 mM aspartate (closed circles) or (d) in 20 mM aspartate (closed squares) and 20 mM aspartate and 1 mM CTP (open squares).

(From Mort & Chan, 1975).
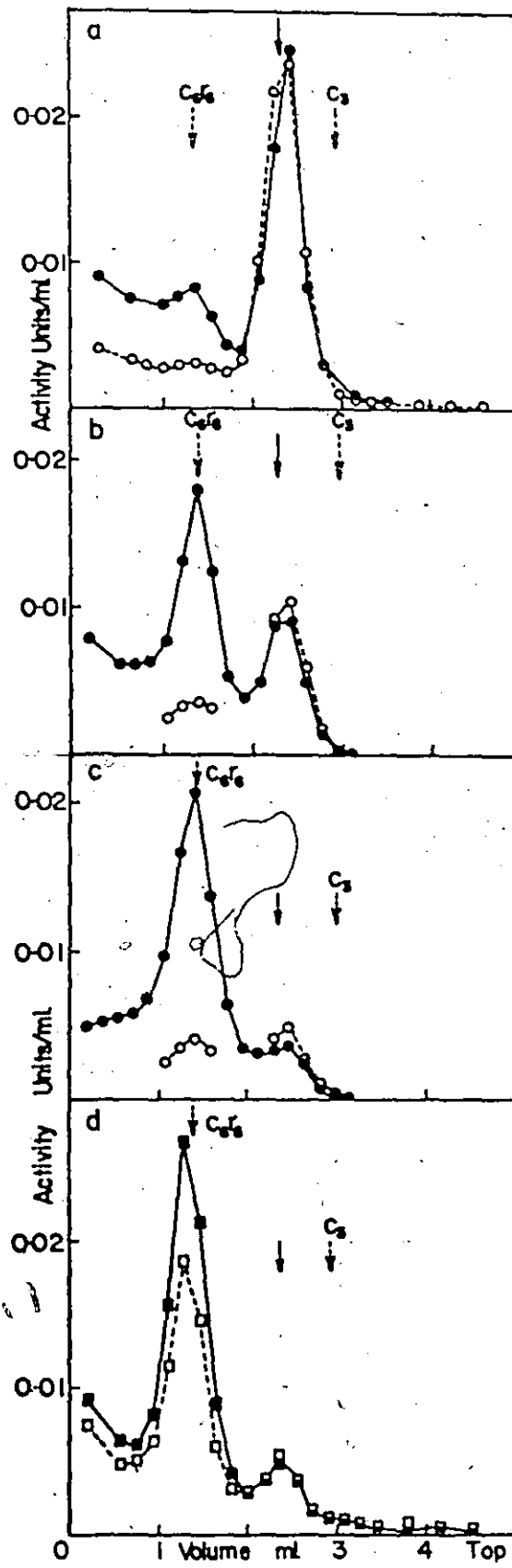
c_3 (0.8 µg/ml) an aliquot removed and treated as described above
gave only one significant peak sedimenting at the position previously
observed for c_3r_6 (Fig. 20a). The ratio of activities at 6 and 25 mM
aspartate for this peak is also characteristic for c_3r_6 (Table II).
As before (Fig. 18a), contaminating activity is seen in the lower
fractions because the gradient contained a large amount of r_2 (see
section 3d).

After 4 minutes of reassociation (Fig. 20b) a markedly lower
peak of activity had sedimented to the c_3r_6 region, while an increased
amount of activity was found at the c_6r_6 position. Again the expected
activity ratios are seen. After 30 minutes of reassociation (Fig. 20c)
essentially all the activity appeared at the c_6r_6 position. This
activity was inhibited by CTP (Fig. 20d).

These results combined with the previous kinetic evidence
(Fig. 15) justify the conclusion that non-saturating concentrations of
r_2 will allow the irreversible reassociation of catalytic and regulatory
subunits to form a species showing the physico-chemical and kinetic
properties of native ATCase.

(d) <u>Characterization of Contaminating Activity in the r_2
Preparation</u>

While r_2 itself is known to have no enzymic activity
(Gerhart & Schachman, 1965), at the extremely high concentrations used
in the above experiments ATCase activity was found to be associated with
the regulatory subunit preparation. To indicate the areas of the pre-
viously discussed gradients (Figs. 18 and 20) that gave ATCase activity
arising from contamination of the r_2 preparation, the gradient shown in

Fig. 18a was repeated except that no c_3 was added. The excess r_2 used to make the c_3r_6 complex (35 µg present initially at a concentration of 500 µg/ml) was however added and as before, r_2 (50 µg/ml) was present, throughout the gradient. The resulting profile (Fig. 21a) shows that when assayed in 6 mM aspartate (circles) there is significant activity at the bottom of the gradient, a very small peak at the position expected for c_6r_6 and low activity extending as far as the position at which c_3r_6 sedimented. Assay in 25 mM aspartate (squares) showed much higher activity for the material at the bottom of the gradient and at the c_6r_6 position, but no significant increase at the c_3r_6 position.

Thus while most of the activity due to the r_2 present in the gradient sedimented to the bottom, the position of the actual peaks in Fig. 21a show that most of the contamination is present in the form of c_6r_6.

The small peak cosedimenting with aldolase however indicates that some of the r_2 itself may contain a small amount of c_3r_6. The activity profile of a sample of r_2 sedimented in a gradient not itself containing r_2 is shown in Fig. 21b. A profile similar to that observed in Fig. 19 resulted but the peak due to c_6r_6 in much greater. Significant activity did however, sediment above the aldolase marker, again indicating that the r_2 preparation was contaminated by c_3 as well as c_6-containing species.

Fig. 21. <u>Control Gradients showing Positions Reached by Impurities</u>
<u>in the r_2 Preparation</u>.

(a) A sample of r_2 (35 µg) and aldolase (33 µg) was
centrifuged in a gradient containing 50 µg/ml r_2.
ATCase activity was assayed in 6 mM aspartate (squares)
and 25 mM aspartate (circles). The arrow represents
the position of the internal marker aldolase.

(b) A sample of r_2 (35 µg) and aldolase (33 µg) was
centrifuged in a gradient containing no r_2. ATCase
activity was assayed in 6 mM aspartate (squares) and
25 mM aspartate (circles). The arrow represents the
position of the internal marker aldolase.
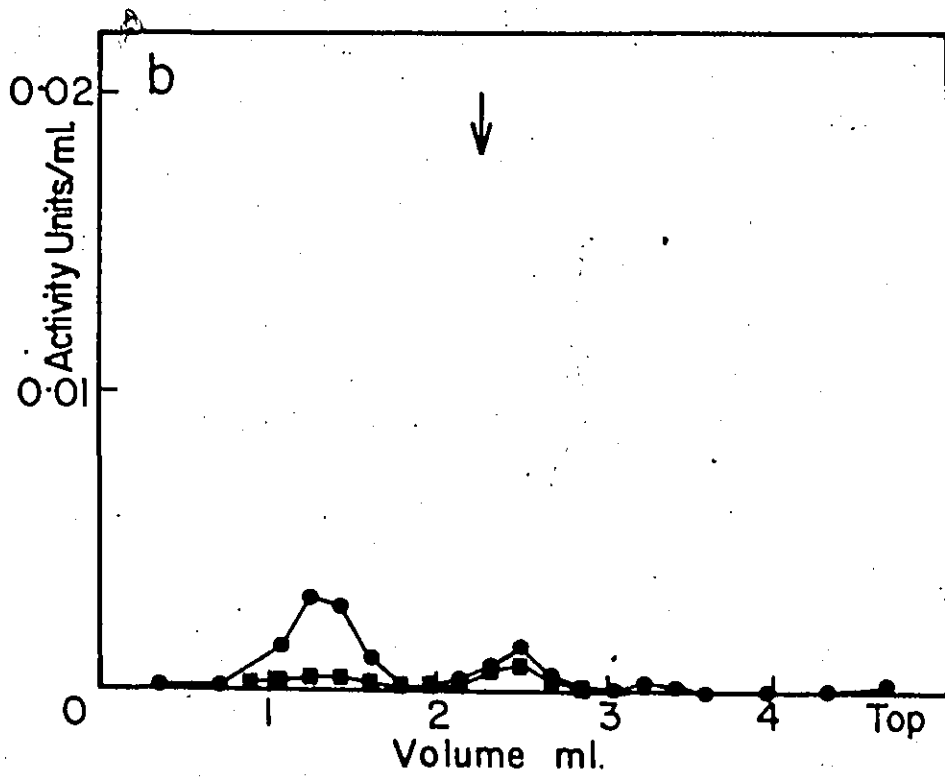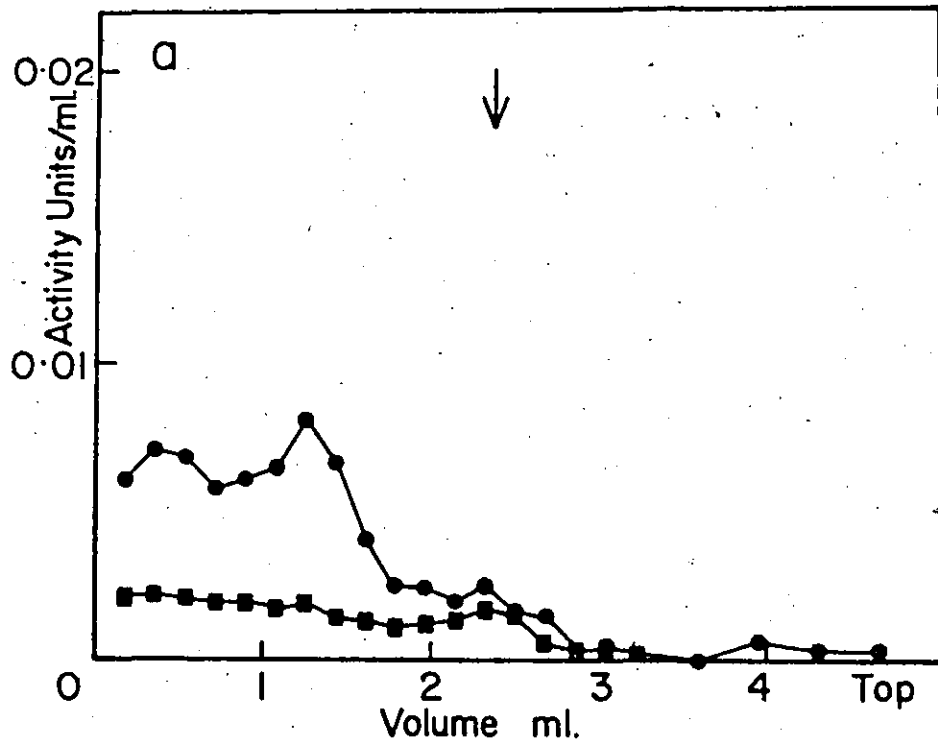
4. Properties of the c_3r_6 Complex

The data presented in the previous sections indicate that the c_3r_6 complex exists as a physically and kinetically distinct species at saturating levels of r_2. A further characterization of the kinetics and possible allosteric properties of c_3r_6 is now presented.

(a) Kinetic Properties of the Complex

From the saturation studies (section 2a) it was concluded that the c_3r_6 complex is stable over a range of aspartate concentrations. A complete initial velocity profile obtained by the continuous titration assay at varied aspartate concentrations in the presence of 40 μg/ml r_2 (a level shown to be saturating) is presented in Fig. 22. The curve for the complex shows no sigmoid character in contrast to the extreme sigmoidicity observed at this pH with the native enzyme. The activity profile for the complex rises rapidly to a maximum at about 20 mM aspartate. At higher concentrations of aspartate lower levels of activity are seen, a property indicative of substrate inhibition by aspartate. Such inhibition has been described in the case of the catalytic subunit by Porter et al. (1969) who attribute it to the formation of a dead-end complex composed of enzyme-aspartate-phosphate. A similar drop off in activity is also seen in the profile of the native enzyme.

The data for the catalytic subunit and the c_3r_6 complex were fitted to the equation for substrate inhibition

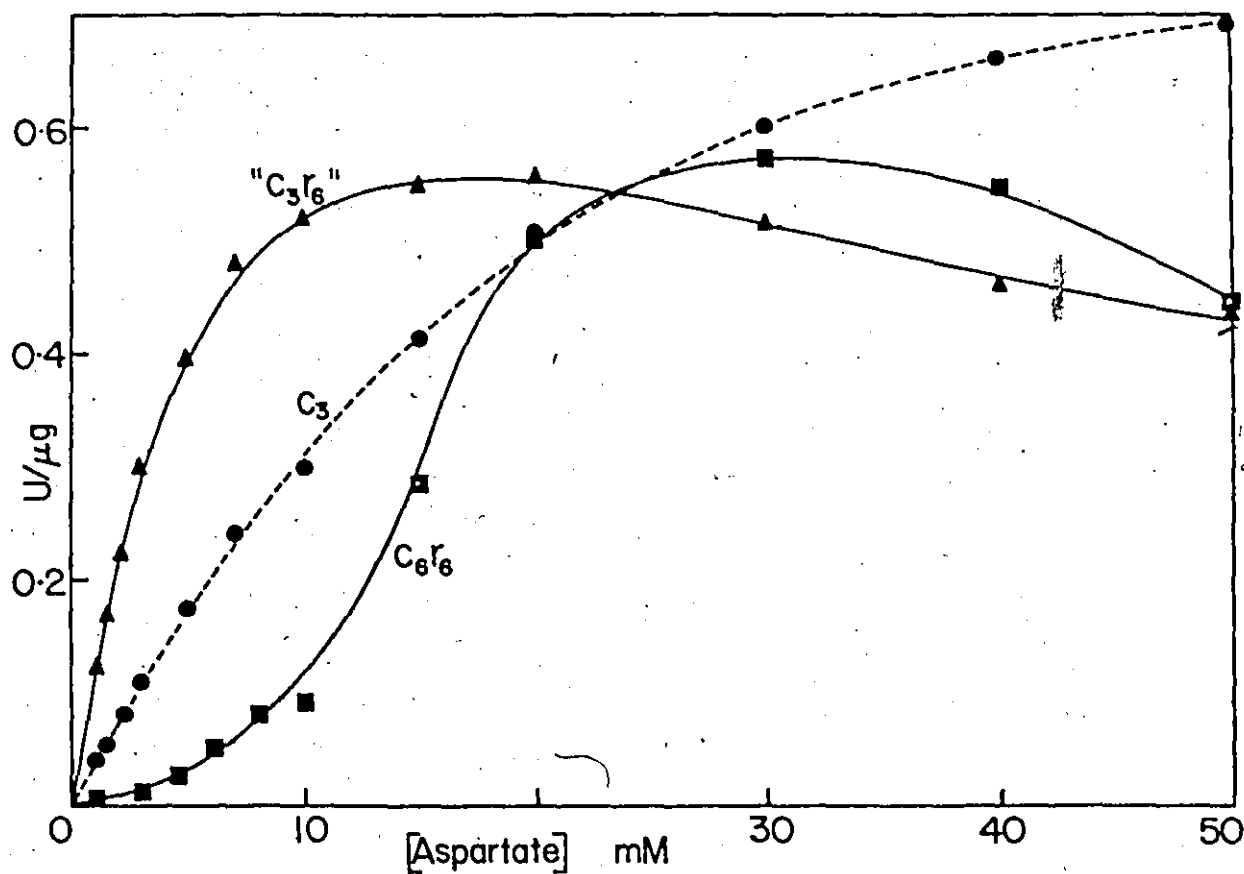$$v = \frac{V_{max}\ S}{K_m + S + \dfrac{S^2}{K_i}}$$

Fig. 22. Initial Velocity Profiles of Various Forms of ATCase
at Various Aspartate Concentrations.
Activity was measured by titration assay at pH 8.5
in the presence of 10 mM carbamyl phosphate. The c_3r_6
complex was assayed by addition of r_2 (40 µg/ml) to
c_3 (0.75 µg/ml). Circles represent c_3, triangles c_3r_6
and squares c_6r_6.

The lines for c_3 and c_3r_6 were calculated from the
equation $v = Vmax \ S/(K_m + S + S^2/K_i)$ using the constants
given in Table III.

(From Mort & Chan, 1975).

by the non linear least squares procedure of Cleland (1967) (See appendix). The results of this fit are shown in Table III.

The K_m for aspartate observed for the c_3r_6 complex is almost six times lower than that for c_3 itself. The Vmax is also somewhat smaller as is the K_i for aspartate. When the experiment was repeated using the radioactive sampling assay (Fig. 23) similar results were obtained. Although substrate inhibition was again observed for c_3r_6 only activities at low aspartate concentrations were analyzed in Fig. 23 where this inhibition is neglegible. The resulting double reciprocal plot gives a linear relationship. The extremely low K_m observed for the c_3r_6 complex has lead to the suggestion (Mort & Chan, 1975) that this complex represents the relaxed, or high affinity form of ATCase as proposed in the two state allosteric model of Monod et al. (1965). Evaluation of the K_m of the relaxed state of c_6r_6 would be expected to be possible by extrapolation of a double reciprocal plot to the X-axis at close to saturating levels of substrate. Fig. 24 shows that even when the native enzyme is maximally activated by ATP this procedure is unfeasible as upward curving of the double reciprocal plot due to substrate inhibition at high aspartate concentrations totally obscures any linearity in this region.

At pH 7 however, substrate inhibition is much less pronounced and has lead Collins (1971) to attempt the above extrapolation: a value of 5.75 mM was calculated for the native enzyme which was slightly reduced (5.15 mM) in the presence of ATP.

In their study of the pH dependence of the K_m for aspartate of the catalytic subunit Porter et al. (1969) found essentially no change

Table III

Kinetic Constants of c_3 and c_3r_6.

	K_m (mM)	V_{max} U/μg c_3	K_i (mM)
c_3 titrator [a]	44.7 ± 5.8	1.73 ± 0.18	80.0 ± 20.5
c_3 radioactive [b]	24.0 ± 2.0	1.50 ± 0.1	
c_3r_6 titrator	7.90 ± 0.65	1.08 ± 0.05	36.2 ± 3.7
c_3r_6 radioactive	6.0 ± 0.4	0.84 ± 0.04	

[a] Titrator data (Fig. 22) fitted to the equation

$$v = V_{max} \times S / (K_m + S + S^2/K_i)$$ by the procedure described by Cleland (1967).

[b] Radioactive data (Fig. 23) fitted to the hyperbola $v = V_{max} \, S / (K_m + S)$.
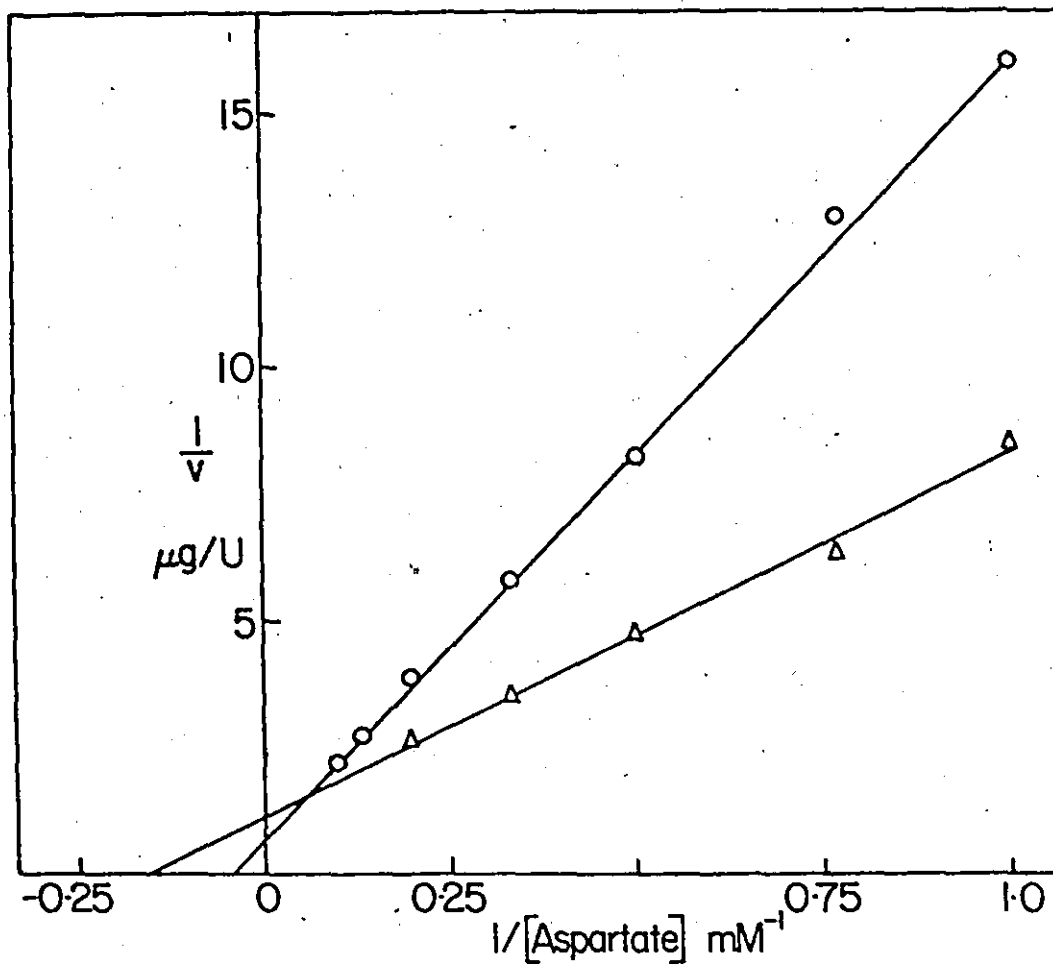
Fig. 23. Double Reciprocal Plot of Aspartate Saturation Curves of c_3 and c_3r_6 Obtained Using the Radioactive Assay.

c_3 (0.08 µg/ml, circles) and c_3r_6 (formed by addition of 50 µg/ml r_2 to 0.08 µg/ml c_3, triangles) were assayed at varied aspartate concentrations and 4 mM carbamyl phosphate at pH 8.5.
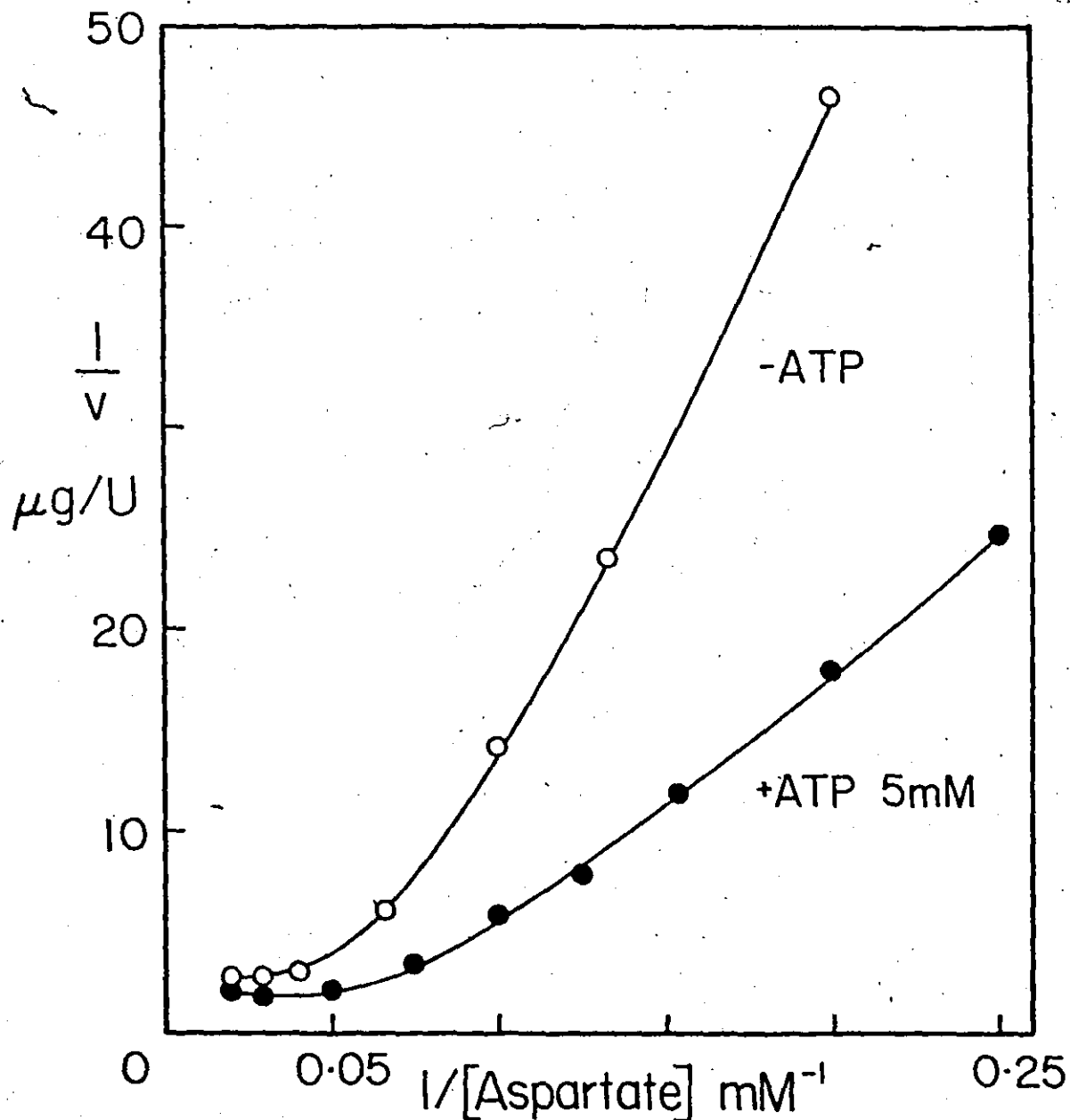
(From Mort & Chan, 1975).

Fig. 24. Double Reciprocal Plot of c_6r_6 with and without ATP at pH 8.5

ATCase was assayed at pH 8.5 by the radioactive procedure with varied aspartate and 4 mM carbamyl phosphate (open circles). As indicated, filled circles denote assays under similar conditions except with 5 mM ATP. Points are averages of two determinations.

between pH 7.0 and 8.7. It seems reasonable therefore, to consider the similarity of the K_m of ATCase at pH 7 with the K_m at pH 8.5 of the c_3r_6 complex as an indication that it represents the relaxed state of c_6r_6.

(b) Effect of CTP and ATP on c_6r_6.

The presence of the regulatory chains in the complex might be predicted to render c_3r_6 susceptible to the action of the allosteric effectors CTP and ATP. Results already presented indicate that, contrary to this expectation, CTP has no significant effect on c_3r_6. The effect of ATP and CTP at various concentrations of aspartate on c_6r_6, c_3 and c_3r_6 are compared in Table IV. While the inhibition of the native enzyme by 1 mM CTP ranged from 44 to 66% depending on aspartate concentration the maximum inhibition observed for c_3r_6 was 2%, slightly less than the maximum of 5% seen for c_3. ATP activated c_6r_6 from 50 to 186% but caused no increase in activity with either c_3r_6 or c_3. Instead slight inhibition was observed for c_3 and c_3r_6.

Kleppe (1965, 1966) has demonstrated that phosphate and particularly pyrophosphate are effective competitive inhibitors with carbamyl phosphate. Similarly Porter et al. (1969) found that many phosphate containing compounds including CTP are competitive inhibitors. ATP also contains a polyphosphate and at the higher concentrations used (5 mM), compared to CTP (1 mM) should be a more effective inhibitor of c_3. Similar inhibition is seen in the case of c_3r_6 indicating that the presence of the regulatory chains had no heterotropic effects.

The slight inhibition by CTP and ATP was consistently lower for c_3r_6 than for c_3. If in addition to a lower K_m for aspartate when compared to c_3, the K_m for carbamyl phosphate for c_3r_6 were also lower, less

inhibition by phosphate containing compounds would be expected.

(c) pH Activity Profiles

Further characterization of the c_3r_6 complex was pursued by determination of the pH-activity profile. At low aspartate concentrations (2 mM) a simple single optimum is obtained with a maximum value at pH 8.5. (Fig. 25a). This is similar to the profile of c_3 (Fig. 25b) except for the generally higher specific activity (Fig. 25b). In contrast c_6r_6 only shows a peak of activity at high pH where Weitzman and Wilson (1966) have found that sigmoid kinetics no longer operate.

At high aspartate concentration (50 mM) c_3 again shows a typical bell-shaped curve with highest activity at pH 8.5 (Fig. 26b). The c_3r_6 complex (Fig. 26a) however, yields a profile very similar in both shape and specific activity to that observed for the native enzyme involving two activity peaks, one around pH 8 the other at pH 10. Both Weitzman and Wilson (1966) and Jacobson and Stark (1973b) have reported the occurrence of a second activity peak at high pH for native ATCase.

The marked similarity between the profiles of c_3r_6 and c_6r_6 at high aspartate concentrations and particularly the existence of the second peak of activity in the pH 10 region might be explained by the spontaneous reassociation of c_3 and r_2 to form c_6r_6 under these conditions. This was shown not to be the case.

The activity of c_3r_6 in 50 mM aspartate at pH 10 was found to be linear with time, therefore, if reassociation were taking place it must occur very quickly. When a sample of c_3r_6 under the above conditions (Fig. 26) was removed after 30 seconds and diluted into a second assay mixture in order to reduce the pH to 8 and the aspartate concentration

Fig. 25.   pH Activity Profiles of the Three Forms of ATCase in 2 mM
Aspartate

   The radioactivity assay was used with saturating con-
centrations of carbamyl phosphate. All activities are ex-
pressed in Units/µg c_3 to facilitate comparison. Filled
symbols indicate assays in Tris acetate.

(a) c_3r_6 complex assayed by addition of 50 µg/ml r_2 to
      0.046 µg/ml c_3

(b) c_3 (circles) 0.075 µg/ml
      c_6r_6 (squares) 0.37 µg/ml (Inset shows this data with the
                                    Y-axis expanded).

Fig. 26.   pH Activity Profiles of the Three Forms of ATCase in 50 mM
Aspartate

   The radioactive assay was used with saturating concentrations
of carbamyl phosphate. All activities are expressed in Units/
µg c_3 to facilitate comparison.
Filled symbols indicate assays in Tris acetate.

(a) c_3r_6 complex assayed by addition of 50 µg/ml r_2 to
      0.45 µg/ml c_3

(b) c_3 (circles) 0.1 µg/ml
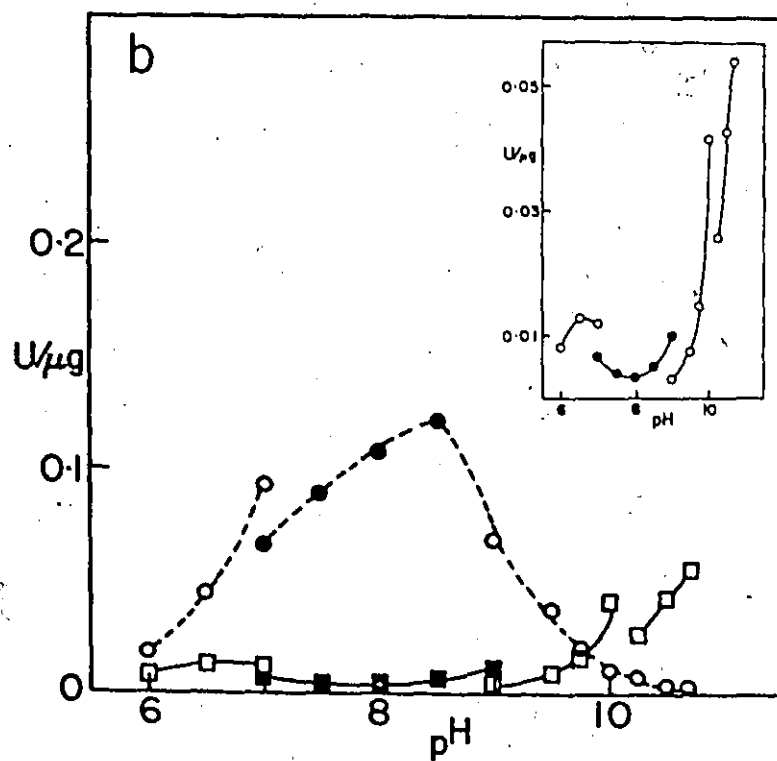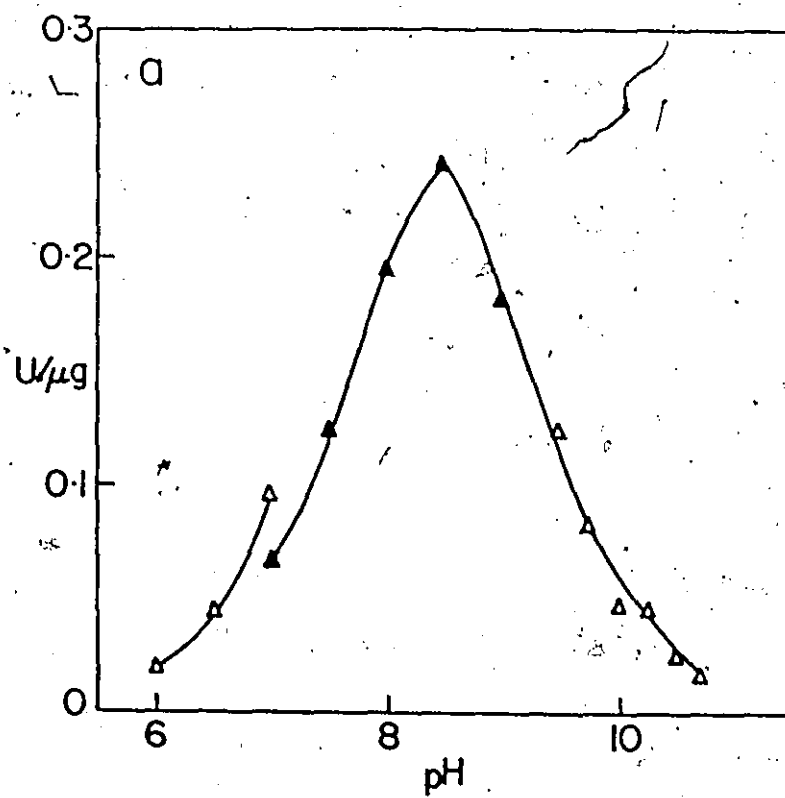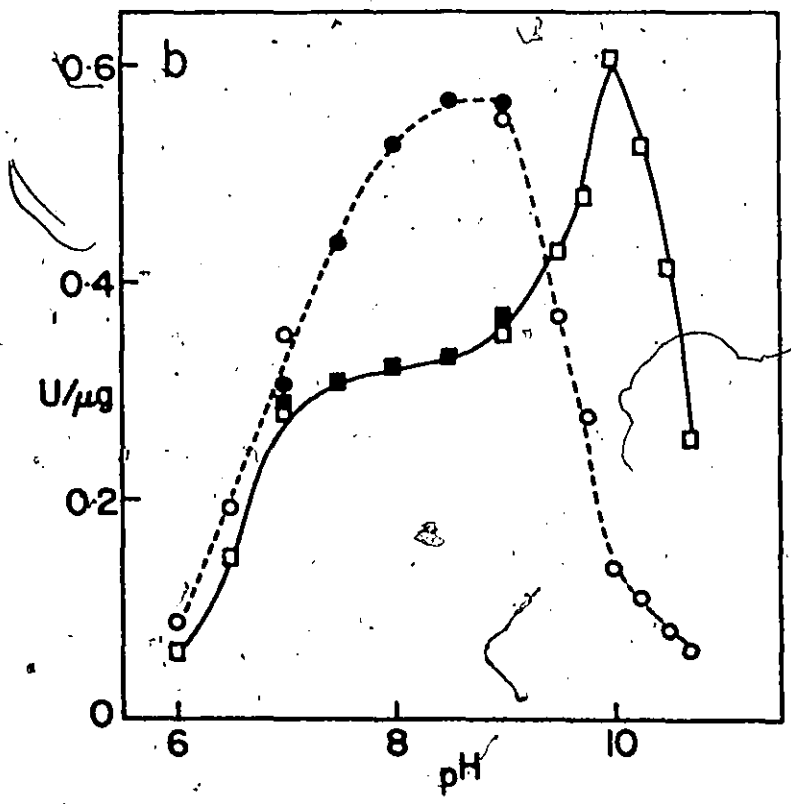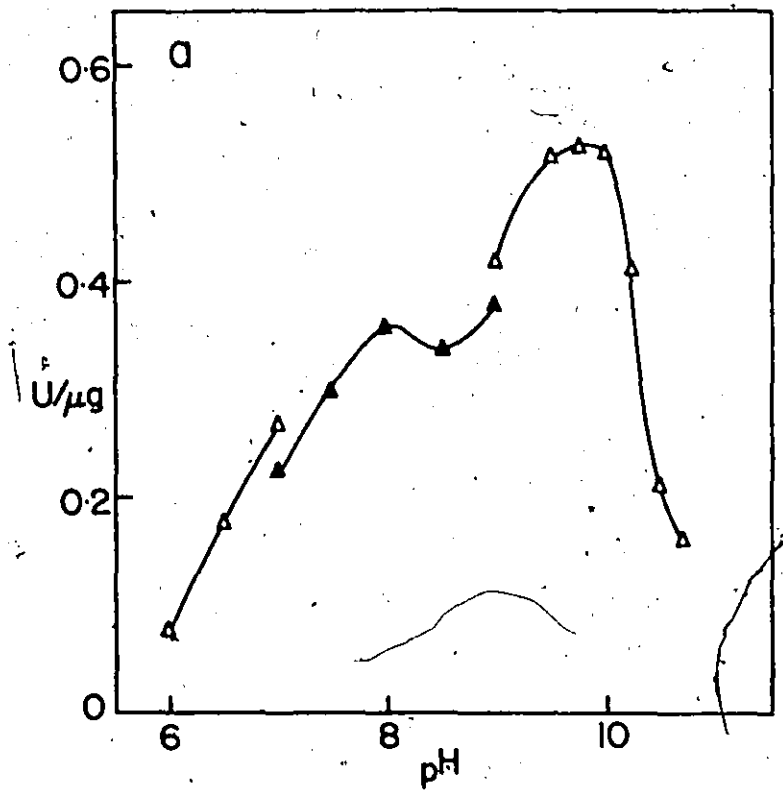 c_6r_6 (squares) 0.25 µg/ml

Fig. 25

Fig. 26

to 6 mM, a specific activity of 0.348 units/µg c_3 was found. When the experiment was repeated with a sample of c_6r_6, however, the change to 6 mM aspartate and pH 8.0 caused the specific activity to decrease to 0.016 units/µg c_3. The specific activity of ATCase at pH 8 and 6 mM aspartate was found to be 0.018 units/µg c_3.

It was therefore concluded that under conditions where c_3r_6 has similar properties to the native enzyme rapid reassembly to the native structure is not taking place.

The similarity of the profiles of c_3r_6 and c_6r_6 in the presence of 50 mM aspartate again argues persuasively that the complex represents the relaxed form of the native enzyme as at this close to saturating substrate concentration c_6r_6 should be predominantly in this conformation. At 2 mM aspartate however, a different conformation would predominate, therefore the pH profiles are different.

5. Investigation of the Kinetic Mechanism of Dead-end Inhibition by Succinate and Maleate

In order to investigate the similarity between the relaxed state of c_6r_6 and the c_3r_6 complex further, native ATCase was studied by an approach which was expected to stabilize the relaxed state and allow characterization of some of its properties.

It is well known that while the aspartate analogues succinate and maleate are simple competitive inhibitors of c_3 (Gerhart & Pardee, 1964), at low concentrations of aspartate these compounds activate c_6r_6. This increase in activity was explained as the result of the substrate analogue mimicking aspartate in exerting a homotrophic effect so that the aspartate

88

actually bound finds the enzyme in the relaxed state. It was therefore

thought that it would be possible to use this approach to determine the

K_m for aspartate of the relaxed state of c_6r_6 at pH 8.5.

While for c_3 and c_3r_6 simple kinetic patterns were found, c_6r_6

gave quite different results. Although these findings are preliminary

they indicate that succinate and maleate cannot be considered as simple

substrate analogues when used in the place of aspartate with c_6r_6. An

extended study following the initial experiments outlined below may yield

interesting information concerning the allosteric mechanism of ATCase.

(a) <u>Effect of Aspartate Analogues on c_6r_6, c_3 and c_3r_6.</u>

At low aspartate concentrations the aspartate analogue

succinate activates c_6r_6 (Fig. 27). As the succinate concentration is

increased a peak of activity is reached beyond which there is a gradual

decline. This decrease in activity at higher succinate levels is to

be expected for if the enzyme is now fully in the relaxed state additional

succinate will simply act as a <u>competitive inhibitor</u>. As expected the

maximal activation is lesser at higher aspartate levels (Fig. 27).

In contrast to the complex pattern seen with c_6r_6 increasing

concentrations of succinate cause a gradual decrease in activity with

c_3 and c_3r_6 (Fig. 28). In both cases double reciprocal plots of activity

at varying aspartate concentrations in the presence and absence of succinate

give patterns characteristic of simple competitive inhibition (Fig. 29),

the pattern well established for c_3 (Gerhart & Pardee, 1964; Porter <u>et al.</u>,

1969; Heyde <u>et al.</u>, 1973). As was observed with the K_m's for aspartate,

the succinate dissociation constant for c_3r_6 was found to be much lower

than for c_3 (Table V) in agreement with the "truncated" form of ATCase.
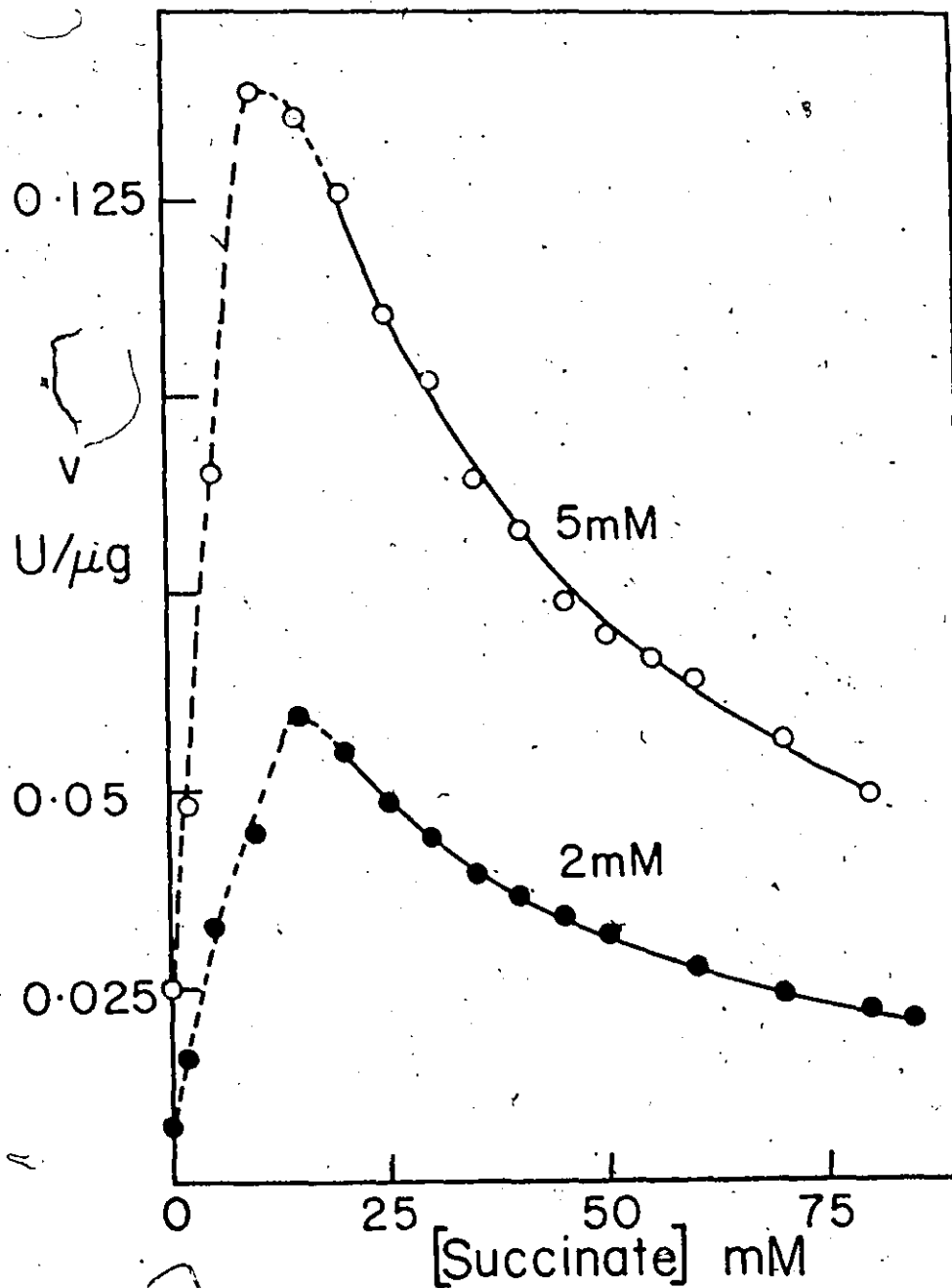
Fig. 27. Effect of Succinate on c_6r_6 Activity at Constant Aspartate Concentrations.

ATCase activity at 2 and 5 mM aspartate and varied succinate concentrations was measured by titration assay at pH 8.5 in the presence of saturating carbamyl phosphate (5 mM). The solid lines were drawn from a least square fit to the equation

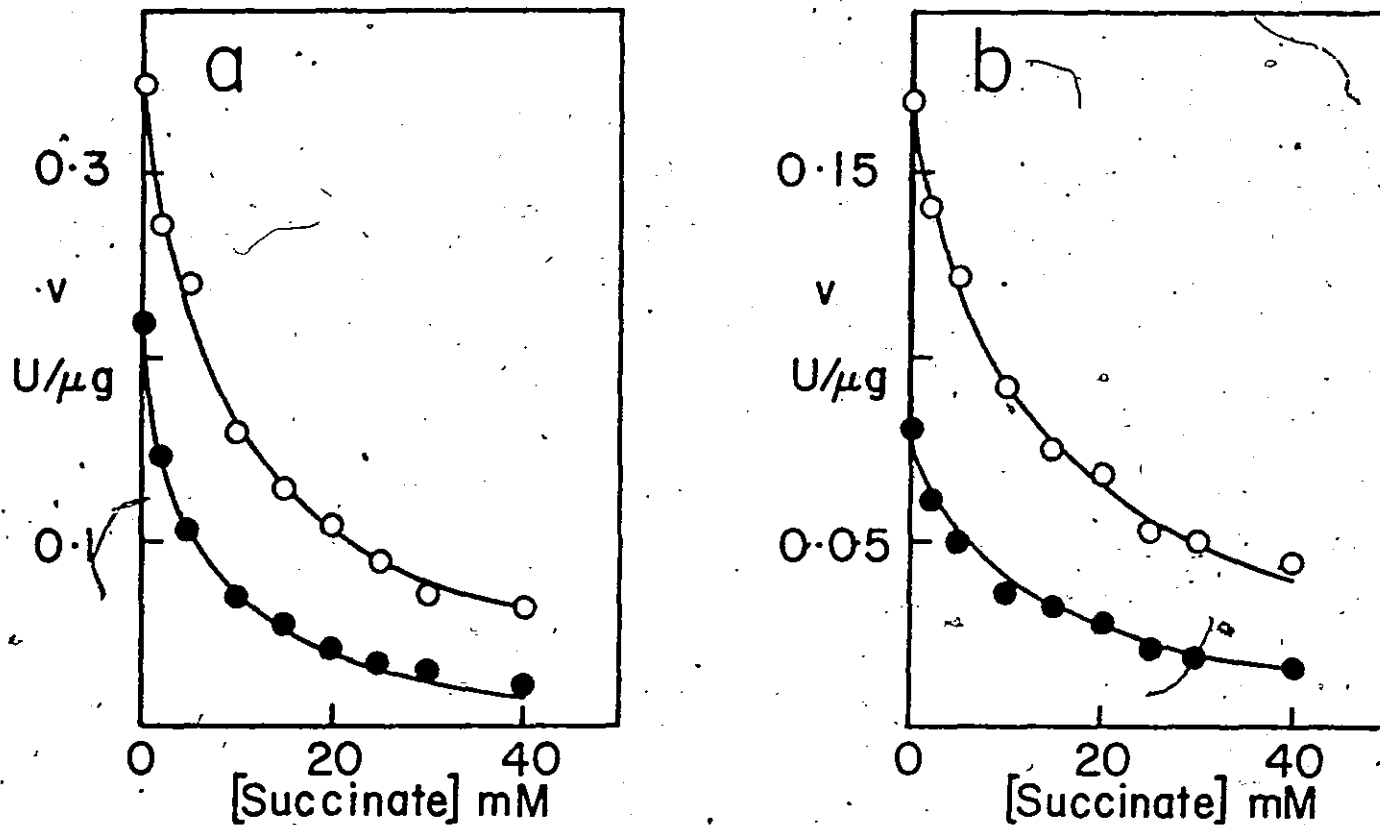$$v = \frac{V_{max}\, S}{(K_m + S)\,(1 + I/K_i)}$$

Fig. 28. <u>Effect of Succinate on c_3 and c_3r_6 Activities at Constant Aspartate Concentrations.</u>

The c_3r_6 complex (a) and c_3 (b) activities at 2 and 5 mM aspartate and varied succinate concentrations were obtained by titrator assay at pH 8.5 in the presence of saturating carbamyl phosphate (5 mM). The c_3r_6 rates were obtained by addition of r_2 (to give a final concentration of 50 µg/ml) to the assay containing c_3 (1.5 µg/ml). The lines are a least square fit to the equation
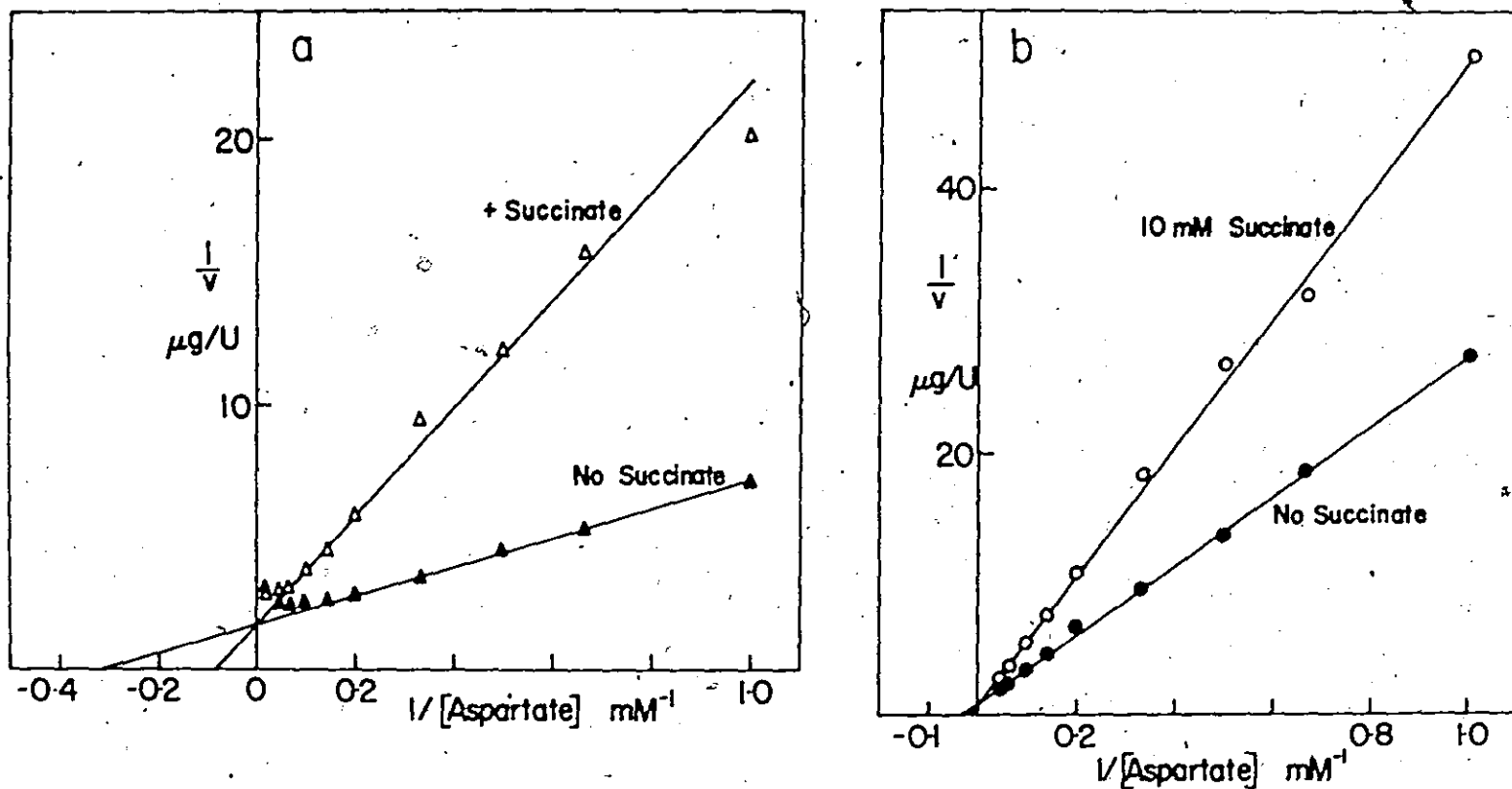
$$v = V_{max} \, S/[K_m \, (1 + I/K_i) + S].$$

Fig. 29. Kinetic Pattern of Succinate Inhibition of c_3r_6 and c_3 with Varied Aspartate.

Double reciprocal plots in the presence and absence of succinate (10 mM) for (a) c_3r_6 and (b) c_3 obtained as in Fig. 18 at pH 8.5. The lines are fits of the original data to hyperbolas. Points at high aspartate concentrations which show upward curvature due to substrate inhibition were excluded from this procedure.