

TO MY WIFE, PAT.

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Prof. J.G. Kaplan for his patience, guidance and encouragement throughout this study and during the preparation of the thesis.

I would like also to thank Dr. P. Fitt, Dept. of Biochemistry and Dr. C. Godin, Dept. of Biology for their suggestions in the preparation of the thesis.

I would like to thank Dr. M. Denis-Duphil for supplying the various mutants used in this study and for the many invaluable discussions we had together. Many thanks are extended to Mrs. M. McIntosh for her expert technical assistance and to the other members of the laboratory for supplying the academic yet friendly atmosphere.

Many thanks are extended to Dr. H. de Robichon-Szulmajster for supplying the E. coli strain used in the preparation of OTCase, to Dr. W.G. Martin for the cells used in gel filtration and for the early analytical ultracentrifuge (Model E) facilities, to Dr. L. Sierens for his assistance in the sucrose density gradient studies and to Dr. K. Kako, Dept. of Physiology, Faculty of Medicine for the use of the analytical ultracentrifuge before one became available in our laboratory.

Finally, I would like to acknowledge the financial assistance of the National Research Council for a post-graduate scholarship.

ABSTRACT

A purification procedure for yeast aspartate trans-carbamylase (ATCase) resulted in co-purification of the carbamyl phosphate synthetase (CPSase) of the pyrimidine pathway; both activities were highly sensitive to feedback inhibition (FI) by UTP. CPSase was generally purified somewhat more than ATCase; this observation was shown to be due to progressive discard with each step of purification of ATCase subunits, present in the crude extract and insensitive to FI by UTP. Co-sedimentation in sucrose density gradient centrifugation and co-elution from Sepharose 6B in the presence of UTP,  $Mg^{++}$  and glutamine further established that both activities together with the feedback site exist in a single complex of molecular weight of approx. 800,000 daltons.

Omission of UTP but in the presence of glutamine and  $Mg^{++}$  in sucrose gradient caused dissociation into a complex of molecular weight 370,000 with both activities but the ATCase was of reduced sensitivity to FI. Further, omission of  $Mg^{++}$  and glutamine in the sucrose gradient resulted in trailing of the CPSase peak behind the ATCase peak. The CPSase remained fully sensitive to FI by UTP.

Chromatography on DEAE-Sephadex and heat caused loss

of CPSase activity and disappearance of FI of the ATCase; the ATCase recovered had a molecular weight of 140,000. A structural model based on these studies and on genetic data was postulated. The  $K_m$  value for CP in the ATCase reaction was  $2 \times 10^{-3}M$  and for aspartate it was  $3.3 \times 10^{-2}M$ . The CPSase reaction showed an absolute requirement for  $K^+$ ,  $Mg^{++}$ ,  $HCO_3^-$ , ATP and glutamine. The pH optimum was 7.4 in the presence of  $Mg^{++}$  and the stoichiometry of the reaction involved 2 moles of ATP and 1 mole of glutamine per mole of CP synthesized.  $Mg^{++}$  seems to serve 2 roles: 1) it binds with free ATP (which by itself inhibits the reaction) to give the true substrate Mg-ATP, and 2) it serves to activate the reaction. The  $K_m$  values for Mg-ATP, glutamine and bicarbonate were  $3.5 \times 10^{-3}M$ ,  $5 \times 10^{-4}M$  and  $3 \times 10^{-3}M$  respectively.

UTP inhibited all the substrates (i.e. of both enzyme reactions) non-competitively and the CPSase reaction was stimulated by XMP. XMP stimulated the CPSase by binding at a separate and distinct site from the UTP site. It is suggested that the physiological role of the CPSase-ATCase complex lies in the preferential channelling of the CP produced into the synthesis of pyrimidines. Preliminary experiments were found to justify this conclusion.

RESUME

La purification de l'ATCase de la levure entraîne la purification simultanée de la CPSase. Les deux activités sont très sensibles à l'inhibition par l'UTP, un produit de la chaîne de synthèse qui intervient dans la régulation par rétro-contrôle de l'activité. L'activité CPSasique est purifiée nettement plus que l'activité ATCasique; ceci est dû à la perte progressive des sous-unités ATCase durant les différentes étapes de la purification.

La co-sédimentation des deux activités et du site de retro-contrôle après centrifugation en gradient de sucrose (en présence d'UTP, de  $Mg^{++}$  et de glutamine) ainsi que leur co-élution après passage sur sepharose 6B (dans les mêmes conditions) sont des arguments en faveur de l'existence d'un seul complexe de poids moléculaire environ 800,000.

La centrifugation en gradient de sucrose en présence de glutamine et de  $Mg^{++}$ , mais sans UTP, entraîne la dissociation du complexe en un complexe plus petit, de poids moléculaire environ 370,000, portant les deux activités mais dont l'ATCase est moins sensible à l'inhibition par UTP. La CPSase reste entièrement sensible à l'UTP. En l'absence de glutamine, de Mg et d'UTP, le pic de la CPase vient légèrement derrière celui correspondant à l'ATCase.

La chromatographie sur DEAE Sephadex et le traitement par la chaleur du complexe, provoquent la disparition de l'activité CPSase et la perte de la sensibilité à l'UTP de l'ATCase. L'ATCase récupérée après ce traitement a un poids moléculaire de 140,000.

Ces données, jointes à des données génétiques, permettent de proposer un modèle de la structure de ce complexe enzymatique.

Pour l'ATCase, le  $K_m$  pour le CP est de  $2.10^{-3}M$  et pour l'aspartate de  $3,3 10^{-2}M$ .

Pour la CPSase, la réaction nécessite absolument  $K^+$ ,  $Mg^{++}$ ,  $HCO_3^-$ , ATP et glutamine. Le pH optimum de la réaction est 7.4. La stochiométrie de la réaction fait intervenir 2 moles d'ATP et 1 mole de glutamine par mole de CP formé. Le Mg semble jouer 2 rôles: d'une part il se lie à l'ATP libre (qui est inhibiteur de la réaction) pour donner le substrat réel Mg-ATP, d'autre part il sert à activer la réaction.

Les  $K_m$  pour le Mg-ATP, la glutamine et le bicarbonate sont respectivement  $3.5 10^{-3}M$ ,  $5.10^{-4}M$  et  $3.10^{-3}M$ .

L'UTP inhibe les deux activités en agissant comme inhibiteur non compétitif des différents substrats. L'XMP stimule la CPSase en se fixant à un site spécial différent du site de l'UTP.

Le rôle physiologique de ce complexe serait d'orienter préférentiellement le CP formé vers la chaîne de biosynthèse des pyrimidines. Des expériences préliminaires confirment cette hypothèse.

TABLE OF CONTENTS

	<u>Page No.</u>
ACKNOWLEDGEMENTS	i
ABSTRACT	ii
RESUME	iv
TABLE OF CONTENTS	vi
LIST OF ABBREVIATIONS	xii
LIST OF FIGURES	xiii
LIST OF TABLES	xix
A. INTRODUCTION	1
I. Literature Review	1
1. Elucidation of Pyrimidine Biosynthetic Pathway	1
a. Tracer and Nutritional Studies	2
b. Enzymatic Studies	5
2. Carbamyl Phosphate Synthetase (CPSase)	15
a. Carbamyl Phosphate Synthesis in <u>E. coli</u>	15
b. Comparative Distribution of CPSase	22
c. Properties of the CPSase Reaction	25
d. Mechanism of the CPSase Reaction	27
3. Aspartate Transcarbamylase (ATCase)	29
a. Introduction	29
b. Aspartate Transcarbamylase of <u>E. coli</u>	30
c. Catalytic Mechanism of Ureidosuccinate Formation	33
d. Allostery in Terms of Quaternary Structure	35
e. Structural Model and Regulatory Mechanism	38
f. Proof of Conformational Change	42
g. ATCase in Terms of the Two Models	45

	<u>Page No.</u>
4. Regulatory Control Mechanisms	48
a. Historical Background and Definitions	48
b. Protein Synthesis	51
c. Regulation of Protein Synthesis with Respect to Repression and Induction	52
d. Regulation of Pyrimidine Biosynthesis	55
II. Introduction to the Problem	59
1. Regulation of Pyrimidine Biosynthesis in Yeast	59
2. Purpose of the Present Studies	63
B. MATERIALS	64
C. METHODS	60
I. Organism	70
1. Baker's Yeast ( <u>Saccharomyces cerevisiae</u> )	70
2. Cultivation of Yeast	70
3. Growth Conditions	71
4. Description of Strains	72
II. Enzyme Assays	75
1. Aspartate Transcarbamylase	75
a. Colorimetric Determination of Ureidosuccinate	76
2. CPSase	79
3. ADP Estimation	85
4. Glutamate Estimation	87
5. Catalase Activity	88
6. Yeast Alcohol Dehydrogenase (ADHase)	88
III. Techniques in Enzyme Purification and Molecular Weight Estimation	89

	<u>Page No.</u>
1. Ammonium Sulphate Fractionation	89
2. Protamine Sulphate Treatment	90
3. Organic Solvent Fractionation	91
4. Column Chromatography	91
a. DEAE Cellulose	92
b. CM Cellulose	93
c. Phosphocellulose	93
d. Gel Filtration	93
5. Gradient Centrifugation	95
IV. Analytical Procedures	97
1. Protein Determination	97
2. Electrophoresis	97
D. RESULTS	98
I. Preliminary Experiments on the Purification of ATCase	98
1. Kinetics of Derepression	99
2. Salt Fractionation	104
3. Organic Solvent Fractionation	106
4. Adsorption and Elution Studies	111
a. Gels and Other Materials	111
b. Column Chromatography	112
5. Stability Studies	114
a. Effect of pH	114
b. Sulfhydryl Reagents	115
c. Effect of High Salt Concentration	115
d. Effect of Substrates, Products and Inhibitors	116
e. Effect of Glycerol	116
6. Conclusion	118

	<u>Page No.</u>
II. Enzyme Purification	119
1. Cultivation of MD169(c) and MD170-7X	119
2. Preparation of Crude Extracts	119
3. Protamine Sulphate Treatment	120
4. Isopropanol-Ammonium Sulphate Fractionation	120
5. Calcium Phosphate Gel Adsorption and Elution and Ammonium Sulphate Fractionation	121
6. Gel Filtration on Sepharose 6B	122
III. Molecular Weight Estimation	129
1. Gel Filtration	129
2. Centrifugation in a Model E Analytical Ultracentrifuge	137
3. Ultracentrifugation in Sucrose Density Gradients	139
IV. Experiments Showing Further Disaggregation	148
1. DEAE Sephadex	149
2. Effect of Heat	152
3. Effect of Heat in the Presence of Bovine Serum Albumin	160
V. Characterization of the 20-fold Purified ATCase	164
1. Effect of pH	164
2. Effect of Pyrophosphate	164
3. Effect of Various Concentrations of Carbamyl Phosphate	167
4. Effect of Various Concentrations of Aspartate	175

	<u>Page No.</u>
5. Inhibition by UTP	179
VI. Carbamyl Phosphate Synthetase - Properties	179
1. Requirements for the Reaction	179
2. Stoichiometry of the CPSase Reaction	183
3. Effect of Avidin and Biotin	185
4. Effect of pH	187
5. Effect of Divalent Ions	187
6. Effect of Substrate Concentration	189
a. ATP and Magnesium ( $Mg^{++}$ )	189
b. Glutamine	200
c. Bicarbonate	200
d. Potassium	200
7. Inhibition by UTP	206
VII. Regulation	209
1. Effect of Purine and Pyrimidine Nucleotides	209
a. Aspartate Transcarbamylase	210
b. Carbamyl Phosphate Synthetase	212
2. Physiological Role of the CPSase-ATCase Complex	222
E. DISCUSSION	238
I. Enzymatic Properties	238
1. ATCase	238
2. CPSase	240
II. Regulation	245
1. Effect of Purines and Pyrimidines	245
2. The Occurrence of Enzyme Aggregates	249

	<u>Page No.</u>
III. Purification	252
1. Molecular Weights and States of Aggregation	252
a. Ultracentrifuge Studies	252
b. Gel Filtration	258
IV. Genetic Data	260
V. Structural Model	263
F. REFERENCES	266

LIST OF ABBREVIATIONS

(A) The Pyrimidine Biosynthetic Pathway

CPSase	- carbamyl phosphate synthetase
CP	- carbamyl phosphate
ATCase	- aspartate transcarbamylase
US	- ureidosuccinate or carbamyl aspartate
DHOase	- dihydroorotase
DHOdehase	- dihydroorotic dehydrogenase
OMPppase	- orotidylic acid pyrophosphate
OMPdecase	- orotidine-5phosphate decarboxylase

(B) Others

BSA	- bovine serum albumin
FI	- feedback inhibition
PRPP	- phosphoribosyl pyrophosphate
pers. comm.	- personal communication
'regulatory function' or 'feedback property'	- capacity of the ATCase activity to be inhibited by UTP
PCMB	- p-chloromercuribenzoate
PMB	- p-hydroxymercuribenzoate

LIST OF FIGURES

	<u>Page No.</u>
1. Structural formula of orotic acid.	6
2. Pyrimidine biosynthesis.	14
3. Regulation of pyrimidine biosynthesis in <u>E. coli</u> .	56
4. Regulation of pyrimidine biosynthesis in yeast.	61
5. Color development with time using three concentrations of standard ureidosuccinate.	78
6. Plot to show linearity with time and enzyme concentration in the ATCase reaction.	80
7. Color development with time using 3 concentrations of standard citrulline.	82
8. Plot to show linearity with time and enzyme concentration in the CPSase reaction (Assay System II).	84
9. Plot to show linearity with time and enzyme concentration in the CPSase reaction (Assay System III).	86
10. Growth behaviour of MD170-7X in G <sub>0</sub> plus various additives.	103
11. Stabilizing effect of various glycerol concentrations on the ATCase activity and feedback inhibition.	117
12. Elution curves of several marker proteins, ATCase and CPSase activities from a 2.5x94 cm. Sepharose 6B column.	130

List of Figures (Cont'd)

	<u>Page No.</u>
13. Elution curves of several marker proteins and the ATCase activity from a 1.5x88.5 cm. Sephadex G-200 column.	131
14. Correlation of the elution data from Sepharose 6B with molecular weight.	133
15. Correlation of the elution data from Sephadex G-200 with molecular weight.	135
16. Sedimentation pattern of a purified preparation.	138
17. Sucrose gradient centrifugation of a purified preparation in the presence of UTP, Mg <sup>++</sup> and glutamine.	140
18. As 17, but in the presence of Mg <sup>++</sup> and glutamine.	142
19. As 17 and 18, but in the absence of Mg <sup>++</sup> , UTP and glutamine.	144
20. As 19, but using a preparation from Strain MD170-7X instead of FL233-3C.	146
21. DEAE-Sephadex column chromatogram.	150
22. Sucrose density gradient centrifugation of a (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> preparation of tubes 6 and 7 (Fig.21).	151
23. Arrhenius plot of ATCase and CPSase activities both in the presence and absence of 2x10 <sup>-3</sup> M UTP.	153
24. The effect of heating at 50°C on the activities and feedback inhibitions of ATCase and CPSase.	154
25. Sucrose gradient centrifugation of a purified preparation which had been heated at 50°C for 5 min.	156

List of Figures (Cont'd)

	<u>Page No.</u>
26. Sucrose gradient centrifugation of catalase and yeast alcohol dehydrogenase.	158
27. Effect of heating at 50° on a DEAE-Sephadex prepared material in the presence and absence of 0.4% BSA.	161
28. As in Fig. 24, but in the presence of 0.4% BSA.	163
29. Dependence of ATCase activity on pH.	165
30. Effect of pyrophosphate on the ATCase activity and inhibition by UTP.	166
31. Effect of CP concentrations on the ATCase activity and also in the presence of pyrophosphate and UTP.	169
32. Lineweaver-Burk plot of the results from Fig. 31.	171
33. Hill plot of the results from Fig. 31.	173
34. Effect of CP concentrations on the disinhibited ARCase activity of a preparation eluted from DEAE-Sephadex in the presence and absence of pyrophosphate.	174
35. Effect of CP concentration on a material heated at 60°C for 3 min. in the presence and absence of pyrophosphate (as a Lineweaver-Burk plot).	176
36. Effect of aspartate concentrations on the ATCase activity in the presence and absence of UTP.	177
37. Lineweaver-Burk plot of Fig. 36.	178
38. Effect of various concentrations of UTP on the ATCase and CPSase activities.	180

List of Figures (Cont'd)

	<u>Page No.</u>
39. Plot of $1/V$ vs. (UTP) for the estimation of $K_i$ in the ATCase reaction.	181
40. Effect of pH on the CPSase activity and inhibition, the ability of $NH_4^+$ to replace glutamine as a function of pH.	188
41. Effect of divalent ions on the CPSase activity as a function of pH.	190
42. Effect of varying concentrations of ATP in the presence and absence of UTP.	191
43. Effect of various concentrations of ATP at three concentrations of $Mg^{++}$ on the CPSase activity.	193
44. Double reciprocal plot of the values from Fig. 43	194
45. Effect of various $Mg^{++}$ concentrations at 3 concentrations of ATP on the CPSase activity.	196
46. Hill plots of the values from Fig. 45.	198
47. Effect of various Mg-ATP concentrations on the CPSase activity in the presence and absence of $Mg^{++}$ and ATP.	199
48. Effect of various concentrations of glutamine on the CPSase activity in the presence and absence of UTP.	201
49. S/V vs. S plot of the values from Fig. 48.	202
50. Effect of various concentrations of $HCO_3^-$ on the CPSase activity in the presence and absence of UTP.	203

List of Figures (Cont'd)

	<u>Page No.</u>
51. (S)/V vs. (S) plot of the values from Fig. 50.	204
52. Effect of various conc. of K <sup>+</sup> ions on the CPSase activity.	205
53. Plot of 1/V vs. (UTP) for the estimation of K <sub>i</sub> for the UTP inhibition of the CPSase activity.	208
54. Effect of various conc. of XMP and UTP on the CPSase activity.	218
55. Effect of various concentrations of XMP in the absence and presence of 3 different concentrations of UTP.	219
56. The data of Fig. 55 are replotted to show the relative activation by XMP, and the effect of XMP on the inhibition by UTP.	220
57. The effect of various <u>conetnrations</u> of ADP on the CPSase activity.	221
58. Effect of various concentrations of ATP in the absence and presence of UTP, XMP and ADP.	223
59. Effect of variation of the ratio of activity of OTCase to ATCase on the direction of utilization of endogenously formed carbamyl phosphate.	233
60. The data from Fig. 59 rearranged as % ureidosuccinate formed.	234
61. Effect of variation of the ratio of activity of OTCase to ATCase on the direction of utilization of endogenously formed CP. Assay of ureidosuccinate was done colorimetrically.	236

List of Figures (Cont'd)

	<u>Page No.</u>
62. Structural model of the ATCase-CPSase complex.	264

LIST OF TABLES

	<u>Page No.</u>
1. Properties of the CPSase reaction from various sources.	26
2. Preparation of OTCase from a derepressed strain of <u>E. coli</u> .	69
3. Description of strains.	73
4. A comparative study of 5 strains of yeast to determine the likely source in the purification of ATCase.	100
5. The effect of growth conditions and certain genetic introductions on the ATCase activity and inhibition of FL233-3C.	102
6. Enzyme purification by ammonium sulphate fractionation using extracts from FL233-3C.	105
7. The effect of protamine sulphate on ammonium sulphate fractionation.	107
8. Purification of ATCase activity by organic solvent fractionation.	108
9. Purification of ATCase by isopropanol fractionation.	110
10. The effect of dialysis and column chromatography on ATCase specific activity and feedback inhibition.	113
11. Purification of yeast ATCase and CPSase from strain MD169(c).	124
12. Purification of yeast ATCase and CPSase from strain MD170-7X.	126

List of Tables (Cont'd)

	<u>Page No.</u>
13. Sensitivity to feedback inhibition of the fractions discarded during purification.	128
14. Molecular parameters of standard proteins and ATCase determined by gel filtration.	136
15. Molecular weights from sedimentation data.	147
16. Molecular weight of non-inhibited material.	159
17. Additive inhibition of ATCase caused by PPI and UTP.	168
18. Requirements for the enzymatic synthesis of citrulline by yeast CPSase.	182
19. Stoichiometry of the carbamyl phosphate synthetase reaction.	184
20. Effect of avidin and biotin on CPSase activity.	186
21. Km's as determined by Assay system I and II.	207
22. Effect of pyrimidine nucleotide, di- and tri-phosphate and uracil on ATCase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	211
23. Effect of purine nucleotide, di- and tri-phosphate on ATCase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	213
24. Effect of pyrimidine nucleotide, di- and tri-phosphate and other compounds on CPSase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	214
25. Effect of purine nucleotide, di- and tri-phosphate on CPSase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	216

List of Tables (Cont'd)

	<u>Page No.</u>
13. Sensitivity to feedback inhibition of the fractions discarded during purification.	128
14. Molecular parameters of standard proteins and ATCase determined by gel filtration.	136
15. Molecular weights from sedimentation data.	147
16. Molecular weight of non-inhibited material.	159
17. Additive inhibition of ATCase caused by PPI and UTP.	168
18. Requirements for the enzymatic synthesis of citrulline by yeast CPSase.	182
19. Stoichiometry of the carbamyl phosphate synthetase reaction.	184
20. Effect of avidin and biotin on CPSase activity.	186
21. Km's as determined by Assay system I and II.	207
22. Effect of pyrimidine nucleotide, di- and tri-phosphate and uracil on ATCase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	211
23. Effect of purine nucleotide, di- and tri-phosphate on ATCase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	213
24. Effect of pyrimidine nucleotide, di- and tri-phosphate and other compounds on CPSase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	214
25. Effect of purine nucleotide, di- and tri-phosphate on CPSase activity and feedback inhibition by $2 \times 10^{-3}M$ UTP.	216

List of Tables (Cont'd)

	<u>Page No.</u>
26. Time course of CP production by CPSase estimated as either citrulline or ureidosuccinate formation; the effect of ornithine on the CPSase reaction.	226
27. The effect of added carbamyl phosphate on ureidosuccinate formation via CPSase-ATCase system.	227
28. The effect of externally added carbamyl phosphate on the utilization of $H^{14}CO_3^-$ in the CPSase reaction.	230
29. Biosynthetic multi-functional enzyme complexes identified in various organisms.	250

A. INTRODUCTION

I. Literature Review

1. Elucidation of the Pyrimidine Biosynthetic Pathway

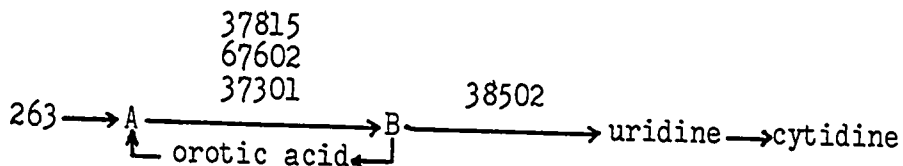
After the isolation of nutritional mutants by Beadle and Tatum (1941), it became obvious that the availability and utilization of the end product (or structural analogue) of a biosynthetic pathway would facilitate greatly the elucidation of the complete chain as it did in purine biosynthesis (Reichard, 1955). However, when the studies of pyrimidine biosynthesis were initiated, such structural analogues and possible intermediates were unknown; thus other techniques had to be employed. These included chase experiments where labelled small precursor molecules were traced into pyrimidine nucleotides, enzymatic studies of the conversion of these small precursor molecules into pyrimidine nucleotides, and studies on the ability of certain substances (presumably intermediates) to satisfy the pyrimidine requirements of microbial auxotrophs (Beadle and Tatum, 1941).

(a) Tracer and Nutritional Studies

Orotic acid was isolated from cow's milk in 1905 by Bicaró and Belloni. However, it was not until the early 1940's that some metabolic significance was attributed to this pyrimidine carboxylic acid. Loring and Pierce (1944), Chattaway (1944), and Rogers (1944) showed that uracil can be replaced by orotic acid as a growth factor in certain microorganisms, suggesting a connection to pyrimidine biogenesis. However, this idea was altered by the discovery that the pyrimidine nucleosides and nucleotides were 10-60 times more effective than orotate (Chattaway, 1944). Also, the fact that uracil replaced orotic acid to the same degree suggested a by-product relationship for orotic acid, since uracil was known not to be on the pathway.

This latter hypothesis was supported by the work of Mitchell and co-workers (1948). They were able to isolate three types of pyrimidine mutants from Neurospora: strain 263, which could utilize orotic acid; strain 38502, which excreted orotic acid; and strains 37815, 67602 and 37301, which could neither excrete nor utilize orotic acid. An analysis of the nutritional requirements of the double mutants obtained by mating 38502 with the other strains established

the following sequence:



Thus, the fact that strains 37815, 67602, 37301 were unable to utilize orotic acid led these workers to conclude that orotic acid was a by product produced by an irreversible reaction. Furthermore, Mitchell and Houlahan (1947) postulated that pyrimidines were formed via oxaloacetic acid and an aliphatic glycoside intermediate, suggesting that ribotidation preceded ring closure. This hypothesis was pleasing at that time due to the analogous situation which was known to exist in purine biosynthesis (Reichard, 1955). Since only three classes of mutants were found, a three step mechanism for the synthesis of pyrimidines was suggested on account of the one gene-one enzyme relationship enunciated by Beadle and Tatum (1941).

In 1949 new interest was aroused in the metabolism of orotic acid after Avidson et al. (1949) demonstrated that 3-N<sup>15</sup> orotic acid was incorporated into the pyrimidine nucleotides of rat liver. Because of the high

specific labelling obtained in these experiments, it was suggested that the whole orotic acid molecule was incorporated. The incorporation of orotic acid into pyrimidines was also confirmed by Weed et al. (1950), and by Reichard and Bergström (1951), where an in vitro rat liver system was used.

These results led to the inescapable question of whether or not orotic acid was a normal intermediate in pyrimidine biosynthesis. This question was tackled by Reichard and co-workers who used rat liver slices and two methods of approach (Reichard and Lagerkvist, 1953; Reichard 1965). The first method consisted of incubating liver slices in the presence of orotate and a suspected labelled precursor. After incubation, orotic acid was re-isolated and the isotopic distribution within the molecule was determined after degradation. The second approach involved the use of labelled  $N^{15}HCl$  incubated with cold precursor, orotic acid and liver slices. It was previously shown by Barnes and Schoenheimer (1943) that  $^{15}N$  label from ammonium citrate was incorporated into the polynucleotide of pigeon and rat liver. Thus, after the incubation in the presence of a true non-labelled precursor, the diluting effect of that precursor on the

incorporation of  $N^{15}$  into orotic acid could be studied (Reichard, 1952).

By using  $N^{15}$  labelled ammonium chloride, bicarbonate  $C^{13}$ , L-aspartate- $N^{15}$ , L-aspartate-1,4- $C^{13}$ , L-aspartate-2,3- $C^{14}$  and ureidosuccinate- $N^{15}$  with the above methods, Reichard and co-workers were able to show that  $N_3$  of orotic acid (Fig.1) was obtained from ammonium,  $C$  from bicarbonate, and the rest of the molecule from aspartic acid. That the orotic acid molecule is subsequently converted into pyrimidine nucleotides was shown by Weed et al. (1950). The precursor relationship of orotic acid to pyrimidine was thus established, confirming results indicated by nutritional experiments with E. coli B (Weed and Cohen, 1951), and yeast (Edmonds et al., 1952). Hence the hypothesis mentioned above, of Mitchell et al. (1948), was rejected on the basis that ring closure occurred before ribotidation. Further clarification came with enzymatic studies (Reichard, 1959).

### (b) Enzymatic Studies

#### (i) Formation of ureidosuccinate

In 1952 Grisolia and Cohen established that in rat liver preparations ATP,  $Mg^{++}$  and acetylglutamate were

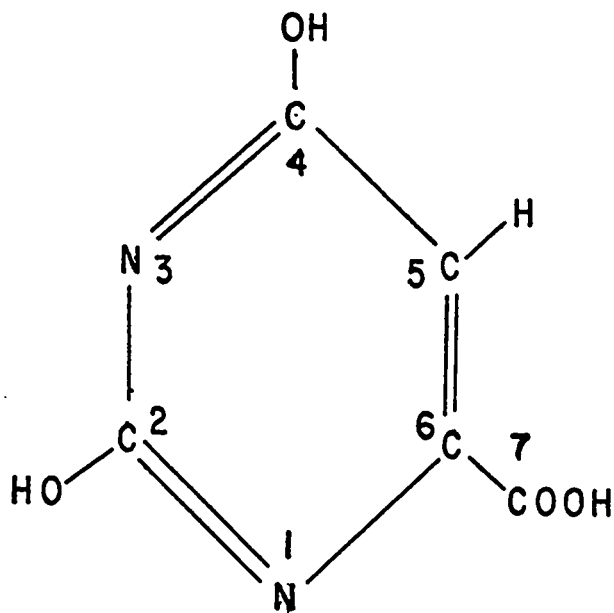
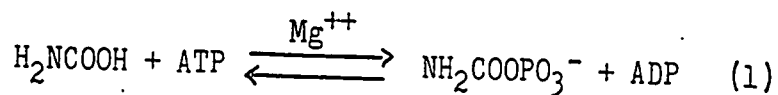


FIG.1 STRUCTURAL FORMULA OF OROTIC ACID

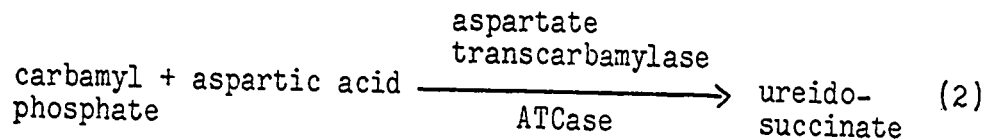
involved in the formation of an activated derivative of CO<sub>2</sub> and ammonia, i.e. "compound X", which condensed with ornithine to form citrulline. Reichard, (1954) and Reichard and Hanshoff, (1955) showed that "compound X" was also involved in the synthesis of ureidosuccinate in rat liver slices. In 1955 Jones et al. succeeded in isolating and identifying carbamyl phosphate from bacterial extracts. They indicated that the reaction catalyzed by the enzyme which they called carbamyl phosphokinase was



They also prepared the dilithium salt of carbamyl phosphate by reacting KCNO with inorganic dilithium phosphate and established the authenticity by showing that this synthesized carbamyl phosphate was also capable of yielding citrulline when incubated in the presence of ornithine and ornithine transcarbamylase from Streptococcus faecalis. The involvement of carbamyl phosphate in the formation of ureidosuccinate was also confirmed by these workers in S. faecalis extracts (Jones et al., 1955), in mammalian systems (Reichard and Hanshoff, 1956),

in E. coli by Yates and Pardee (1956a).

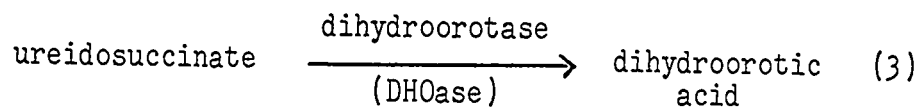
Yates and Pardee (1956b) also showed that the activity of aspartate transcarbamylase, the enzyme which catalyzed the formation of ureidosuccinate from aspartate

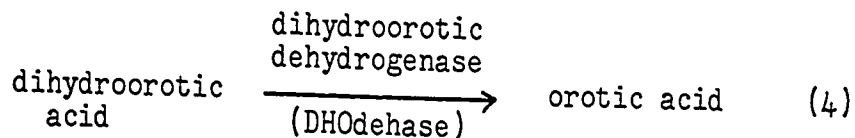


and carbamyl phosphate, was inhibited by CTP, an end product of the pathway, and suggested feedback inhibition as a possible control mechanism. Regulatory control mechanisms will be considered later on.

(ii) Formation of orotic acid

The conversion of ureidosuccinate to orotic acid was demonstrated by Lieberman and Kornberg (1954). In extracts made from Zymobacterium oroticum and Corynebacteria grown on enrichment media containing orotic acid they were able to show two enzymatic activities. These were dihydroorotase, which by a dehydration reaction effected ring closure forming dihydroorotic acid from ureidosuccinate and dihydroorotic dehydrogenase, an NAD



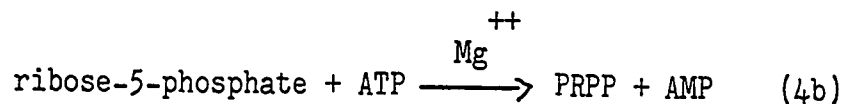


linked reaction catalyzing the formation of orotic acid from dihydroorotic acid. It was also shown that although the reverse dehydrogenase reaction was favored, a high degree of specificity was implicated. Among the compounds tested as substrates and which were found to be inactive were uracil, cytosine, thymine and 5-methyl cytosine. The fact that these activities were discovered in bacteria grown on orotic acid as a carbon source opened the possibility that these could be inducible degradative enzymes and not involved in the biosynthetic sequence. Yates and Pardee (1956a) confirmed the presence of these enzymes in actively growing E. coli. They demonstrated that mutants lacking dihydroorotate dehydrogenase required orotic acid, uracil or cytosine for growth; and found that another pyrimidine-requiring mutant, which could not utilize orotic acid for growth, instead excreted this carboxylic acid together with dihydroorotic acid and ureidosuccinate into its growth medium. Such mutants would therefore be blocked at a step subsequent to orotic acid formation. The presence of these enzymes

in rat liver homogenate was also demonstrated by Cooper et al. (1955).

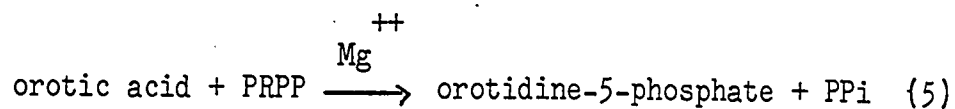
(iii) Formation of uridine monophosphate from orotic acid

Clarification of the mechanism of conversion of orotic acid to a nucleotide linkage was due to the brilliant work of Kornberg and co-workers. The problem of whether or not orotic acid was a true intermediate led Kornberg et al. (1955) to the isolation and identification of phosphoribosylpyrophosphate (PRPP), the formation of which was demonstrated with a purified pigeon liver enzyme according to the reaction:



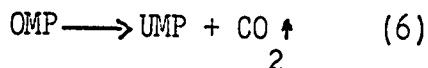
With respect to pyrimidine biosynthesis, they succeeded purifying orotidylic pyrophosphorylase and orotidylic decarboxylase from yeast autolysates, a most active source.

The former enzyme catalyzed the reaction:



This reaction was found to be reversible, to require  $\text{Mg}^{++}$ ,

to be inhibited by OMP and to have an equilibrium constant of 0.1. Since, as we shall see below, OMP is converted directly into the authentic precursor UMP, and since the above reaction (5) exhibited a very high degree of specificity for orotic acid, it was thus established beyond any doubt that orotic acid was a true intermediate. Other substances tested as substrates but without effect were ureidosuccinate, dihydroorotic acid, uracil, cytosine, orotidine and adenine. The latter enzyme, orotidylic decarboxylase, catalyzed the reaction:



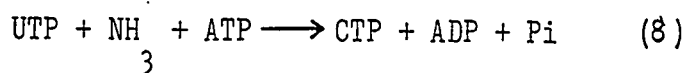
This reaction was irreversible and specific.

It must be noted that from the equilibrium constants of the two steps prior to this irreversible decarboxylation reaction the equilibrium did not favor the forward reaction leading to pyrimidine biosynthesis. However, when coupled with this irreversible reaction, the two steps led smoothly to uridine monophosphate synthesis. These two enzymatic reactions have also been observed in other microorganisms and in avian and mammalian systems (Magasanik, 1962). The conversion of UMP to UDP

and UTP was shown to involve reactions with ATP catalyzed by specific kinases (Berg and Joklik, 1954; Lieberman et al., 1955).

(iv) Formation of cytidine triphosphate

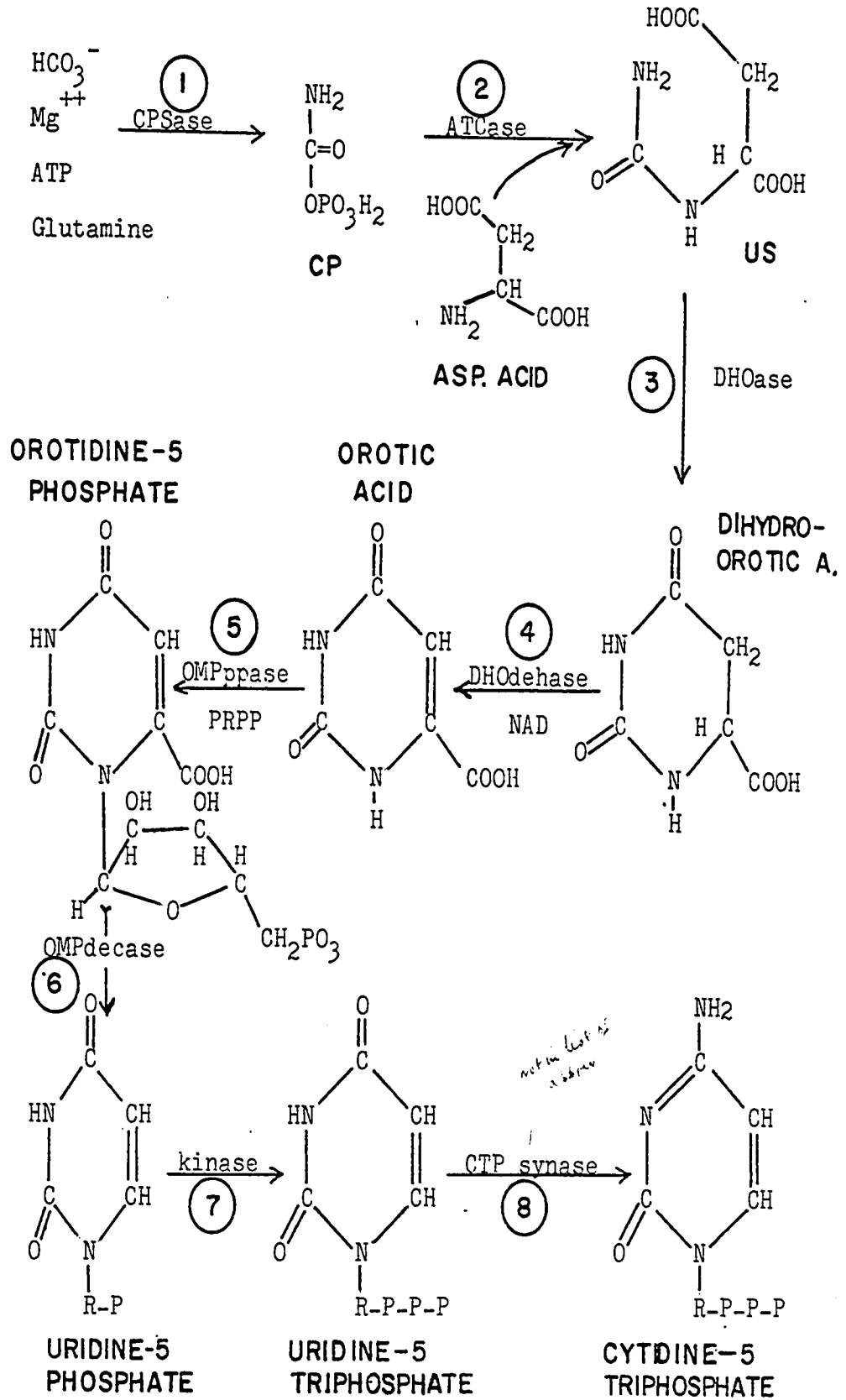
It has been noted in chase experiments involving labelled orotic acid and in vivo animal systems that uridine nucleotides were labelled earlier than cytidine nucleotides (Rutman et al., 1954), and that the specific labelling of RNA-uracil was much greater than RNA-cytosine (Weed et al., 1950; Weed and Wilson, 1951; Hurlbert and Potter, 1952). These data of course indicated a sequence whereby uridine nucleotides are formed first from orotic acid and subsequently cytidine nucleotides from uridine nucleotides. Direct demonstration of the conversion of uridine nucleotides to cytidine nucleotides was found by Lieberman (1955, 1956), to occur primarily at the triphosphate level in extracts of E. coli by a reaction depending upon ammonia and ATP. The reaction catalyzed by cytidine triphosphate synthetase was:



UDP was capable of replacing UTP; however, the reaction

rate was only 50% of that obtained with UTP. UMP, uridine or uracil failed to react. Glutamine, asparagine, glutamic acid and aspartic acid could not replace ammonia in E. coli extracts. In mammalian systems, however, it was found that glutamine replaced ammonia as the amino donor and that GTP stimulated the reaction (Kammen and Hurlbert, 1958). In 1961, contrary to the findings of Lieberman, Chakraborty and Hurlbert showed that in E. coli glutamine was indeed the amino donor in cytidine nucleotide synthesis and not ammonia. However, ammonia was able to substitute for glutamine at higher pH and concentration. Cytidine triphosphate synthetase was later purified and characterized in E. coli B (Long and Pardee, 1967). The discrepancy in the results of these two groups of workers was explained by Long and Pardee as a modification in the structure of the enzyme, caused by the extraction and purification procedure by Lieberman.

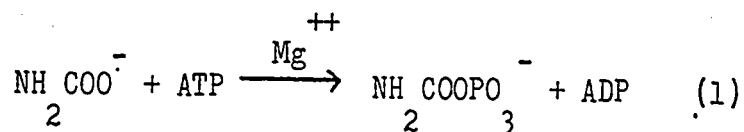
### FIG. 2 PYRIMIDINE BIOSYNTHESIS



## 2. Carbamyl Phosphate Synthetase

### (a) Carbamyl Phosphate Synthesis in E. coli

In the previous section it was pointed out that a carbamyl derivative ("compound X") formed from  $\text{NH}_2^+$ , ATP,  $\text{Mg}^{++}$  and  $\text{CO}_2$ , was involved in the synthesis of citrulline, (a precursor of arginine) and ureidosuccinate, (a precursor of pyrimidines). Grisolia and Cohen (1953) showed that glutamate derivatives served a catalytic role. Furthermore, "compound X" was identified as carbamyl phosphate which was synthesized by carbamyl phosphokinase via the reversible reaction:



(Jones and Lipmann, 1960; Jones et al., 1955).

For a long time this multifunctional capacity of carbamyl phosphate to serve as a common precursor in the biosynthesis of arginine and pyrimidines and in the production of ATP, led to some confusion as to the identity of the enzyme responsible for the synthesis of carbamyl phosphate for each of these functions. Moreover, in vitro studies indicated that glutamine

could serve as the amino donor (Levenberg, 1962; Pierard and Wiame, 1964), and that carbamyl phosphate synthetase, an enzyme discovered in frog liver by Marshall et al. (1958), and carbamyl phosphokinase from bacteria possessed a reversible acetyl-(formyl-)phosphokinase activity (Raijman and Grisolia, 1964; Grisolia and Harmon, 1962). This plurality of functions led Thorne and Jones (1963) to a comparative study on carbamyl phosphokinase and acetyl phosphokinase in S. faecalis and E. coli. They were not able to separate these activities in E. coli, but in S. faecalis they separated the acetyl phosphokinase activity into two fractions, one of which had no carbamyl phosphokinase activity. Because of the low affinity for acetate of the fraction with carbamyl phosphokinase activity, they concluded that the formation of acetyl phosphate via this enzyme was of little biological importance in vivo. In addition, they found that the carbamyl phosphokinase activity of S. faecalis was inducible by arginine and that the equilibrium of the reaction favored ATP formation. In recent years this confusion has been cleared up in studies with E. coli. It was pointed out that carbamyl phosphate was utilized both in the synthesis of arginine and pyrimidines; indeed, the

isolation of one-step mutants simultaneously requiring arginine and uracil indicated that both biosynthetic chains share a common enzymatic activity (Kanazir et al., 1959; Beckwith et al., 1962). It was suggested that carbamyl phosphate was formed by the phosphorylation of carbamate which was produced non-enzymatically from ammonium carbonate (Jones et al., 1955; Thorne and Jones, 1963; Beckwith et al., 1962). This inconsistency was shown to be due to the presence of two carbamate phosphorylating systems separable by protamine fractionation. Fraction II, which contained acetyl phosphokinase or acetokinase, (as it was called by these workers) was found to be relatively stable at pH 5.6, to have an affinity for acetate and carbamate and to be active in extracts of all the strains studied (Yasphe and Gorini, 1965). Carbamyl phosphokinase (Fraction I) was found to be relatively labile at pH 5.6, to have an affinity for carbamate but not for acetate and to be absent in the double auxotrophs, thus corresponding to the results obtained genetically. Similar genetic results were obtained by Pierard and Wiame (1964) except that, instead of ammonia-utilizing carbamyl phosphokinase, these workers identified the enzyme as a glutamine-dependent carbamyl phosphate

synthetase. This glutamine utilizing enzyme was first identified by Levenberg (1962) in the mushroom system. Furthermore, by utilizing antibodies to acetokinase, Brzozowski and Kalman (1966) were able to show that all the ammonia-dependent carbamyl phosphate synthetase activity (or carbamyl phosphokinase activity) observed in the double auxotrophs was due to acetokinase.

The final solution came with the findings of Kalman et al. (1965) who demonstrated that carbamyl phosphokinase and glutamine carbamyl phosphate synthetase are the same enzyme. These workers demonstrated both activities in extracts of E. coli and showed co-purification, co-sedimentation, identical behaviour in electrophoresis and similar response to guanidine, formamide, thiol reagents and inhibitors, thus indicating beyond any doubt that both activities are functions of the same protein. Furthermore, analysis with the purified enzyme showed that glutamine as well as ammonia may serve as a nitrogen donor in carbamyl phosphate synthesis, but ammonia utilization is best under conditions of higher pH and concentration (Anderson and Meister, 1965; Kalman et al., 1966). The failure of Yasphe and Gorini (1965) to identify the glutamine-dependent carbamyl phosphate

synthetase was explained by these workers as being due to a preferential inactivation of the glutamine site during purification.

Similar effects have been observed in other glutamine utilizing systems (Moyed and Magasanik, 1957; Long and Pardee, 1967). The presence of a glutamine-dependent carbamyl phosphate synthetase as the true system in vivo was confirmed by Levenberg (1962) in the mushroom and by Lacroute et al. (1965) in yeast, where there is virtually no acetokinase activity. Similar results were obtained in B. subtilis (Reissig et al., 1967).

What might seem contrary to the idea of the presence of one carbamyl phosphate synthetase present in E. coli was the isolation of certain mutants which were independent of arginine when grown in the absence of uracil, but which were dependent on arginine (a strict requirement) when uracil was present in the medium (Novick and Maas, 1961; Gorini and Kalman, 1963). These data suggest either of two hypotheses:

- 1) There are two enzymes, one specific for the arginine pathway, and another for the pyrimidine pathway. The mutants lack the former and thus become dependent on arginine in the presence of excess uracil due to FI and

repression of the latter. Indeed, such is the case in yeast (Lacroute et al., 1965). This hypothesis is in fact excluded by the finding mentioned above, that a single mutation results in double auxotrophy.

- 2) There is but one enzyme which in the mutant becomes extremely sensitive either to FI or repression or both. Thus in the presence of excess uracil a requirement for arginine develops.

All the authors agree that the first hypothesis is excluded; there is but one CPSase. On the other hand Gorini and Kalman (1963), Yasphe and Gorini (1965) attributed the requirement for arginine as being due to repression by uracil, whereas Pierard et al. (1965) attributed the phenomenon to the enhancement of FI by uracil or CTP; their conclusion was based on the fact that in this mutant repression was found to be cumulative, dependent on both arginine and uracil. The conclusion is further supported by the fact that the mutation causing hypersensitivity is located within the locus responsible for double auxotrophy.

It now becomes obvious that much of the problem stemmed from the identification of an ammonia dependent carbamyl phosphokinase activity in which the glutamine

dependent activity had been eliminated by the method of purification (Yasphe and Gorini, 1965; Kalman et al., 1965). Similar instability of glutamine dependent carbamyl phosphate synthetase has been observed in the soluble fraction of ascites or fetal rat liver cells (Jones and Hager, 1966), and in Neurospora where it was shown to be cold sensitive (Williams and Davis, 1968). Also, the fact that acetokinase\* (the preferred nomenclature for acetyl phosphokinase) was able to synthesize carbamyl phosphate in vitro contributed to the confusion. Thus it would appear that the normal physiological mechanism for carbamyl phosphate synthesis in E. coli is via a glutamine utilizing carbamyl phosphate synthetase.\*\*

\* Acetate kinase (ATP: acetate phosphotransferase  
EC 2.7.2.1)

\*\* Carbamoylphosphate synthetase (ATP: carbamate  
phosphotransferase (dephosphorylating) EC 2.7.2.5)

(b) Comparative Distribution of CPSase

In E. coli there is only one glutamine-dependent carbamyl phosphate synthetase (Pierard and Wiame, 1964) which synthesizes CP for both the arginine and pyrimidine pathways and which is controlled by one genetic locus (Pierard et al., 1965). This is indicated by the genetic finding that a one-step mutation leads to the simultaneous auxotrophic requirement for both arginine and uracil and that reversion yields a wild-type prototype (Roepke et al., 1944). However, in Neurospora crassa there are two independent and specific carbamyl phosphate synthetases (Davis, 1963; Lou and Hermann, 1967), one specific for the pyrimidine and one for the arginine pathway. In this organism a one-step mutation leads to a single requirement for either uracil or arginine, but not for both. Thus, CP synthesized by the arginine-specific synthetase is not available for the synthesis of pyrimidines and vice versa. In other words, there is a strict channelling of the CP into the pathway for which it has been produced (Davis, 1967).

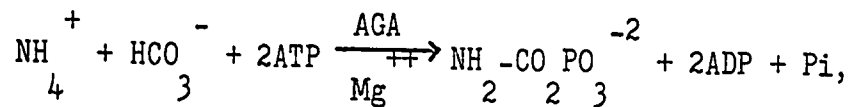
In yeast, Saccharomyces cerevisiae, a different situation exists. As in Neurospora, there are two carbamyl phosphate synthetases, one specific for arginine and the

other for pyrimidine. However, the activities are not completely independent since the CP synthesized by either enzyme is available for utilization in both pathways (Lacroute, 1964; Lacroute et al., 1965). This interesting observation was made by Lacroute in the following way:

1. Each CPSase is repressible by its own end product (arginine or uracil).
2. He discovered a class of mutations which were prototrophs in minimal medium, but which were auxotrophic for arginine in the presence of excess uracil in the growth medium. He discovered the reciprocal class which became auxotrophic for uracil in the presence of excess arginine.
3. His conclusion was that there were 2 CPSases, 1 specific for arginine and 1 for pyrimidines, but that sufficient overflow occurs to prevent the loss of either from causing an auxotrophic phenotype. When excess end-product is added and the activity of the corresponding CPSase is reduced to a minimum level, insufficient CP is produced for the heterologous chain and consequently auxotrophy is observed (Lacroute, 1966).

In the mitochondrial fraction of mammalian and amphibian liver (Marshall et al., 1958; Guthohrlein and Knappe, 1968), the reaction catalyzed by carbamyl

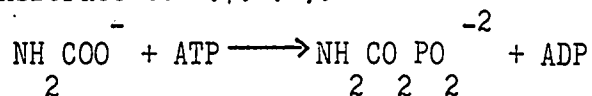
phosphate synthetase is:



an irreversible reaction where N-acetyl-l-glutamate (AGA) acts as an allosteric effector (Jones, 1963).

Recently a glutamine-dependent carbamyl phosphate synthetase has also been identified in the soluble supernatant of liver extracts. It is interesting to note that ATCase (aspartate transcarbamylase) was also found in this supernatant fraction whereas OTCase (ornithine transcarbamylase) was found largely in the mitochondrial fraction (Jones and Hager, 1966). In E. coli, the CPSase also preferentially utilizes glutamine but N-acetyl-l-glutamate has no effect (Kalman et al., 1966; Anderson and Meister, 1965).

In degradative pathways where arginine is used as a source of energy, the following reaction is catalyzed by the enzyme carbamate kinase: (ATP: carbamate phosphotransferase EC 2.7.2.2).



This reaction is reversible and requires magnesium ions.

(Marshall and Cohen, 1966); the equilibrium favors the synthesis of ATP.

This enzyme was inducible by arginine in S. faecalis and in lactic acid bacteria (Slade and Slamp, 1952; Thorne and Jones, 1963), and catabolically repressible in some Pseudomonas strains (Ramos et al., 1967).

(c) Properties of the CPSase Reaction

Table 1 summarizes some of the properties of CPSase in a variety of organisms. Generally, glutamine serves as the amino donor except in the mitochondrial enzymes from rat and frog liver where  $\text{NH}_4^+$  replaces glutamine and N-acetylglutamate is required for maximum activity. CPSase has also been identified in the soluble fraction of extracts from the gut tissue of the earthworm (Bishop and Campbell, 1963), and in the mouse spleen (Tatibana and Ito, 1967). The former resembles the liver enzyme in requirements except that  $\text{Mn}^{++}$  was found to be three times as effective as  $\text{Mg}^{++}$ . The latter was similar to the glutamine enzyme identified by Jones and Hager (1966) in liver extracts.

Table 1  
Properties of the CPSase Reaction from Various Sources

Organism	Purity	M.W.	pH optm.	ATP Mx10 <sup>3</sup>	K <sub>m</sub> values			NH <sub>4</sub> <sup>+</sup> Mx10 <sup>3</sup>
					Mg-ATP Mx10 <sup>3</sup>	HCO <sub>3</sub> <sup>-</sup> Mx10 <sup>3</sup>	Glun. Mx10 <sup>4</sup>	
1. <u>E. coli</u> B	H(450x)	7x10 <sup>5</sup>	8.5	C	C	C	6.0	120
2. <u>E. coli</u> B	300x	(13.6S)		8.0		1.2	3.8	
3. <u>S. typhimurium</u>	320x		7.6	3.2			2.6	
4. <u>Agaricus bispora</u>	14x		7.6				8.0	
5. Alaska pea	45x		8.2	0.39	1.5		1.2	
6. Rat liver mitochondria	H	2.5x10 <sup>5</sup>	7.6		5.3			1.1
7. Frog liver	315	3.15x10 <sup>5</sup>		0.48				2.3
8. <u>Neurospora</u>	8x		7.5		3			0.54

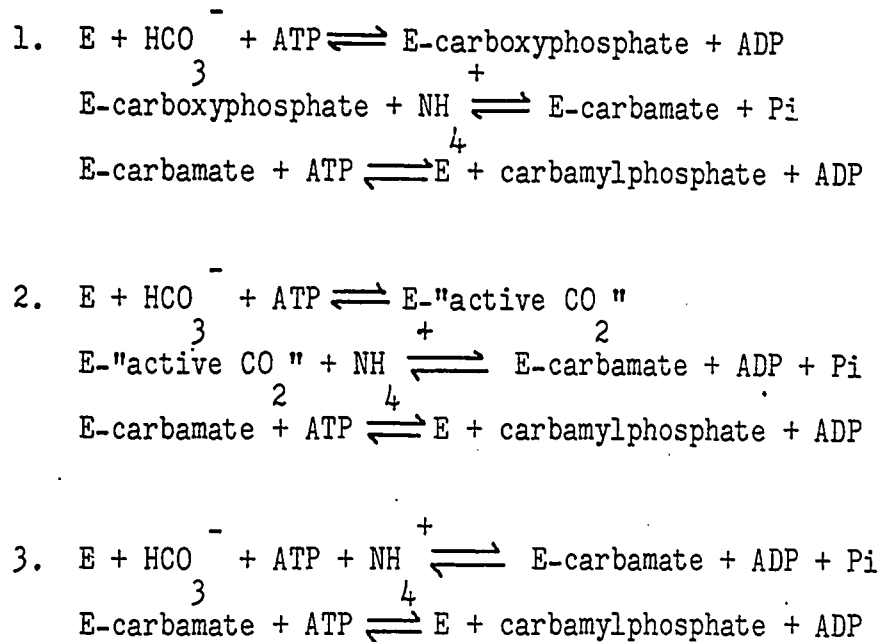
1. Kalman et al. (1966).
2. Anderson and Meister (1965), (1966).
3. Abd-El-Al and Ingraham (1969).
4. Levenberg (1962).
5. O'Neal and Naylor (1969).
6. Guthohrlein and Knappe (1968), (1969).
7. Marshall et al. (1961)
8. Lou and Hermann (1967).

C - curvilinear

H - homogeneous

(d) Mechanism of the CPSase Reaction

An essential in the study of the mechanism of an enzyme reaction is the availability of pure enzyme. The CPSase from E. coli and from liver mitochondria fulfil this requirement. The following possible mechanisms have been proposed from isotopic exchange studies (Metzenberg et al., 1958; Jones, 1965):



It is well known that biotin is involved in the fixation of CO<sub>2</sub> in several enzymatic systems (Mahler and Cordes, 1966). Recently Wellner et al. (1968) claimed that biotin was present in bacterial CPSase, suggesting

the possible formation of N-carboxy-biotin in the "CO<sup>2</sup> activities" step above. However, attempts by other workers to repeat this finding in the bacterial as well as the liver system established that CPSase contains no biotin (Peng and Jones, 1969; Huston and Cohen, 1969). Kinetic studies by Guthohrlein and Knappe (1969) of the variations in initial velocities as function of HCO<sup>-</sup><sub>3</sub> and NH<sup>+</sup><sub>4</sub> concentrations permitted them to exclude mechanism 1. Mechanism 2 was preferred over mechanism 3 because the former is bimolecular compared to the latter which is termolecular. An addition product of the terminal phosphate group with bicarbonate was suggested as a possible intermediate.

To date, the data are limited; thus an accepted mechanism for the synthesis of carbamyl phosphate by carbamyl phosphate synthetase awaits further experimentation.

### 3. Aspartate Transcarbamylase (ATCase)

#### (a) Introduction

Aspartate transcarbamylase (carbamoyl phosphate-L-aspartate carbamoyl transferase, EC2.1.3.2) catalyzes the formation of carbamyl aspartate from carbamyl phosphate and aspartic acid. This enzyme was demonstrated in E. coli (Reichard and Hanshoff, 1956; Yates and Pardee, 1956) and in higher organisms (Lowenstein and Cohen, 1954) and in a variety of other sources (Neuman and Jones, 1964; Bethel and Jones, 1969). In E. coli, ATCase may be considered as the first enzyme leading to the synthesis of pyrimidines since, as has been shown above, this organism possesses only one carbamyl phosphate synthetase for the biosynthesis of arginine and pyrimidines. In contrast, the existence of a pyrimidine-specific carbamyl phosphate synthetase in yeast and Neurospora indicates that this is the first enzyme of pyrimidine biogenesis. E. coli ATCase has been purified and crystallized (Sheperdson and Pardee, 1960) and is now available in gram quantities (Gerhart and Holoubek, 1967). Because of the availability and the importance of this enzyme in regulation, extensive studies have been carried out in various laboratories on its

properties, structure, catalytic and regulatory mechanisms. An extensive review of the earlier work has been published (Stadtman, 1966).

(b) Aspartate Transcarbamylase of E. coli

The enzyme was shown to catalyze the condensation of L-aspartate (apparent  $K_m$  65mM) and carbamyl phosphate (apparent  $K_m$  0.45mM) to form ureidosuccinate by a mechanism typical of transferase reactions involving sulfhydryl groups (Reichard and Hanshoff, 1956). The utilization of carbamoyl phosphate is inhibited competitively by certain anions, especially pyrophosphate (Kleppe, 1965 ; Kleppe, 1966), whereas aspartate utilization is inhibited competitively by certain divalent cations, e.g.  $Mg^{++}$  and  $Mn^{++}$ . These apparently function by binding the aspartate, thus giving an apparent competitive inhibition (Kleppe and Spaeren, 1966). Moreover, the activity is inhibited pseudo-competitively by CTP, an end-product of the biosynthetic sequence, and stimulated by ATP (Yates and Pardee, 1956; Gerhart and Pardee, 1962). ATCase exhibits very complex kinetics. The saturation curve for aspartate is sigmoidal, indicating cooperative effects similar to those observed in the binding of

oxygen to haemoglobin, where the binding of one molecule of oxygen facilitates the binding of the next (Morgan and Chichester, 1935; Wyman, 1948). Such interaction between like ligands is termed homotropic (Monod et al., 1965). At higher pH values (contrary to the Bohr effect in haemoglobin), or in the presence of the inhibitor CTP, the saturation curve for aspartate becomes even more sigmoidal; however, the presence of the activator ATP during assay normalizes the kinetics to the Michaelis-Menten type. The activation by ATP (or inhibition by CTP) was shown to be associated with an apparent increase (or decrease) in the affinity for aspartate (Gerhart and Pardee, 1962). Such 'interaction' between unlike ligands is termed heterotropic (Wyman, 1963). Recently it has been shown that the saturation curve of this enzyme for carbamyl phosphate is also sigmoidal, indicating that the carbamyl phosphate sites also show homotropic 'interactions' (Bethel et al., 1968).

That CTP, the competitive inhibitor, binds at a site completely different from the catalytic site has been established. This is indicated by studies where the inhibition by CTP was abolished without affecting the catalytic activity (in fact the activity was found to

increase two-fold in some cases). Such studies include proteolytic digestion (McClintock and Markus, 1968), heating at 60°C for 4 minutes, and treating the native enzyme with 0.8M urea, mercurials (Gerhart and Pardee, 1962) or with x-rays (Kleppe et al., 1966; Kleppe and Spaeren, 1967). Some discrepancy with respect to whether or not some of these treatments completely abolish inhibition by CTP has been indicated (Weitzman and Wilson, 1966). Most convincing however, is the actual separation of the catalytically-active fraction (catalytic subunits) from a fraction with the binding site for CTP (regulatory subunits), by treatment with p-chloromercuribenzoate (PCMB). When the two fractions were recombined in the presence of  $\beta$ -mercaptoethanol after removal of PCMB, reaggregation to the native enzyme resulted, as indicated by molecular weight determination and restoration of the feedback inhibition site of CTP (Gerhart, 1964; Gerhart and Schachman, 1965). Since ATP antagonizes the inhibition by CTP in a typical competitive fashion, and desensitization of the native enzyme to CTP leads to desensitization to stimulation by ATP, the indication is that ATP binds at the same or overlapping site as CTP.

Recently a method for the preparation of large

amounts of isolated catalytic subunits has been described (Gerhart and Holoubek, 1967). In this regard, it must also be pointed out that the kinetics of the catalytic subunit are completely different from those of the native enzyme. The catalytic subunit shows Michaelis-Menten kinetics with aspartate and the pH optimum is 8.5, compared to 7.0 for the native enzyme (Gerhart and Pardee, 1963, 1964). Weitzman and Wilson (1966) also identified another peak at pH 10.2 for the native enzyme.

(c) Catalytic Mechanism of Ureidosuccinate Formation

Porter et al. (1969) carried out a detailed study of the mechanism of synthesis of ureidosuccinate using isolated catalytic subunits. They found by steady state kinetics that the reaction is ordered: carbamyl phosphate binds before aspartate and ureidosuccinate dissociates before the inorganic phosphate. By using the techniques of nuclear magnetic resonance (Schmidt et al., 1969) and UV difference spectroscopy (Collins and Stark, 1969) to study the dissociation of carbamyl phosphate, L-aspartate and their analogues, a detailed mechanism based on general acid-base catalysis was worked out. The working hypo-

thesis suggested is that the isolated subunit in its natural form assumes a configuration with some steric hindrance to the binding of aspartate. However, carbamyl phosphate on binding removes this hindrance by a shift in conformation which results from the binding of the phosphate dianion and the carbonyl group to specific sites (general acid site). L-aspartate on binding causes a large change in conformation which forces the amino group of L-aspartate and the carbonyl group of carbamyl phosphate together. The energy for this conformational change is obtained from electrostatic binding energy. During this transition a positive charge is developed as a result of proton abstraction by a general base. Cleavage of the bond between the carbonyl carbon and inorganic phosphate of carbamyl phosphate results in the formation of ureidosuccinate and inorganic phosphate. These substances are released sequentially as the subunit regains its original configuration. Support for certain aspects of Stark's mechanism has been indicated in the studies of Gerhart and Schachman (1968), and Changeux et al. (1968).

(d) Allostery in Terms of Quaternary Structure

It may now be asked: how can an inhibitory substance (CTP), chemically unlike the substrates (carbamyl phosphate and aspartate) of the enzyme (ATCase) and binding at a site completely separate from the catalytic site, cause apparent competitive inhibition? An explanation was offered by Monod et al. (1963) that binding of the inhibitor at an allosteric site causes a conformational change to a state of decreased affinity for the substrates. The enzyme is said to have undergone an allosteric transition; thus, the homotropic and heterotropic effects are viewed as allosteric effects. Indeed, many of the characteristics of ATCase have also been observed in other regulatory enzymes (Stadtman, 1966). These characteristics include homotropic effects in the binding of a substrate or substrates, heterotropic effects in the binding of the inhibitor at a specific site and the physical and/or genetic demonstration that the enzyme contains subunits.

Two general models relating to the subunit structure of protein with its allosteric and cooperative properties have been proposed. The model of Monod et al. (1965) was designed primarily to explain the cooperative

effects in the binding of like ligands (homotropic effects) and based on the example of haemoglobin where it was shown that saturation with oxygen is accompanied by rearrangement of its 4 subunits (Muirhead and Perutz, 1963). This model in essence assumes that: (1) allosteric proteins are oligomers consisting of two or more identical subunits (i.e. protomers) which are arranged with an internal plane of symmetry; (2) each protomer may exist in at least two conformational states (R and T) which are in dynamic equilibrium with each other; (3) each protomer has a binding site for the substrate and another for the allosteric effector, but in a given conformational state, all the protomers have equal affinity for a specific ligand; (4) transition of one protomer by ligand binding results in equal conformational change of the other protomers which occur in such a manner that symmetry is conserved. Homotropic and heterotropic effects were explained in that the R state preferentially binds substrate whereas the enzyme exists primarily in the T state which binds the inhibitor preferentially. Thus, the substrate saturation curve will be sigmoidal since addition of small amounts of substrate will first shift the equilibrium toward the

R state, which has a greater affinity for the substrate.

The second model is the "induced fit" model of enzyme catalysis, which is extended to include enzymes containing interacting subunits. This model was proposed by Koshland (1958) and is based upon "the flexible interaction between ligand and protein which may induce a new conformation of the subunit. This deformation may, in turn, affect the shape and stability of neighboring subunits, the extent of the change being a consequence of the nature of the interaction between the subunits and the amount of distortion induced by the ligand. If the conformation change in one subunit is very loosely coupled to the conformation of the neighboring subunits, the change may not affect its neighbours and Michaelis-Menten kinetics will be observed. If there are closely coupled subunit interactions, a change induced in one subunit will cause partial or equal changes in the neighboring subunits and, in general, the changes will occur sequentially with ligand addition, i.e., hybrid conformational states will be observed" (Koshland and Neet, 1968).

Indeed, other models capable of explaining homotropic effects have been proposed (Atkinson, 1966; Sweeny and Fisher, 1968). However, ATCase will be dis-

cussed only in relation to the two models outlined above.

From the explanations above it can be seen that the two hypotheses are not entirely mutually exclusive; e.g., both predict that enzymes showing homotropic and heterotropic effects must contain subunits and that these effects are explained as a result of subunit interaction. The model of Monod et al. (1965) postulates an axis of symmetry, hence this model would not apply to an enzyme with an odd number of subunits. To date, no such regulatory enzyme has been discovered. This model also predicts a 'concerted' mechanism for the subunit interaction, thus a small amount of substrate would suffice for a complete shift in conformation. On the other hand, the 'induced fit' model of Koshland predicts a 'sequential' mechanism; thus a larger amount of substrate would be required for complete conformational change ( $T \rightleftharpoons R$ ). Experiments in an attempt to select between the two models will be treated later on.

(e) Structural Model and Regulatory Mechanism

Gerhart and Pardee (1964), as a result of the observed kinetic properties of the native enzyme and the fact that heat caused disaggregation into 4 one-quarter

molecules, viewed the native enzyme as a tetramer consisting of four monomers. They also suggested that as a result of the interaction between the monomers, the enzyme in its native form has a low affinity for aspartate; however, binding of one molecule of aspartate facilitates the binding of the next, etc. (thus, homotropic effects as in haemoglobin). CTP by its binding at an allosteric site, serves to strengthen the interaction of the subunits and thus lessen the affinity for aspartate (i.e. heterotropic effects). ATP, by its binding at the same or overlapping site as CTP, tends to weaken this interaction, resulting in activation of the catalytic activity. Proof of interaction came with the observation that maleate acts as an activator at low concentrations or as a competitive inhibitor at higher concentrations in the presence of a small amount of aspartate.

Indeed, homotropic or cooperative effects in the actual binding of succinate, an aspartate analogue, and heterotropic effects in the antagonism by succinate of CTP binding have been demonstrated by equilibrium dialysis techniques (Changeux et al., 1968). These workers also identified four binding sites for succinate and four binding sites for the inhibitor CTP or analog,

BrCTP, per molecule of native enzyme.

Physical measurements of molecular weights indicate 310,000 for the native enzyme (11.7S), 96,000 for the catalytic subunit (5.8S) and 30,000 for the regulatory subunit (2.8S) (Gerhart and Schachman, 1965). These data, by calculation, culminated in a structural model for the native enzyme which consists of two catalytic subunits and four regulatory subunits instead of the model consisting of four monomers (Gerhart and Pardee, 1964).

However, binding studies showed the presence of two binding sites for succinate per catalytic subunit and one for CTP per regulatory subunit. Furthermore, Herve and Stark (1967) by amino-terminal analyses, found the catalytic subunits to consist of 1.92 moles of alanine, and the regulatory subunits 0.93 moles of threonine. Thus, the native enzyme would, by deduction, consist of four catalytic and four regulatory chains. A binding site was assigned to each chain (Herve and Stark, 1967).

Changeux et al. (1968), because of the tetrameric binding characteristics of the native enzyme, adapted the principles of Monod et al. (1965), and postulated a more refined structural model for ATCase. The enzyme is viewed as an isologous tetramer, with each of the pro-

tomers being composed of one-half catalytic subunit and one regulatory subunit. In other words, each protomer consisted of one binding site for succinate and one for CTP. They also pointed out that the observed heterotropic and homotropic effects are indirect since the binding sites are located on different subunits and therefore must depend on the conformational change of the protein itself.

Recently, however, Weber (1968) studied the primary structures of the regulatory (R) and catalytic (C) polypeptides. He found that the molecular weight of the polypeptide chains, as estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulphate solution, was 33,000 for the C chain and 17,000 for the R chain. He was able to sequence the amino acid of the R chain; a molecular weight of 17,000 for the R chain was also confirmed by summation of its known amino acid constituents. He further concluded that the tetrameric model of Changeux et al. (1968) must be rejected for a model consisting of  $C_6R_6$  for ATCase.

Further support for Weber's model came from studies of the water content, and density of two crystalline forms of ATCase. Wiley and Lipscomb (1968) found that the

trigonal crystal form (obtained in the presence of CTP and pH 5.9) exhibits a three-fold axis of symmetry indicating that the number of identical subunits must be multiples of three. The tetragonal crystal form (obtained by ammonium sulphate crystallization technique) showed a two-fold axis of symmetry. Thus, it was concluded that the ATCase molecule must contain at least six copies of each polypeptide chain, which again is incompatible with the tetrameric model as proposed by Changeux et al. (1968), but consistent with that of Weber (1968).

(f) Proof of Conformational Change

It is known that sulfhydryl bonds are involved in the formation of tertiary and quaternary structure of proteins (Berhard, 1968). Gerhart and Schachman (1968) by spectrophotometric titration with p-hydroxymercuribenzoate (PMB) identified a total of  $27 \pm 1$  sulfhydryl groups for the intact ATCase enzyme. These workers utilized the reaction rate of these sulfhydryl groups with PMB as an estimate of the conformational change occurring in the presence of various ligands. They found that the reaction rate which is of the pseudo-first order

type increased six-fold in the presence of succinate and carbamyl phosphate. Either of the two substrates by itself or CTP alone had very little effect, but the increased reaction rate in the presence of the two substrates was partially antagonized by CTP. Similar conclusions were obtained with studies with the ultracentrifuge where a decrease in the sedimentation coefficient of 3.6-5% was observed in the presence of both carbamyl phosphate and the substrate analogues, maleate or succinate (Gerhart and Pardee, 1963; Gerhart and Schachman, 1968).

More evidence for conformational change came with immunological techniques, proteolytic studies and optical rotation measurements. Bethel et al. (1968) studied the dissociation caused by PMB by using antibodies to the native enzyme. The subunit did not react with the antibodies. They also showed that the effect of carbamyl phosphate or succinate was to increase dissociation in the presence of PMB and that the maximal effect of each ligand was additive. The allosteric effectors and pyrophosphate were found to stabilize the enzyme. By using antibodies to the isolated regulatory and catalytic subunits, it was also shown that the greatest confor-

mational change of the isolated subunits occurred in the presence of their specific ligand. McClintock and Markus (1968) studied the rate of digestion of the native enzyme with several proteolytic enzymes. They found that the rate increased in the presence of aspartate in a sigmoidal fashion similar to the substrate saturation curve; however, the substrate analogue, succinate, had very little effect; the allosteric effectors, ATP and CTP, resulted in a decrease relative to control. Conformational changes were also indicated by changes in optical rotation in the binding of succinate in the presence of carbamyl phosphate (Dratz and Calvin, 1966). No optical change was observed in the presence of succinate or CP alone, indicating that CP is required for the binding of succinate.

Therefore, it can be concluded from these studies that the effect of substrate is to 'loosen' the enzyme structure, thus exposing more sulfhydryl groups and making it more susceptible to dissociation by PMB or tryptic digestion, and decreasing the sedimentation coefficient of the enzyme. On the other hand, the ATP and CTP effectors have the opposite effect. These studies do not explain the fact that ATP caused stimulation of the

catalytic activity.

(g) ATCase in Terms of the Two Models

By use of the data in the first two papers of the series, Changeux and Rubin (1968) argued in favor of the "two-state" model as postulated by Monod et al. (1965). As suggested by the model, Changeux and Rubin showed by binding studies that the native enzyme exists largely in one (T) of the two states (in a ratio of 4T:1R), which preferentially binds the inhibitor and not the substrate. A comparative study of the state function (as revealed through PMB reaction rates with sulfhydryl groups and changes in sedimentation coefficient) with the saturation function showed that conversion of the enzyme into the substrate binding (R) state requires only a fraction of the binding sites to be filled. This, therefore, indicated that a concerted mechanism is involved in the transition, contrary to the induced fit model where a sequential mechanism is predicted. Moreover, the R state was found to bind substrate exclusively with an affinity a thousand-fold greater than its affinity in the T state (Changeux and Rubin, 1968).

However, McClintock and Markus (1968, 1969) pointed

out that the effect of aspartate on the digestibility of native ATCase is contrary to the model of Monod et al. (1965). This model suggests that binding of small amounts of aspartate should stabilize the enzyme in one conformation (R state), i.e. via a concerted mechanism. But the fact that there was direct relationship between aspartate concentration (saturation function) and digestibility of native ATCase (state function) suggests a sequential mechanism thus confirming a prediction of the 'induced fit' model.

The question is: can we choose between the two models? The dissociation constant studies of Collins and Stark (1969) indicated that aspartate and succinate bind poorly in the absence of carbamyl phosphate; however, in its presence succinate readily binds but aspartate still binds relatively poorly as shown by its high dissociation constant. Changeux and co-workers <sup>(reference?)</sup> studied the effects of succinate binding in the presence of carbamyl phosphate on conformational changes and the results confirmed a concerted mechanism of interaction upon the binding of succinate. However, succinate is not the true substrate; thus, whether or not the same mechanism occurred in the presence of aspartate remains to be shown. Now,

how does one account for the experiments of McClintock and Markus (1968, 1969) which support a sequential mechanism of interaction? These data can be explained on the assumption that the R state is more susceptible to tryptic digestion and that the increased aspartate concentration results in increasing the amount of enzyme in the R state: therefore, a homology between the state and saturation functions is to be expected if such assumptions are true.

Thus, the answer to the question asked above is that a definite choice between the two models is at this time premature.

4. Regulatory Control Mechanisms

(a) Historical Background and Definitions

A most remarkable feature of living organisms is their ability to regulate and control cellular metabolism so that a constant condition (homeostasis) is maintained between the various catabolic and anabolic processes. One possible basis of such mechanisms was recognized when it was observed that labelled amino acids, when added to the growth medium of E. coli, were preferentially utilized to amino acids, synthesized de novo from glucose. Indeed, the incorporation was found to be almost immediate (Roberts et al., 1955). However, the mechanisms involved in this self-regulation became clear only after the demonstration that the end product of a biosynthetic pathway inhibits the activity (feedback inhibition) of one of the early enzymes, usually the first, (Umbarger, 1956; Yates and Pardee, 1956b) and leads to a reduction of the biosynthesis of the enzymes (repression) involved in the synthesis of the end-product (Monod and Cohen-Bazire, 1953; Cohn et al., 1953; Gorini and Maas, 1957; Vogel, 1957). These mechanisms are usually separate and distinct with respect to their physiology and control

and are usually involved in the regulation of a single biosynthetic sequence in microorganisms (Maas and McFall, 1964).

What may have been a case of feedback inhibition, in fact was demonstrated as early as 1940 by Dische, who showed that the phosphorylation of glucose in erythrocyte hemolysates could be prevented by the addition of phosphoglycerate (Dische, 1940, as cited by Umbarger, 1964). Unfortunately, the regulatory significance of Dische's findings was not appreciated at that time. Umbarger is usually regarded as the discoverer of feedback inhibition; he showed that the end product, isoleucine, inhibits specifically the first enzyme unique to its synthesis (threonine deaminase) and pointed out the advantages of such a regulatory mechanism at the molecular level. Simultaneously the same type of regulation was shown to exist in pyrimidine biosynthesis in the case of CTP inhibition of ATCase (Yates and Pardee, 1956b).

This work served to ignite the fuse for the explosion of research in the field of regulatory control mechanisms. As a result a greater understanding of how the cell maintains homeostasis has been achieved. It is impossible at this time to discuss the research which led to the classification of various regulatory mechanisms

into types and subtypes; however, excellent review articles have appeared: Maas and McFall (1964), Umbarger (1964), Atkinson (1965), Stadtman (1966), Atkinson (1966), Cohen (1968), Datta (1969).

Thus, only those mechanisms which are known to be involved in pyrimidine biosynthesis will be discussed here.

#### Definitions

Feedback inhibition: a decrease in the activity of a regulatory enzyme (usually the first enzyme of a biosynthetic sequence) effected when the end product binds at a regulatory site which, at least in all cases so far reported, is distinct and separate from the catalytic site.

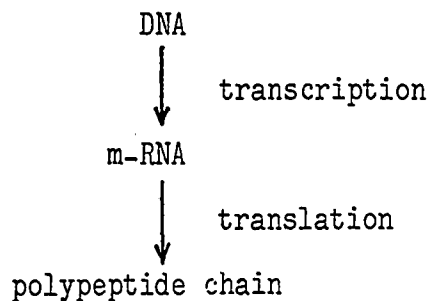
Repression: "a decrease, resulting from the presence in cells of a given substance (repressor), in the rate of synthesis of a particular enzyme or group of metabolically related enzymes." The opposite effect is termed derepression (Maas and McFall, 1964).

Induction: "a relative increase, resulting from the exposure of cells to a given substance, in the rate of synthesis of a particular apoenzyme" (Maas and McFall, 1964).

Operon: "consists of a cluster of genes. These genes have related functions and are regulated together" (Ames and Martin, 1964).

(b) Protein Synthesis

It is known that genes control the structure of proteins by a sequence of steps which is often referred to as the dogma of molecular biology. Encoded in the nucleotide sequence of the gene is the information dictating the primary structure of protein. This information is deciphered in the following manner:



Here the nucleotide sequence in the DNA is transcribed to give a messenger-RNA which is subsequently translated yielding the completed polypeptide chain. After completion and release, the polypeptide chain assumes a three dimensional structure determined by the amino acid

content and sequence of that protein (Epstein et al., 1963). The intricacies involved in the mechanisms of protein synthesis have been reviewed (Arnstein, 1965; Moldave, 1965; Schweet and Heintz, 1966).

(c) Regulation of Protein Synthesis with Respect to Repression and Induction

Various review articles have been published: Ames and Martin (1964), Horowitz and Metzenberg (1965), Brenner (1965), Vogel and Vogel (1967), Epstein and Beckwith (1968), Geiduschek and Haselhorn (1969). Thus the following describes some highlights which resulted in the early theory of the mechanism of regulation.

Genetic studies of the enzymes involved in various biosynthetic pathways e.g. arginine, tryptophane, histidine and inducible system e.g. lactose breakdown in E. coli revealed that the genes (structural genes) are closely linked and coordinately expressed as an 'operon' (Jacob et al., 1960). In the lactose system regulatory mutants were isolated where a single mutation led to a lack of, or alteration in repressibility. This condition was found to be recessive to wild type and the mutants were shown to map at a position distinct from the structural genes,

called a regulatory gene (i). These findings culminated in the concept that the i gene produces a "repressor" substance which controls the expression of the structural genes (Jacob and Monod, 1961).

How does the repressor substance act? In addition to the characteristics mentioned above, further studies with the lactose inducible systems led to the isolation of constitutive mutants. These mutants are identified by the fact that the inducible enzymes are produced in concentrations comparable to fully induced levels in the absence of the inducer. Genetic studies indicate cis dominance and trans recessiveness for such mutants and map them adjacent to the structural genes, in a region called the operator. This region is identified as the site of action of the "repressor" substance.

End-product repression as opposed to catabolic repression or glucose effect is viewed to occur in the following manner: The repressor (produced by the regulatory gene) per se, has no affinity for the operator of the structural gene, and thus the operon is in its activated state; however, on binding with the end product (co-repressor), the combined repressor acquires this affinity and thus on binding to the operator prevents

further synthesis of the m-RNA and thus synthesis of the corresponding polypeptide.

In inducible systems, the repressor has the opposite capacity, i.e. in its uncombined state it has affinity for the operator, but combined with the inducer (apo-enzyme) it has no affinity. This model was proposed by Jacob and Monod in 1961, who also speculatively viewed the repressor as a ribonucleic acid. However, good indirect evidence (Sadler and Novick, 1965) suggested that the repressor must be protein, and in recent years the lac repressor (Gilbert and Muller-Hill, 1966) and phage repressor (Ptashne, 1967a) have been isolated and shown to be protein and to bind specifically to DNA (Ptashne, 1967b). These results are in good agreement with the prediction of Jacob and Monod (1961) who suggested that the control regulation occurred at the level of transcription. To date, there is strong evidence for transcriptional control in certain systems; in others a translational control mechanism seemed to be prevalent (Cline and Bock, 1966). There is also the hypothesis that both transcription and translation might be simultaneously involved (Stent, 1964). Thus, the mechanism of regulation has been resolved in

a few cases but studies seem to indicate that the mechanism of regulation will differ depending on the system under study.

(d) Regulation of Pyrimidine Biosynthesis

Regulation of pyrimidine biosynthesis must be considered with respect to cell economy, precursor availability (since carbamyl phosphate is also utilized in arginine biosynthesis) and coupled utilization with purines in the synthesis of RNA and DNA.

Genetic studies of the enzymes involved in pyrimidine biosynthesis in E. coli (Fig.3 ) identified the four loci controlling the latter steps (three to six) as an operon since these were found to be closely linked and coordinately expressed under conditions of repression and derepression . The loci controlling CPSase (1) and ATCase (2) were found to be separate and non-coordinately controlled. It was also suggested that a common repressor was involved in regulating the synthesis of enzymes 2-6 (Beckwith et al., 1962). Taylor et al. (1964) showed that the gene controlling OMPppase (5) is not located within the postulated operon; in addition, similar results were obtained in S. marinosruba (Belser, 1961) and

Regulation of Pyrimidine Biosynthesis in E. coli

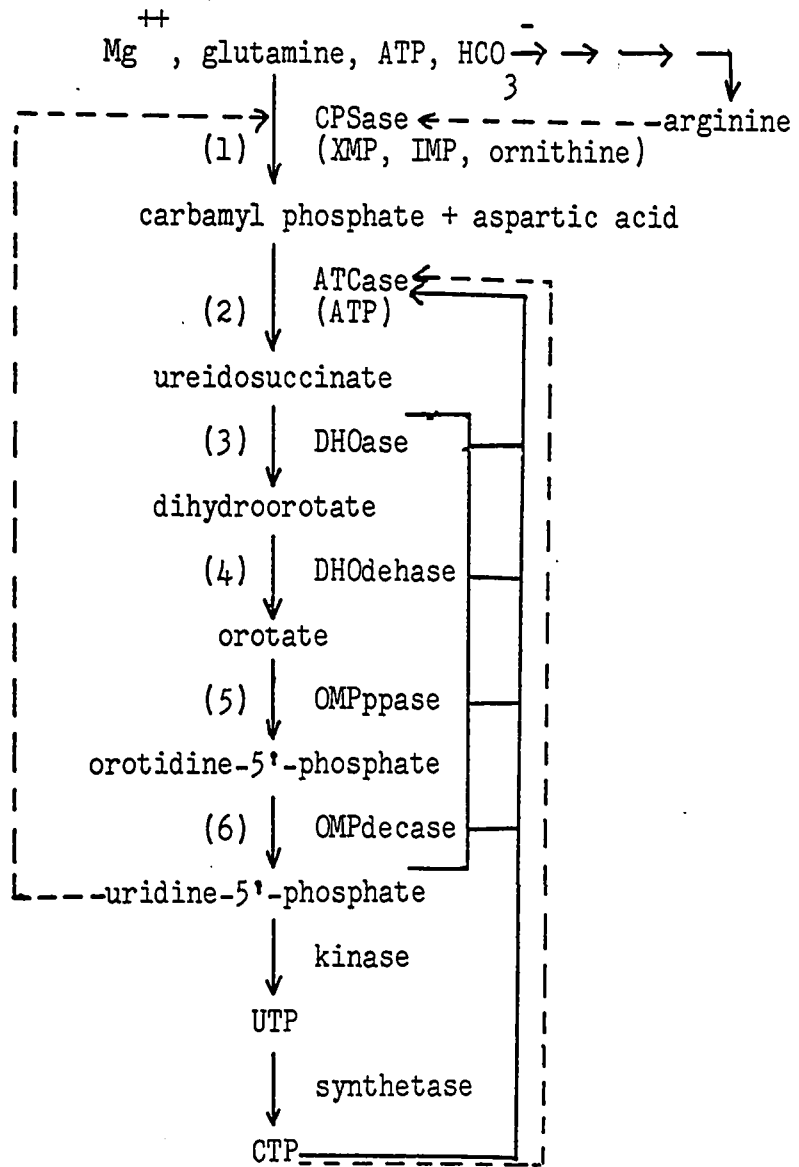


Figure 3 . Solid lines indicate repression where the enzymes in squared bracket are coordinately expressed; dashed lines show feedback inhibition; and the compounds listed in round bracket under the enzyme represent the activators of the enzyme. Abbreviations on page xii

S. marescens (Hayward and Belser, 1964). In order to preserve the operon concept, it was suggested by Hayward and Belser (1964) that OMPpase (4) might be a two protein enzyme, one of which is coded within the postulated operon.

CPSase (1), a common enzymatic activity to pyrimidine and arginine synthesis is cumulatively repressed by arginine and uracil (Pierard et al., 1965). ATCase (2) is inhibited by CTP and stimulated by ATP (Gerhart and Pardee, 1962), the latter indicating coupling with purine utilization. This coupling is strengthened by the fact that CPSase is stimulated by UMP and IMP (Anderson and Meister, 1966). UMP (95%) and arginine (5%) inhibited in an additive fashion the CPSase activity (Pierard et al., 1965); on the other hand, under conditions where nucleotide concentration is high, the availability of carbamyl phosphate for arginine biosynthesis is ensured by the fact that ornithine overcomes UMP inhibition of CPSase and also causes stimulation in the absence of UMP (Pierard, 1966; Anderson and Marvin, 1968).

In studies of other biosynthetic pathways, where a metabolite is common to more than one chain, other mechanisms to ensure availability have been encountered.

These include enzyme multiplicity where more than one enzyme synthesizes the common precursor, but each is independently regulated (Datta, 1969); and preferential channelling or confinement of a common metabolite into the pathway in which it has been produced (Davis, 1967). Such is the case in Neurospora where there are two independent and specific CPSases. However, in yeast where the carbamyl phosphate synthesized by either of the two CPSase enzymes is not completely compartmented, there is as yet no evidence for channelling.

## II. Introduction to the Problem

### 1. Regulation of Pyrimidine Biosynthesis in Yeast

Early studies of the pyrimidine biosynthetic pathway in baker's yeast, Saccharomyces cerevisiae, by mutant methodology, led Mortimer and Hawthorne (1966) to the identification of 4 loci ( $ur_1$ ,  $ur_2$ ,  $ur_3$ ,  $ur_4$ ) responsible for pyrimidine biosynthesis. Lacroute (1968) by genetic as well as biochemical studies was able to identify the enzyme corresponding to each locus. He found that the  $ur_1$  locus controls dihydroorotic dehydrogenase (DHO Dehase), the  $ur_2$  carbamyl phosphate synthetase (CPSase) and aspartate transcarbamylase (ATCase), the  $ur_3$  dihydroorotase (DHOase) and  $ur_4$  orotidine-5-phosphate decarboxylase (OMP Decase). The genetic loci controlling orotidylic pyrophosphatase (OMP ppase) and cytidine triphosphate synthetase (CTPase) still remain unidentified. Furthermore, Lacroute showed that the  $ur_2$  locus is controlled by end product repression and that the enzymes, ATCase and CPSase, are inhibited by one of the end products, UTP (Lacroute, 1964A; Lacroute et al., 1965). He also showed that the other enzymes of the biosynthetic pathway were not controlled by repression or feedback

inhibition, but rather by a sequential induction mechanism in which certain of the intermediary products were the inducers (Lacroute, 1964b; Lacroute, 1968). The regulation of the pyrimidine biosynthetic pathway in baker's yeast is summarized in Fig. 4 .

The evidence which led Lacroute to conclude that the  $ur_2$  gene controls both ATCase and CPSase is as follows: It was observed that single-step mutations which cause the loss of either ATCase activity or CPSase activity or which cause the simultaneous loss of both activities all map within the  $ur_2$  locus. It was further observed that mutations which disinhibited ATCase to UTP also resulted in disinhibition of the CPSase (Lacroute and Slonimski, 1964); these mutants also map at the  $ur_2$  locus. These results led Lacroute to speculate that these two enzyme activities might be associated in a single complex. He also suggested that there are three possibilities compatible with the above genetic data: "(i) There is one operon corresponding to three polypeptide chains, one for ATCase, one for CPSase, and one for the UTP site. (ii) There is one operon corresponding to two polypeptide chains, one for both ATCase and CPSase activity and one for the UTP site. (iii) There is only one polypeptide

Regulation of Pyrimidine Biosynthesis in Yeast

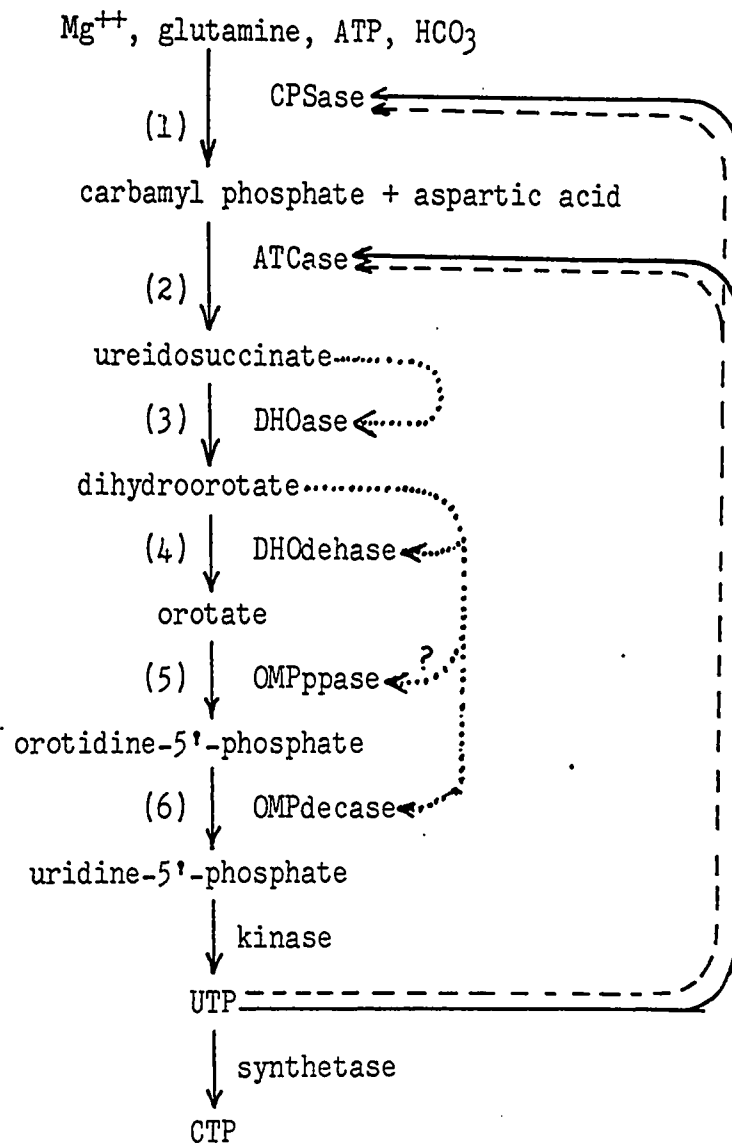


Fig. 4 . Solid lines indicate repression; dotted lines, induction; and dashed lines, feedback inhibition.

chain, bearing ATCase and CPSase activities and the UTP site." (Lacroute, 1968).

Kaplan and Messmer (1969) presented a fourth hypothesis, that the  $ur_2$  gene codes for but one peptide chain which as a single subunit possesses only ATCase activity not subject to feedback inhibition; upon aggregation of the subunits, the CPSase and regulatory sites would be formed or become active.

Interallelic (intragenic) complementation studies (Lacroute, 1966) and the combined effects of temperature and dilution on ATCase (Kaplan and Messmer, 1969) have suggested that this enzyme has a subunit structure. Furthermore, it has been shown that feedback inhibition of ATCase is rapidly lost during heating of semi-purified preparations at 50 C (Kaplan et al., 1967) and that the feedback site of this enzyme is stabilized during extraction from the cell, and possibly within the cell, by UTP (Kaplan et al., 1969).

Burns (1966), by using a yeast mutant deficient for uracil, adenine and histidine, was able to show by physiological studies that the regulation of pyrimidine biosynthesis is strongly coupled to the purine system. Furthermore, Wong and Burns (1969) found that ATP at

$2 \times 10^{-2}$  M caused 30% inhibition of the ATCase activity of this mutant; however, this inhibition could be reversed by the addition of equimolar concentrations of glutamine and bicarbonate. They concluded that these results are in agreement with the speculation that both ATCase and CPSase are functions of the same enzymatic complex. Kaplan et al. (1967) on the other hand, found that ATP and GTP at  $10^{-2}$  M were largely without effect on the ATCase activity. However, ATP was able to prevent the inhibition by UTP.

## 2. Purpose of the present studies

My research had the following aims:

1. to test the hypothesis that the CPSase and ATCase activities together with the regulatory site are present in or associated with a single molecular complex;
2. to purify the ATCase and CPSase activities;
3. to obtain direct biochemical evidence of the existence of a subunit structure of such a complex;
4. to study the physical and enzymatic properties of the complex and its subunits;
5. to study the possible physiological significance of the enzyme aggregate;
6. to study the regulation of these enzymes; and
7. to propose a plausible model of its structure.

RESEARCH LABORATORY  
DEPARTMENT OF CHEMISTRY  
UNIVERSITY OF CALIFORNIA  
SAN DIEGO, CALIFORNIA 92161

B. MATERIALS

Medium and Vitamins

Yeast extracts, Bacto-peptone and agar were products of Difco Laboratories. Thiamine HCl, l-inositol, pyridoxine, nicotinic acid (niacin) and d-biotin were obtained from Nutritional Biochemicals Corp., and calcium pantothenate from J.T. Baker Co.

Chemicals

Reagent grade chemicals were used throughout these studies except where otherwise specified.

Ammonium hydroxide (NH OH), ammonium phosphate (NH H PO ), calcium chloride (CaCl ), citric acid (C H O .H O), diacetyl monoxime (CH C:NOHCOCH ), ferric chloride (FeCl ), magnesium sulphate (MgSO .7H O), manganese sulphate (MnSO .H O), potassium chloride (KCl), potassium hydroxide (KOH), potassium persulphate (K S O ), sodium chloride (NaCl), sodium citrate (Na C H O .7H O), sodium hydroxide (NaOH), sodium molybdate (NaMoO .2H O), zinc sulphate (ZnSO .7H O) were purchased from Fisher Scientific Company. Boric acid (H BO ) and potassium pyro-

RESEARCH LABORATORY  
STATIONERY AND SUPPLIES  
GENERAL CHEMICALS DIVISION

phosphate ( $K_4P_2O_7$ ) were obtained from British Drug Houses. Potassium iodide (KI) was obtained from J.T. Baker and copper sulphate ( $CuSO_4$ ) from Merck, U.S.A.. Ammonium sulphate (enzyme grade) was obtained from Nutritional Biochemicals Corp. (N.B. C.) and p-diphenylaminesulfonic acid sodium salt ( $C_6H_5NHC_6H_4-SO_3Na$ ) from Eastman Organic Chemicals.

#### Nucleotides

Adenosine monophosphate (AMP), ADP, ATP, cytidine monophosphate (CMP), CDP, CTP, guanosine monophosphate (GMP), GDP, GTP, inosine monophosphate (IMP), IDP, ITP, thymidine monophosphate (TMP), TDP, TTP, were purchased from Calbiochem. Uridine monophosphate (UMP), UDP, UTP, were obtained from Boehringer (Mannheim). Uridine-5-diphosphoglucose (UDPG), xanthosine monophosphate (XMP), XDP, XTP and cyclic-AMP were products of the Sigma Chemical Co.

#### Buffers

Potassium dihydrogen phosphate ( $K_2H_2PO_4$ ), potassium hydrogen phosphate ( $KH_2PO_4$ ), Tris (hydroxymethyl-)amino-methane (Trizma Base) were also products of Sigma.

### Standards

Carbamylaspartate acid and citrulline were from Calbiochem. Bovine serum albumin was obtained from Nutritional Biochemicals.

### Substrates

Carbamyl phosphate dilithium salt, L-aspartic acid, L-ornithine, L-glutamate, phosphoenol pyruvate, L-glutamine were products of Calbiochem. NAD, NADH were obtained from Sigma.  $\text{NaH}^{14}\text{CO}_3$  was purchased from Nuclear Chicago Corp.

### Marker Proteins and Enzymes

Yeast alcohol dehydrogenase and ribonuclease were purchased from Calbiochem. Thyroglobulin, catalase and glutamate dehydrogenase were from Sigma, whereas ovalbumin and pyruvate kinase were products of General Biochemicals. Lactate dehydrogenase was from Boehringer and blue dextran 2000 from Pharmacia Inc. A sample of lyophilized E. coli cells (strain #9001 from Servac Laboratories) was a gift from Dr. W.G. Martin of the National Research Council, Ottawa laboratories.

Miscellaneous Chemicals and Reagents

Glycerol, isopropanol, ethanol, methanol and acetone were purchased from Fisher Laboratories. Sucrose, ethylenediaminetetracetic acid (EDTA) and Norite were also from Fisher. Mercaptoethanol, N-acetyl-ornithine and protamine sulphate were from Calbiochem. N-acetyl-glutamic acid was from Sigma. Concentrated sulfuric acid ( $H_2SO_4$ ) and hydrochloric acid were obtained from Mallinckrodt Chemical Works. Avidin was purchased from Nutritional Biochemicals and Atlas BRIG-35 from Atlas Chemicals. Phosphorus pentoxide ( $P_2O_5$ ) came from British Drug House.

Chromatographic Materials

Diethylaminoethyl cellulose (DEAE), carboxymethyl cellulose (CMC) and phosphocellulose were obtained from Bio-Rad Laboratories. Sephadex gel beads (G-25, G-200), Sepharose 6B, 4B, DEAE Sephadex, CM Sephadex were products of the Pharmacia Inc. Dowex 50-W-X12 was purchased from J.T. Baker Co.  $CaPO_4$  gel was from Calbiochem. MN-cellulose Pulver 300g was obtained from Machinerey, Nagel and Co., Germany, and silica gel from Merck, Germany.

RESEARCH LABORATORY  
UNIVERSITY OF MICHIGAN  
ANN ARBOR, MICHIGAN 48106

Preparation of E. coli ornithine transcarbamylase (OTCase)

OTCase was prepared from E. coli (vit. B<sub>1</sub>, met.) by a modification of the method by Rogers and Novelli (1962). A typical purification is outlined in Table 2. The preparation after ammonium sulphate fractionation was completely devoid of ATCase or CPSase activities.

TABLE 2

PREPARATION OF OTCase  
from a derepressed strain of E. coli

Purification Steps	Volume (ml.)	Total Units	Enzyme Activity		
			Specific Activity	Purification	Recovery (%)
1. Crude extract	60.0	9,459,360	7,166	-	100
2. Heated supernatant	59.0	9,276,216	19,604	2.7	98
3. Protamine sul- fate supernatant	56.0	8,316,672	20,570	2.9	88
4. (NH) <sub>4</sub> SO <sub>4</sub> 50-80%	8.0	7,891,696	27,402	3.8	83

The assay was carried out at 25 °C and the reaction mixture contained in a final volume of 0.5 ml., enzyme, Tris-HCl buffer (50 u moles, pH 7.6), ornithine (6 u moles) and carbamyl phosphate (10 u moles). A unit of activity is defined as 1 mu mole citrulline formed per min. The derepressed strain of E. coli was a gift from Dr. H. de Robichon-Szulmajster, Laboratoire D'Enzymologie, Gif-sur-Yvette, France.

C. METHODS

I. Organism

1. Baker's Yeast (*Saccharomyces cerevisiae*)

The wild type and certain mutant strains of baker's yeast were stocks originally obtained from Dr. Francois Lacroute (FL) and grown under conditions appropriate for their genotype (Lacroute, 1966). Other mutants used were obtained from Dr. M. Duphil-Denis (M.D.), of this laboratory. These strains are all closely related.

2. Cultivation of Yeast

All cells were grown at 25°C with vigorous shaking. Growth was initiated by inoculating 100 ml. of complete medium (including supplement if necessary) with a loopful of stock culture. After 24 hours, 5.0 ml. of this preculture was used to inoculate each litre of growth medium with or without supplement. Cells were allowed to grow to the end of log phase, after which time they were harvested by centrifugation and washed twice with distilled water. The cell paste thus obtained was either used immediately or frozen and kept at -20°C until needed.

Crude extracts were prepared as described by Kaplan et al. (1967); greater detail will be given under results.

### 3. Growth Conditions

• Complete medium for the growth of precultures consisted of yeast extract (1%), Bacto-peptone (1%), and glucose (2%). For making plates for the growth of stock cultures, 3% agar was included. Minimal medium ( $G_0$ ) was made as described by Galzy and Slonimski (1957) and as modified by Kaplan et al. (1967). The following stock solutions were prepared. Mineral salts (A) 60 g.  $NH_4H_2PO_4$ , 5 g.  $MgSO_4 \cdot 7H_2O$ , 20 g.  $(NH_4)_2SO_4$ , 10 g.  $KH_2PO_4$ , 1 g. NaCl and 1 g.  $CaCl_2$  were dissolved and made up to one litre with distilled water. To prevent formation of insoluble calcium salts the separately dissolved  $CaCl_2$  was added last when the solution was almost to volume.

Trace elements (B) 500 mg.  $H_3BO_3$ , 40 mg.  $CuSO_4$ , 100 mg. KI, 400 mg.  $MnSO_4 \cdot H_2O$ , 200 mg.  $Na_2MoO_4$ , 400 mg.  $ZnSO_4 \cdot 7H_2O$  were dissolved and made up to 1 litre with distilled water.

Ferric chloride solution (C) 20 mg.  $FeCl_3$  were dissolved in 100 ml. distilled water and stored in a dark bottle.

Vitamin solution (D) 80 mg. each of calcium pantho-

thenate, thiamine HCl, l-inositol, and pyridoxine, 20 mg. nicotinic acid (niacin), 0.8 mg. d-biotin were dissolved and made up to 200 ml. with distilled water. This solution was sterilized by filtration for it was found that autoclave sterilization caused some of the vitamins to break down.

Citrate buffer (E) 17 ml. 1M sodium citrate  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$  and 8 ml. 1M citric acid  $\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$  were combined. This mixture yielded a final pH of 5.0.

The following constituted 1 litre of minimal medium ( $G_0$ ): 100 ml. solution A; 1 ml. each of solutions B and C; 5 ml. solution D; 25 ml. citrate buffer (E); 20 g. glucose and 868 ml. distilled water. To prevent caramelization, glucose was autoclaved separately as a 20% solution. In cases where supplement was required, e.g. uracil and/or arginine, these were added before sterilization. Sterilization was carried out for 20 minutes at  $121^\circ\text{C}$ , 15p.s.i. in an American Sterilizer Company autoclave.

#### 4. Description of Strains

Table 3 lists the strains in this study, their genotypes and phenotypes with respect to the supplement

Table 3  
Description of Strains

Strain	Ploidy	Genotype	Phenotype	Growth Condition
FL90	2N	+/+	wild type	G <sub>0</sub>
FL100	N	+(a)	wild type	G <sub>0</sub>
FL80-2A	N	cpa <sub>2</sub> (α)	wild type	G <sub>0</sub>
FL14	N	ur <sub>3-2</sub> /ur <sub>3-2</sub>	uracil requiring	G <sub>0</sub> + 8 ug/ml.
FL233-3C (MD165-1C)	N	ur <sub>3-2</sub> Fur <sub>4-1</sub> (a)	uracil requiring	G <sub>0</sub> + 200 ug/ml.
MD170-7X	N	ur <sub>3-2</sub> Fur <sub>4-1</sub> cpa <sub>2</sub>	uracil requiring	G <sub>0</sub> + 225 ug/ml.
MD166-7B	N	ur <sub>3-2</sub> Fur <sub>4-1</sub> (α)	uracil requiring	G <sub>0</sub> + 200 ug/ml.
MD169(c)	2N	ur <sub>3-2</sub> Fur <sub>4-1</sub> (a) <u>ur<sub>3-2</sub>Fur<sub>4-1</sub>(α)</u>	uracil requiring	G <sub>0</sub> + 200 ug/ml.

REPRODUCED FROM THE ORIGINAL DOCUMENT

required for growth.

FL80-2A is a haploid which lacks the carbamyl phosphate synthetase of the arginine pathway. Since the carbamyl phosphate synthesized by the pyrimidine specific CPSase is also available for arginine biosynthesis, wild type growth conditions suffice for this mutant strain.

FL14, a diploid lacking orotidine-5-phosphate decarboxylase (OMP deCase) was grown under conditions of derepression for ATCase in the presence of 8 ug/ml uracil (Kaplan et al., 1967); 80 ug/ml of uracil causes repression.

FL233-3C (MD165-1C) as in FL14, lacks OMP deCase but in addition lacks a pyrimidine permeation mechanism. Because of the latter mutation, a constant but limiting concentration of uracil gets into the cell, although the cells are grown in presence of high concentrations of uracil (200 ug/ml). Thus derepression of ATCase occurs, simulating growth conditions in a chemostat.

MD170-7X results when the mutation of FL80-2A is introduced into FL233-3C. Thus growth conditions are the same as FL233-3C.

MD166-7B is the ( $\alpha$ ) haploid strain as compared to FL233-3C (MD165-1C) which is the (a) haploid strain.

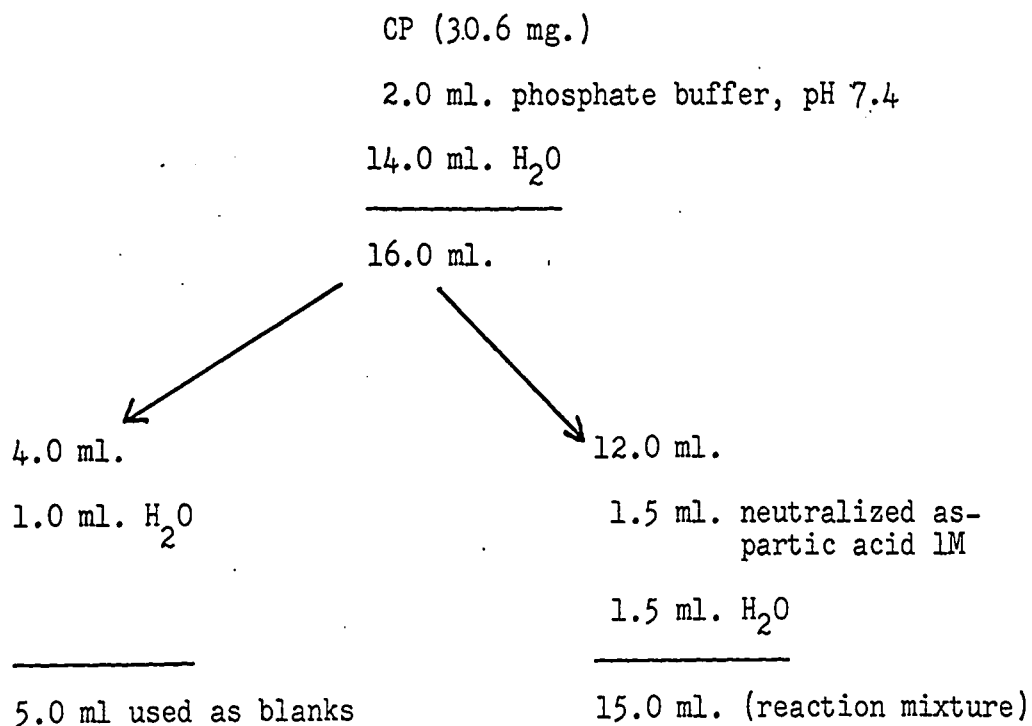
Mating of these two strains results in the diploid MD169<sub>(c)</sub>. Thus growth conditions for MD166-7B or MD169<sub>(c)</sub> are the same as FL233-3C.

## II. Enzyme Assays

All assays were carried out at 25<sup>o</sup> C, the temperature at which the cells were grown.

### 1. Aspartate Transcarbamylase

Aspartate transcarbamylase activity (ATCase) was assayed by the method of Koritz and Cohen (1954), as modified by Gerhart and Pardee (1962), and based upon the colorimetric determination of ureidosuccinate, the product of the reaction. The standard reaction mixture (final volume 0.5 ml.) contained enzyme, potassium phosphate buffer (50 μ moles, pH 7.4), aspartate (50 μ moles) and carbamyl phosphate (10 μ moles). Stock solutions of 1M aspartic acid neutralized with KOH was used in making up the reaction mixture. Since carbamyl phosphate (CP) develops a colour with the reagent mixture (Koritz and Cohen, 1954), CP was always included in the blank. A typical preparation is given in the following scheme:



A unit. is defined as the amount of enzyme capable of forming 1 mu mole of ureidosuccinate per minute at 25°C in the above reaction mixture. Specific activity is expressed as units per milligram protein.

## 2. Colorimetric Determination of Ureidosuccinate

The following stock solutions were prepared:

- A. 66 ml. conc H<sub>2</sub>SO<sub>4</sub> diluted to 100 ml. with H<sub>2</sub>O while in ice;
- B. 2.25 g. diacetylmonoxime (2,3 butanedione-2-oxime) made up to 100 ml. with H<sub>2</sub>O;

- C. 114 mg. diphenylamine-p-sulphonate sodium salt and  
0.4 g. Atlas BRIJ 35 dissolved in 100 ml. 0.1 N HCL;
- D. 250 mg. potassium persulphate in 100 ml. H<sub>2</sub>O.

Solutions B, C, and D were stored in dark bottles and all were kept under refrigeration until needed.

Just before the determinations, the mixture was prepared by mixing the above stock solutions at 0°C in the ratio 3A:1B:1C by volume and in the indicated sequence. The enzymatic reaction was stopped by adding 2.5 ml. of the mixture to each tube and mixing thoroughly. The ice cold tubes were then placed in a 60°C water bath for 30 minutes and shielded from light. After incubation the tubes were cooled in ice water.

To commence color development, the tubes were first equilibrated at 30°C for 15 minutes, then 0.5 ml. solution D was added to each tube and the solution was mixed thoroughly for 30 seconds. The tubes were then placed in the water bath at 30°C. After the mauve color developed for 30 minutes (at which time the color was found to be maximum and linear up to 300 n moles, Fig. 5 ), the optical density was read at 560 mμ in a Zeiss spectrophotometer. The amount of ureidosuccinate produced was calculated from the absorbance of standard solutions

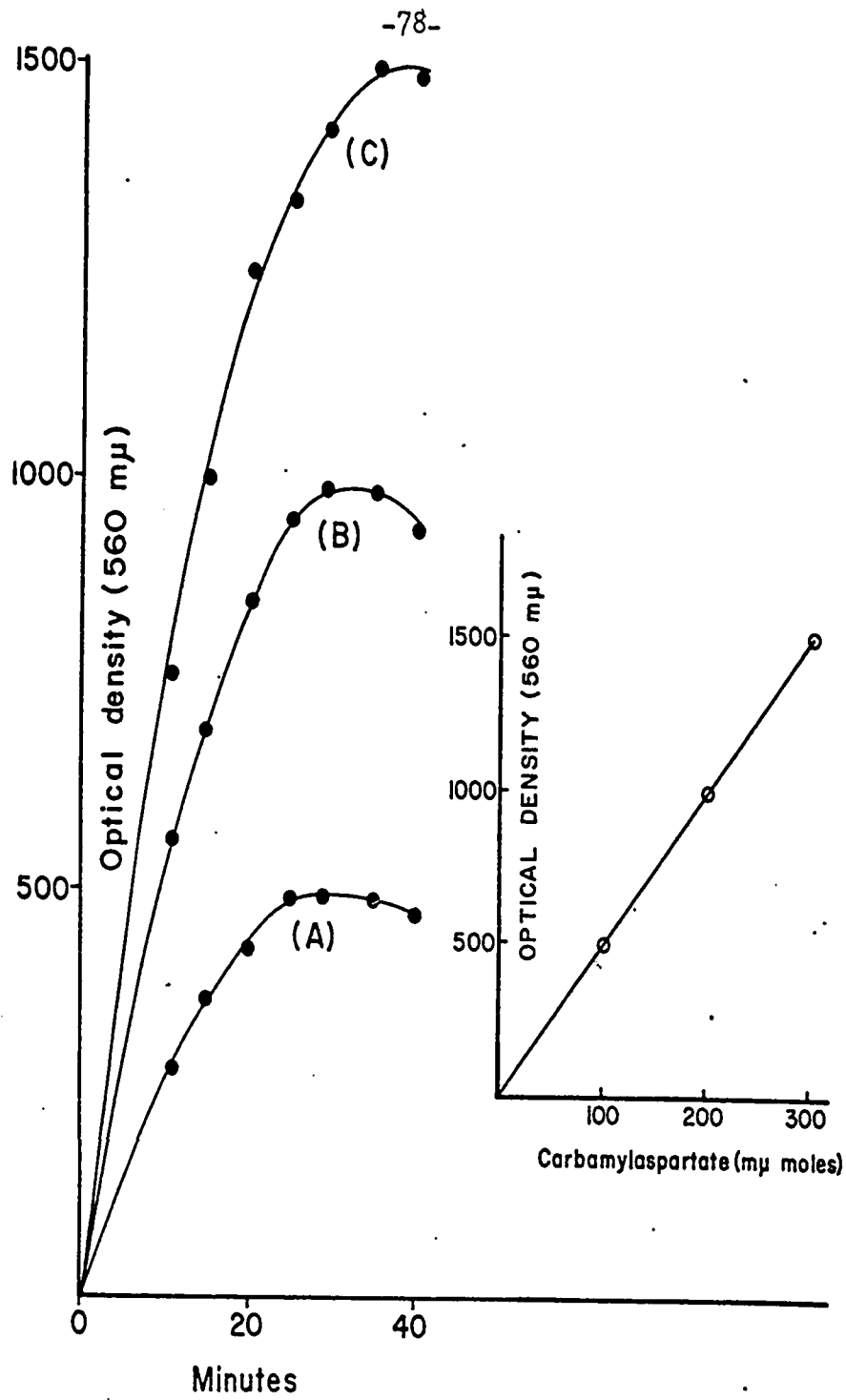


Fig. 5 . Color development with time using three concentrations of standard ureidosuccinate (A. 100  $\mu$  moles, B. 200  $\mu$  moles, C. 300  $\mu$  moles). Inset shows the color was linear after 30 min. at 30°C.

of ureidosuccinate, which were always determined simultaneously.

The ATCase activity was shown to produce ureidosuccinate from aspartate and carbamyl phosphate as a linear function of time through 20 minutes of incubation (Fig. 6, left) and enzyme concentration up to 20 ul. (Fig. 6, right). The boiled enzyme gave no reaction. The enzyme used in this experiment was partially purified from FL90 according to Kaplan et al. (1967).

### 3. Carbamyl Phosphate Synthetase

Carbamyl phosphate synthetase activity (CPSase) was estimated by condensing the carbamyl phosphate synthesized into either citrulline in the presence of ornithine and ornithine transcarbamylase (OTCase), or ureidosuccinate in the presence of aspartate and ATCase. Citrulline or ureidosuccinate was colorimetrically determined by a modification of the method of Gerhart and Pardee (1962). In these determinations 5.0 ml. of the mixture was added to the 1.0 ml. reaction mixture or standards and color development was initiated by adding 1.0 ml. solution D. Ureidosuccinate was estimated after 30 minutes of incubation at 30°C, as in the

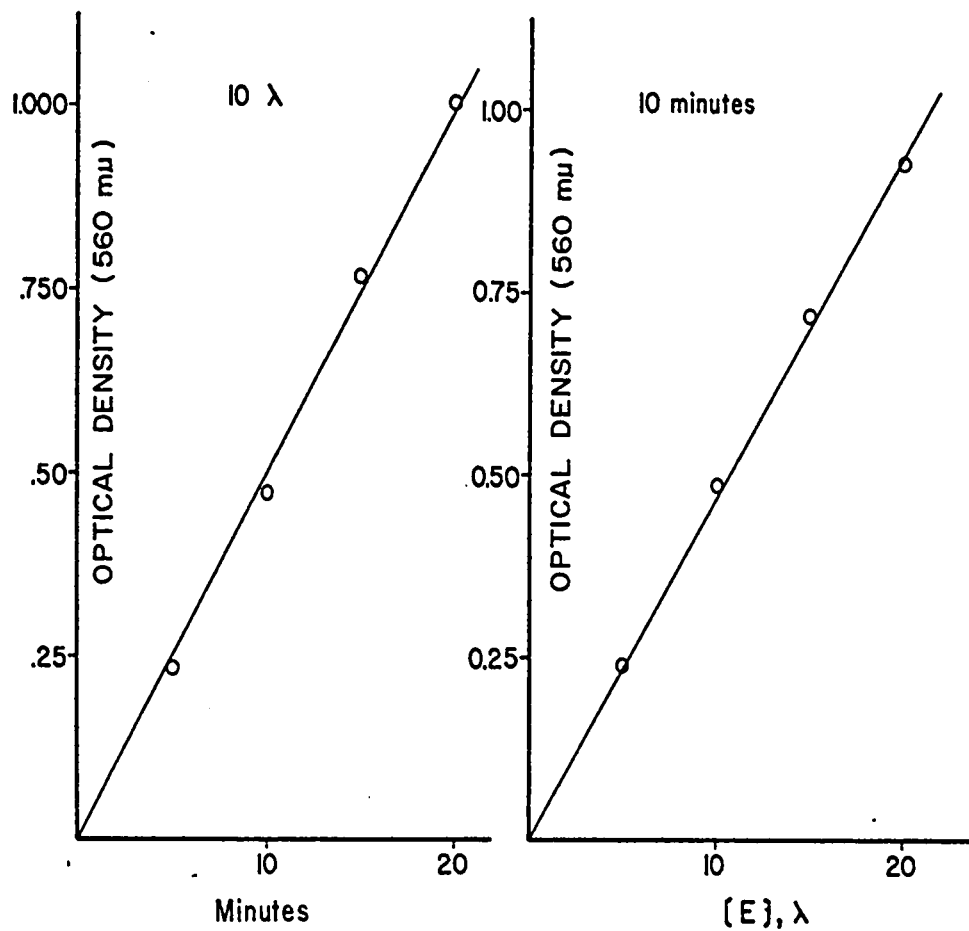


Fig. 6 . Plot to show the linearity with time (right) and enzyme concentration (left) in the ATCase reaction. The complete reaction mixture contained in 0.5 ml.: enzyme, phosphate buffer (50 u moles, pH 7.4), aspartate (50 u moles) and carbamyl phosphate (10 u moles).

ATCase reaction, where only one-half the volume was used. The incubation time required for maximum color development at 30°C using authentic citrulline standards was 20 minutes and linear to a concentration of 150  $\mu$  moles (Fig. 7 ). Three assay systems were used for estimating the CPSase activity:

Enzyme Assay I In the earlier studies where potassium phosphate buffer was used, the reaction mixture (final volume 1.0 ml.) contained enzyme, potassium phosphate buffer (100  $\mu$  moles, pH 7.6), glutamine (0.5  $\mu$  moles),  $\text{NaHCO}_3$  (50  $\mu$  moles),  $\text{MgSO}_4$  (30  $\mu$  moles), ATP (7.5  $\mu$  moles), ornithine (6  $\mu$  moles) and an excess of purified OTCase preparation, added in 5  $\mu$ l.

Enzyme Assay II The standard reaction mixture contained in a final volume of 1.0 ml.: enzyme, Tris- $\text{SO}_4$  buffer (100  $\mu$  moles, pH 7.6), glutamine (6  $\mu$  moles), ATP (20  $\mu$  moles),  $\text{MgSO}_4$  (20  $\mu$  moles),  $\text{NaHCO}_3$  (20  $\mu$  moles), ornithine (6  $\mu$  moles) and excess purified E. coli OTCase in 5  $\mu$ l. When ureidosuccinate was to be determined, 50  $\mu$  moles aspartate was included instead of ornithine and OTCase. ATCase was supplied in the enzyme system.

Enzyme Assay III This assay system was based on the fact that the synthesized products of assay II (citrulline or

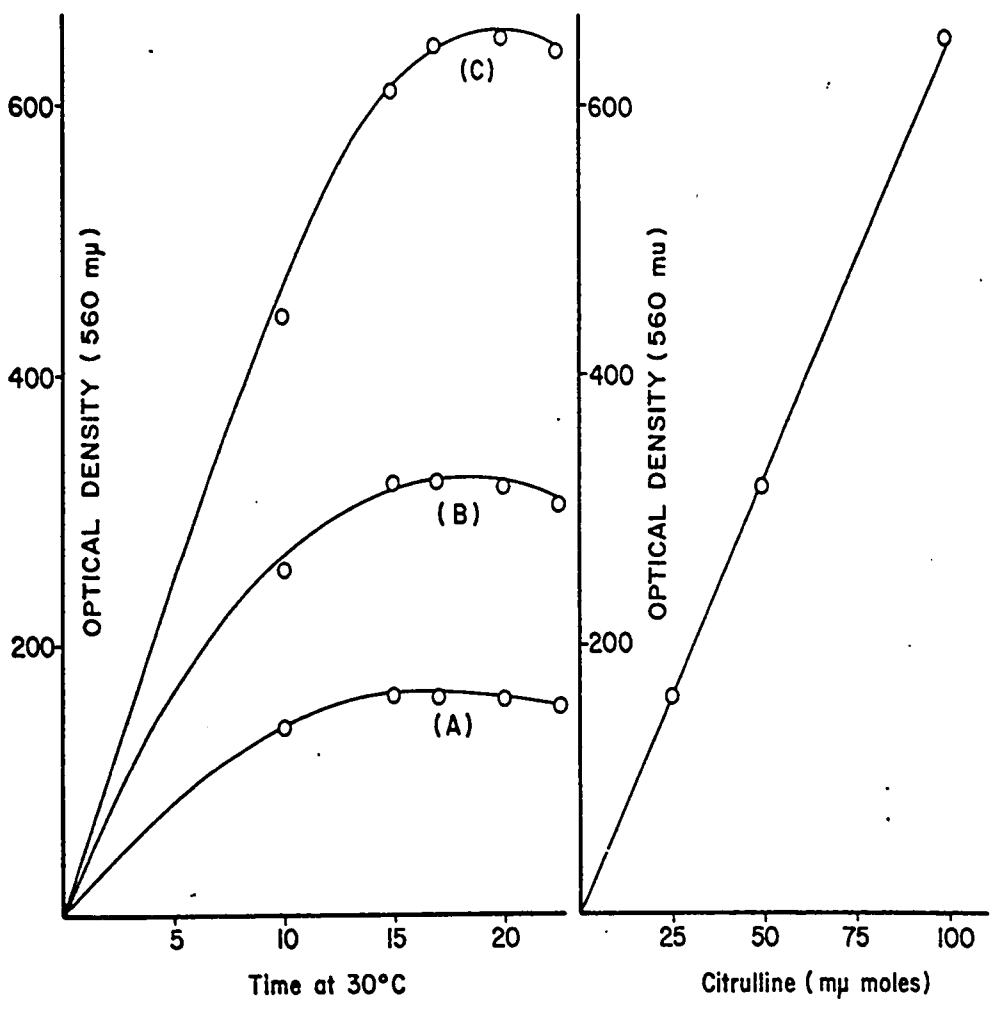


Fig. 7. (left) Color development with time using 3 concentrations of standard citrulline (A. 25 μ moles, B. 50 μ moles, C. 100 μ moles). (right) Plot shows that the color was linear after 18 min.

REPRODUCED FROM THE JOURNAL OF BIOLOGICAL CHEMISTRY

ureidosuccinate) are acid stable. This system (III) differed from assay system II in that it utilized  $\text{NaH}^{14}\text{CO}_3$  (Nuclear Chicago) ( $2.5 \times 10^5$  cpm) with 10  $\mu$  moles  $\text{NaHCO}_3$ , and had only a 0.5 ml. reaction volume. After the incubation, the reaction was stopped by adding 0.1 ml. 0.4N HCl. 300  $\mu$ l of the reaction mixture was then pipetted on a glass planchet, which was placed on a hot plate and under an infra-red lamp. To this sample was added 300  $\mu$ l. 4N HCl and the solution was heated to dryness and then counted on a Nuclear Chicago gas-flow counter. The CPSase activity was estimated for the amount of radioactivity incorporated. Reactions where 0.1N HCl was added before the enzyme sample served as blanks. One unit was defined as the amount of enzyme which would form one  $\mu$  mole of citrulline or ureidosuccinate per hour at 25°C. Specific activity was expressed as units per milligram protein.

Under these assay conditions, systems I, II and III gave similar results. Fig. 8 using assay system I, shows that citrulline formation was linear with time and protein concentration. Boiled enzyme preparation gave no reaction and samples lacking ATP served as blanks. The enzyme used here and in all further studies

UNIVERSITY OF CHICAGO  
LIBRARY

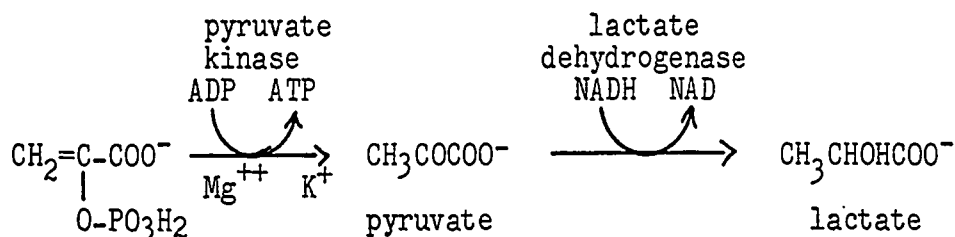


of the CPSase reaction, unless otherwise specified, was purified through the calcium phosphate gel-ammonium sulphate step (Table 11, step 4).

Fig. 9 shows that similar results were obtained with assay system III. The amount of  $^{14}\text{C}$ -citrulline produced was calculated on the basis of the amount of radioactivity (cpm) per  $\mu\text{mole NaHCO}_3$  used in the assay.

### 3. ADP Estimation

The amount of ADP was estimated by extrapolation from a standard curve based upon the following reactions:



A direct relationship exists between ADP utilization and decrease in absorbance at 340  $\mu\text{m}$  as a result of NAD formation. The reaction was assayed by a modification of the method of Anderson and Meister (1965). The

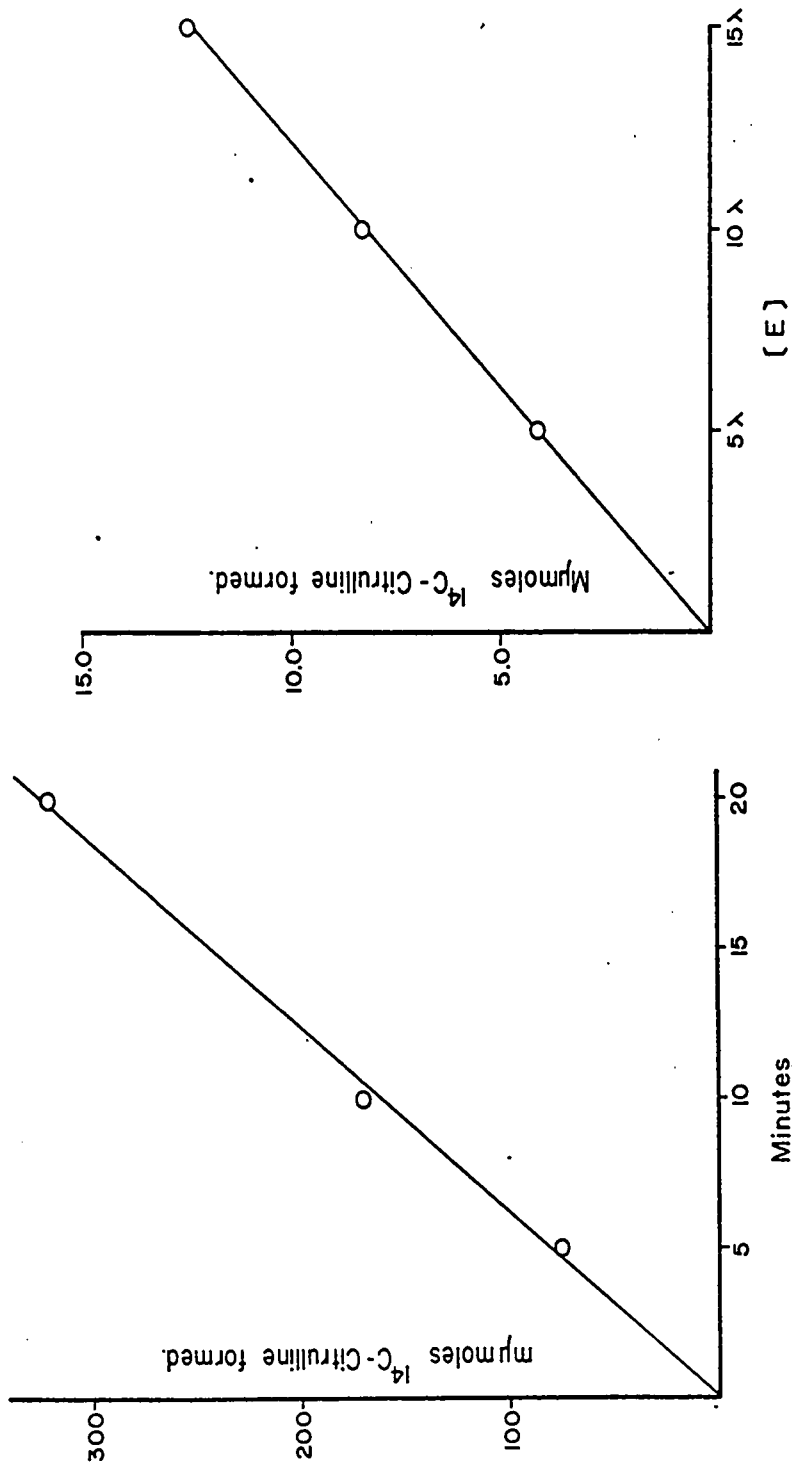


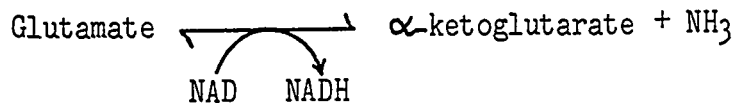
Fig. 9. Plot to show the linearity with time (left) and enzyme concentration (right). The complete reaction mixture (assay system III) contained in a final volume of 0.5 ml.: enzyme, Tris-SO<sub>4</sub> buffer (5 u moles, pH 7.6), glutamine (3 u moles), ATP (10 u moles), MgSO<sub>4</sub> (10 u moles), NaHCO<sub>3</sub> + NaH<sup>14</sup>CO<sub>3</sub> (5 u moles, 1.25x10<sup>5</sup> cpm), ornithine (3 u moles) and excess purified OTCase.

reaction mixture contained 50 u moles Tris-SO<sub>4</sub> buffer pH 7.6, 60 u moles KCl, 1.5 u moles MgCl<sub>2</sub>, 0.3 u moles phosphoenol pyruvate, 0.17 u moles NADH and excess lactate dehydrogenase (3 units/ml.) and pyruvate kinase (6 units/ml.) in 0.9 ml. Either standard ADP solution in buffer or the solution to be tested was added in 0.1 ml. to make up a final volume of 1.0 ml.

A linear relationship was obtained up to 120 mu moles of ADP.

#### 4. Glutamate Estimation

Glutamate was determined according to the method of Strecker (1955) which was based on the following reaction:



The increase in absorbance at 340 mu (after equilibrium was established) was related to glutamate concentrations. The assay system consisted of 0.5 ml. of solution A which contained 400 u moles phosphate buffer pH 7.6, 2.0 u moles NAD, 2.4 units glutamate dehydrogenase and 0.5 ml.

solution B containing standard glutamate or solution to be tested. Glutamate content was determined by extrapolation from a standard curve.

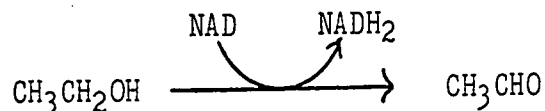
The standard relationship was obtained up to 80 mu moles of glutamate. A similar curve was obtained by Hartman and Buchanan (1958) in their studies of 5-phosphoribosylpyrophosphate amidotransferase.

#### 5. Catalase Activity

Catalase activity was assayed by a modification of the method by Chantrenne (1955). The concentration of  $H_2O_2$  used was 82 mu moles/2.0 ml. An enzyme unit catalyzed the splitting of 1 mM  $H_2O_2$  per min. at 25°C.

#### 6. Yeast Alcohol Dehydrogenase (ADHase)

Yeast ADHase was determined by the method of Vallee and Hoch (1955) in which the initial rate of increase in absorbancy at 340 mu was measured. The reaction is:



The complete reaction mixture contained in a final volume of 3.2 ml.: pyrophosphate buffer, 16 u moles pH 8.8; ethanol, 312.5 u moles; NAD, 7.8 u moles; and enzyme

REPRODUCED BY LIBRARY OF PARLIAMENTARY CANADA

in 0.2 ml. One unit reduces 1 u mole NAD/min. at 25°C under the above conditions.

### III. Techniques in Enzyme Purification and Molecular Weight Estimation

#### 1. Ammonium Sulphate Fractionation

Salt fractionation of the enzyme solution was carried out using a saturated ammonium sulphate solution neutralized with  $\text{NH}_4\text{OH}$ . Although this method had the disadvantage that it required working with larger volumes than did the use of solid ammonium sulphate, it was necessary because of the instability of the ATCase enzyme at low pH's.

The amount of saturated ammonium sulphate solution used was calculated from the following formula:

$$\frac{A}{100} = \frac{X}{B + X}$$

where X is the amount of ammonium sulphate solution (ml.)

B is the volume of enzyme solution (ml.)

A is the saturation required (%)

REPRODUCED FROM  
ATCASA ONLINE CANADA

## 2. Protamine Sulphate Treatment

Protamine sulphate fractionation for removal of nucleic acids (Felix, 1960) was introduced when it was observed that ammonium sulphate as well as alcohol precipitated the enzyme over a wide range and the results were not reproducible (Inamdar, pers. comm.).

Two methods of approach were used.

Method A. Protamine solution was prepared by dissolving the dry powder in distilled water to a concentration of 20 mg/ml and the pH was adjusted to  $7.0 \pm 0.5$  with NaOH. The resultant solution was slightly turbid, but no attempt was made to remove the turbidity since it was found not to interfere with the purification. Typically, enough protamine sulphate solution was added to the crude extracts to make a final concentration of 0.1%, which was sufficient to precipitate most of the nucleic acids.

This method was used primarily in preliminary experiments; however, when it was found that method B coupled with isopropanol fractionation gave a better purification, this method was dropped altogether.

Method B. Solid protamine sulphate was added to a final concentration of 1%. The coarse substance was crushed to a fine powder with a spatula and subsequently added very slowly to the rapidly stirred crude extract.

### 3. Organic Solvent Fractionation

The use of organic solvents as protein precipitants in enzyme purification is a common practice. Usually, the solvents acetone, methanol and ethanol are used at a very low temperature ( $-20^{\circ}\text{C}$ ), which is attained by using salted ice. The use of isopropanol here represents a departure from the usual procedure.

The precooled organic solvent was added dropwise to the rapidly stirred enzyme extract. After mixing for an additional 10 minutes, the mixture was centrifuged at  $-15^{\circ}\text{C}$  for 15 minutes at 20,000 g. This step resulted in the formation of gummy precipitate which was only soluble with difficulty in buffer (0.1M phosphate pH 6.8), but readily soluble in buffer containing 30% ammonium sulphate.

### 4. Column Chromatography

The procedures for handling and working with cellulosic ion-exchangers were derived from the work of

BRITISH COLUMBIA  
UNIVERSITY LIBRARY  
VICTORIA, BRITISH COLUMBIA

Peterson and Sober (1962).

(a) DEAE Cellulose

Before use, this anionic exchanger (capacity 0.87 meq./dry g.) was regenerated by washing with 0.5N HCl followed by 0.5N NaOH and then with distilled water until the washings were almost neutral. Most of the fines were removed during this washing process. The exchanger was subsequently suspended in 0.05M phosphate buffer pH 7.4, and then packed into a chromatographic column (1.5 cm. diameter by 30 cm. long). These chromatographic columns are equipped with a reservoir attachment so that much of the ion exchanger can be added at once to fill the column completely. After allowing the resin to settle overnight, the effluent was attached to a peristaltic pump which was adjusted to 10-15 ml. per hour. The column was then washed overnight with buffer and additives before use.

Elution procedure on DEAE cellulose: In early experiments the stepwise elution method was used starting from 0.05M phosphate buffer and increasing by increments of 0.05M up to 0.5M buffer. After it was observed that the enzyme could be eluted by 0.25M buffer, linear gradients of 0.2-0.25 and 0.2-0.3M were utilized for elution as recommended by Peterson and Sober (1962).

EMERSON'S OF CANADA  
OTTAWA, ONTARIO, CANADA

The enzyme could also be eluted with 0.3M KCl in 0.01M imidazole-HCl buffer, pH 7.0, which was determined by stepwise elution.

(b) CM-cellulose

This cation exchanger (capacity 0.7meq./dry g.) was regenerated by washing in 0.5N NaOH, distilled water, 0.5N HCl, distilled water and finally suspended in phosphate buffer 0.05M pH 6.8. The procedure for packing the column was similar to that for packing the DEAE column.

(c) Phosphocellulose

This cationic exchanger (0.88 meq./g.) was used without a regeneration procedure. After washing with 0.05M phosphate buffer pH 6.8 to remove fines, the column was packed as described for DEAE column.

(d) Gel filtration

Sephadex G-200 was used for the molecular sieving of enzyme preparations. This can be used as a method of purification and as a means of estimating the molecular weight of a protein. In general, the use of Sephadex materials was according to the recommendations of Sephadex Gel Filtration in Theory and Practice (Pharmacia Inc., Uppsala, Sweden).

The dry Sephadex beads (G-200) were swollen in 0.05M

phosphate buffer pH 7.4 containing  $2 \times 10^{-3} \text{M}$  B-mercaptoethanol for 7 days, after which the swollen gel was washed to remove fines. The suspended gel particles in a ratio of 1/1 with buffer were then used to pack the column (1.5 cm. by 88.5 cm.) following the procedure outlined in the manufacturer's booklet (above).

A sample of swollen Sephadex G-25 (0.5 cm. in height) was used to stabilize the surface of the G-200 material. The packed column was then washed for 4 days with buffer at a flow rate of 9.0 ml. per hour. A sample size of 0.6 ml. and fractions of 2.1 ml. were collected. Before the molecular weight estimation, the column was standardized by applying markers both separately and together. Thyroglobulin (20mg/ml), ovalbumin (20mg/ml), bovine serum albumin (20 mg/ml) and E. coli cells (1 mg/ml) were assayed by their absorbance at 280 mu. Catalase (15mg/ml) and ATCase were estimated both by their absorbance at 280 mu and by their activities (see enzyme assay).

Sepharose 6B. Sepharose 6B (agarose gel beads) was obtained as a suspension. After washing with 0.05M phosphate buffer pH 7.6 containing 10% glycerol,  $2 \times 10^{-3} \text{M}$  B-mercaptoethanol and  $2 \times 10^{-3} \text{M}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  to remove the fines, the gel was used as in the case of swollen

G-200, except that a column of 2.5x94 cm. equipped with flow adaptors was used. A flow rate of 10.0 ml/hr was maintained by means of a pressure head of 30 cm. The sample volume was 2.0 ml. and fractions of 5.0 ml. were collected. Standardization of the column for molecular weight estimations was carried out as described previously for G-200.

In order to test the effect of UTP and glutamine, these compounds, at concentrations of  $3 \times 10^{-3} \text{M}$  and  $5 \times 10^{-4} \text{M}$  respectively, were included in the buffer system, 250 ml. of which were used to pretreat the column prior to adding the enzyme solution. 250 ml. was used so that the elution of the UTP solution preceded slightly the elution of the ATCase activity. This procedure was adapted in order to minimize the amount of UTP used.

Sephadex G-25: Sephadex G-25 (medium) was also used to desalt enzyme preparations. Columns of 0.85x15 cm. in gel bed heights were sufficient to desalt a 0.4 ml enzyme sample.

##### 5. Gradient Centrifugation

Linear gradients (4.6 ml.) using 5 and 20% (w/v) sucrose in 0.02M phosphate buffer (pH 7.6) containing

various additives were prepared as described by Martin and Ames (1961). A sample of 0.1 ml. containing catalase as an internal marker was layered on each gradient. After centrifugation using the SW-65 rotor in Beckman model L2-65 ultracentrifuge at 5°C, fractions containing 13 drops (0.22 ml. approx.) were collected. These fractions were then assayed for the various activities as described previously.

Sedimentation coefficients were calculated according to the method of Bishop (1966), which is a modification of the method described by Martin and Ames (1961). The density and viscosity were calculated by the empirical method of Barber (1966) for the various sucrose fractions at 5°C. The amount of sucrose in each fraction was measured by refractometry. The centre of gravity of the activities was calculated as described by Shumaker (1967).

Molecular weights were estimated by comparison to the internal marker catalase, MW 250,000, by use of either the calculated sedimentation coefficients and/or the distance travelled from the meniscus during centrifugation, as described by Martin and Ames (1961):

EMERSON COLLECTING  
MATERIALS  
ONTARIO CANADA

$$R = \left( \frac{MW_1}{MW_2} \right)^{2/3} \quad \text{where } R = \frac{\text{sedimentation coefficient of sample}}{\text{" " catalase}}$$

or  $R = \frac{\text{distance travelled by sample}}{\text{" " catalase}}$

MW<sub>1</sub> = molecular weight of sample

MW<sub>2</sub> = " " catalase

#### IV. Analytical Procedures

##### 1. Protein Determination

Protein was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a standard.

##### 2. Electrophoresis

Electrophoresis was carried out on cellulose acetate strips according to the method outlined in the company's handbook "Gelman Procedures, Techniques and Apparatus for Electrophoresis" (Ann Arbor, Michigan, U.S.A.). Gel electrophoresis was carried out as described by Davis (1964).

REPRODUCED FROM ORIGINAL COPY

D. RESULTS

I. Preliminary Experiments on the Purification of ATCase

When this study was initiated, attempts to purify the enzyme aspartate transcarbamylase (ATCase) beyond ammonium sulphate fractionation had been unsuccessful (Kaplan et al., 1967). Repeated attempts to purify this enzyme using various ion exchangers resulted in either loss of activity or feedback properties with no increase in specific activity (Inamdar and Kaplan, pers. comm.). It was the hope of this study to reinvestigate some of these studies in an effort to purify ATCase to a greater degree in order to prove or disprove by co-purification and MW estimation techniques the existence of CPSase activity and the ATCase activity in a single enzyme complex.

Because of the ease in detecting the ATCase activity as compared to the CPSase activity, the purification procedure was worked out for the ATCase activity, and then when the purification steps were established, both activities were studied concomitantly.

RESEARCH COUNCIL OF CANADA  
FUNDING OF RESEARCH

1. Kinetics of Derepression

The derepression kinetics of ATCase was investigated by comparison of the specific activities of the enzyme in crude extracts of different strains. Table 4 shows that, in strain FL14, a 5-6-fold increase in specific activity of ATCase as compared to wild types was observed. However, the amount of inhibition by  $2 \times 10^{-3} \text{M}$  UTP was very low (18% in derepressed FL14) compared to 70-80% in the wild types. FL80-2A, a strain lacking the CPSase of the arginine pathway, showed higher specific activity compared to the wild types. Strain FL233-3C in addition to having a high specific activity of ATCase comparable to that of the derepressed strain FL14, yielded an enzyme which was highly susceptible to inhibition by UTP. Hence FL233-3C was used as the source for the purification of ATCase. Since the ur-2 gene in this strain is the same as wild type (Lacroute, 1966), the ATCase should also be wild type. It was deduced that the specific activity of FL233-3C could be further increased by repression of the enzymes involved in other biosynthetic pathways if the cells were grown in partially enriched medium, or by increasing the ploidy of the cells.

EMILIO G. GILBERT  
MILWAUKEE  
BOTANICAL GARDEN CANADA

Table 4

A Comparative Study of 5 Strains of Yeast to Determine  
the Likely Source in the Purification of ATCase

Strain and Growth Conditions	Genotype	ATCase Specific Activity	% Inhibition by $2 \times 10^{-3} M$ UTP
FL90 in G <sub>0</sub>	a/α	40.0	71
FL100 in G <sub>0</sub>	a	41.0	78
FL80-2A in G <sub>0</sub>	cpa2	48.6	75
FL14 in G <sub>0</sub> + 200 ug/ml.U.	$\frac{a \text{ ur}_{3-2}}{\alpha \text{ ur}_{3-2}}$	224	18
FL233-3C in G <sub>0</sub> + 200 ug/ml.U.	a ur <sub>3-2</sub> <sup>FUR</sup> <sub>4-1</sub>	192	75

U - Uracil

UNIVERSITY OF CALIFORNIA  
 LIBRARY

The results presented in Table 5 show that the addition of peptone and adenine had no increasing effect on ATCase specific activity but served only to decrease the time taken to reach stationary phase from 40 hours to 24 hours. On the other hand, MD169<sub>(c)</sub> (diploid of FL233-3C) gave a substantial increase in ATCase specific activity compared to the parent strains MD166-7B and MD165-1C. Table 5 also shows a strain (MD170-7X) in which the  $cpa_2$  mutation was introduced into FL233-3C. Here the specific activity was considerably increased above both parent strains up to a value of 301. This strain had the added advantage in that only the pyridine specific CPSase was present, thereby eliminating any interference by the arginine specific CPSase activity and a clearer picture with respect to the purification could be obtained. Thus it was seen that the repression of the enzymes of other biosynthetic pathways did not result in any increase of the ATCase specific activity: however, introduction of the  $cpa_2$  mutation or increase of the ploidy resulted in a substantial increase of ATCase.

Figure 10 shows the growth behaviour of MD170-7X using various concentrations of uracil. It can be seen that uracil is an absolute requirement (curve A) and

Table 5

The Effect of Growth Conditions and Certain Genetic In-  
 troductions on the ATCase Activity and Inhibition of FL233-3C

Strain and Growth Conditions	Genotype	ATCase Specific Activity	% Inhibition by $2 \times 10^{-3} M$ UTP
FL233-3C (MD165-1C) in $G_0$ + 200 ug/ml.U.	$a \text{ ur}_{3-2} \text{FU}_{4-1}^r$	163	78
MD166-7B in $G_0$ + 200 ug/ml.U.	$\alpha \text{ ur}_{3-2} \text{FU}_{4-1}^r$	192	90
MD166-7B in $G_0$ + 200 ug/ml.U. + peptone (1%) + adenine 200 ug/ml. U.	"	180	80.5
MD169(c) in $G_0$ + 200 ug/ml.U.	$\frac{a \text{ ur}_{3-2} \text{FU}_{4-1}^r}{\alpha \text{ ur}_{3-2} \text{FU}_{4-1}^r}$	266	78
MD170-7X in $G_0$ + 225 ug/ml. U.	$\alpha \text{ cpa}_2 \text{ ur}_{3-2} \text{FU}_{4-1}^r$	301.1	84

U - Uracil

SWINERTON & SUTHERLAND  
 BREDAVA, ONTARIO, CANADA

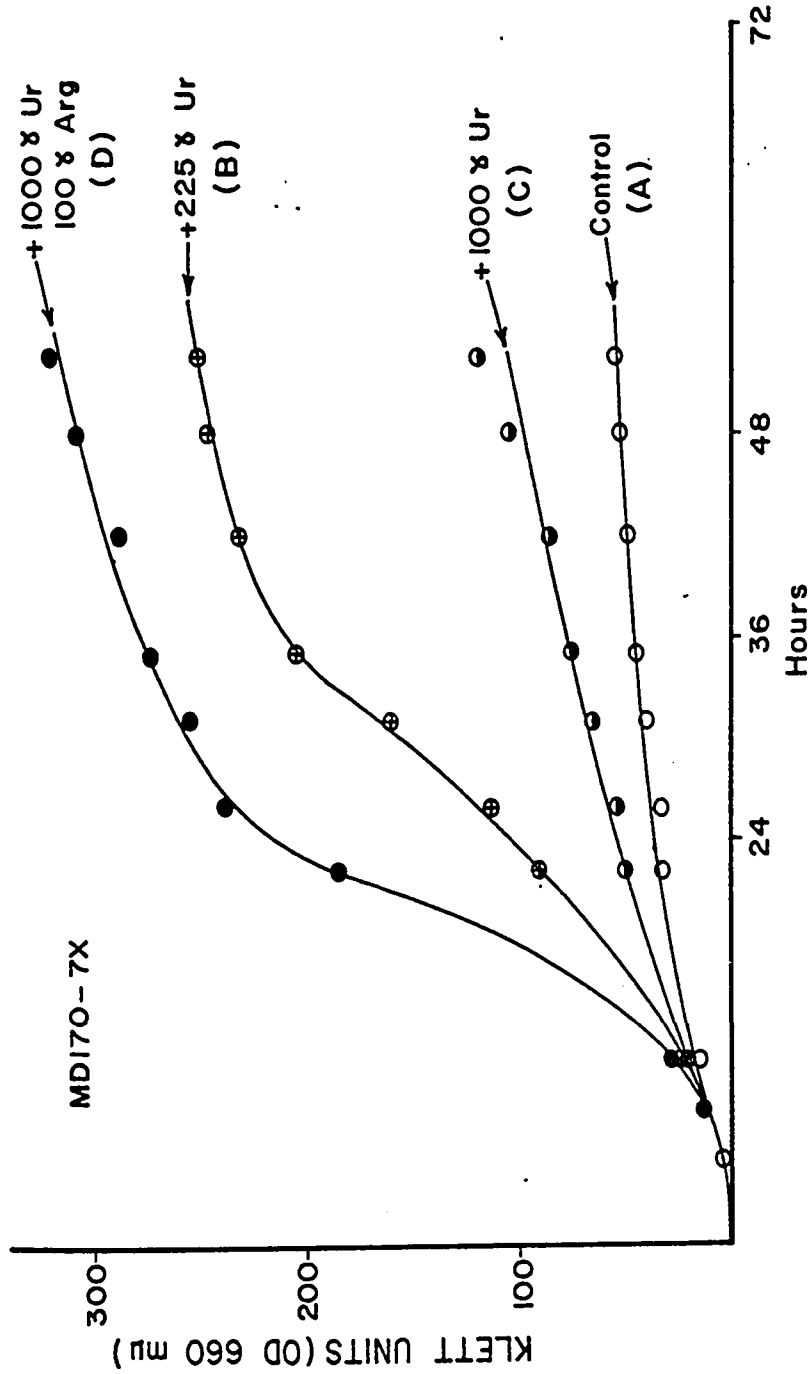


Fig. 10. Growth behaviour of MD170-7X, a haploid strain lacking the arginine specific CPSase and having a limited uracil permeation mechanism, in G<sub>0</sub> (curve A) and G<sub>0</sub> plus various additives (B, C, and D as indicated).

optimal growth occurs at 225 ug/ml uracil (curve B) However, at very high concentrations of uracil (1000 ug/ml) growth is inhibited (curve C) but is reversed in the presence of arginine (curve D).

These results confirm the genetic observations and are explained on the basis that the high concentrations of uracil cause repression of the CPSase of the uracil pathway; as a result, a requirement for carbamyl phosphate for arginine biosynthesis develops. This requirement is overcome by the arginine added in the growth medium.

## 2. Salt Fractionation

Kaplan et al. (1967) found that the ATCase activity was salted out between 33-45% ammonium sulphate with about 3-4-fold increase in specific activity. Hence, the salt concentration limits were adjusted in hope of increasing the purification factor by narrowing the ammonium sulphate range.

Table 6 gives the results of several fractionation steps increasing by increments of 10%. It can be seen that most of the ATCase activity was precipitated between 30-45% ammonium sulphate and a 2-fold increase in specific activity resulted with this strain. Narrower ranges

Table 6

Enzyme Purification by Ammonium Sulphate Fractionation  
Using Extracts from FL233-3C

Specific Activities

Crude Extract	Ammonium Sulphate Fractions (% saturation)						Super- natant
	0-25	0-30	25-35	30-40	35-45	40-50	
151	53	69	308 (2.0)	363 (2.5)	253 (1.7)	115 (0.7)	22 10
158							
169	31	39	288 (1.7)	285 (1.6)	165 (1.0)	76 (0.4)	6 8
174							

The purification factor relative to crude extract  
is given in brackets below the various specific activities.

(than 10%) of ammonium sulphate concentration did not increase the purification factor significantly and only 50% of the total activity was recovered. Table 7 shows the effect of treating the crude extract with protamine sulphate (as a solution or solid) before ammonium sulphate fractionation. This treatment resulted in only a slight increase in specific activity; however, the significance of this step lies in the alcohol fractionation procedure which follows.

### 3. Organic Solvent Fractionation

The results of organic solvent fractionation of the protamine sulphate treated crude extract are given in Table 8. It can be seen that fractionation with 20-40% acetone or methanol caused precipitation of most of the ATCase activity; however, acetone had an additional detrimental effect on the inhibitory site of the enzyme, and 20-30% methanol gave a 3-4-fold purification over crude extracts. Ethanol on the other hand, was observed to have two effects as compared to methanol: it decreased the alcohol fractionation range in which the ATCase activity was found and caused a large amount of other proteins to precipitate below 20% saturation (v/v).

RESEARCH REPORT  
BIOCHEMISTRY  
UNIVERSITY OF TORONTO

Table 7

The Effect of Protamine Sulphate on Ammonium Sulphate  
Fractionation

Treatment	Specific Activity	Purification	Recovery (%)
none (crude extracts)	191.1	-	100
$(\text{NH}_4)_2\text{SO}_4$ (33-45%)	262	1.4	83
protamine sulphate solution (0.1%) (method A) $(\text{NH}_4)_2\text{SO}_4$ (33-45%)	371	1.9	70
protamine sulphate solid (1%) (method B) $(\text{NH}_4)_2\text{SO}_4$ (33-45%)	485	2.5	65

LIBRARY OF THE  
NATIONAL INSTITUTE OF  
HEALTH  
OTTAWA, ONTARIO, CANADA

Table 8  
Purification of ATCase Activity by Organic Solvent  
Fractionation

Treatment	Protein (mg/ml)	Specific Activity	Inhibition (%)
none	29.0	95	70
Acetone 0-20%	4.35	97	35
20-30%	4.9	407	17
30-40%	25.2	84	8
40-50%	12.0	4	11
none	31.5	98	78
Methanol 0-20%	10.4	82	63
20-30%	13.0	367(3.8)	80
30-40%	3.1	235	68
40-50%	9.3	30	56
none	31.5	98	78
Ethanol 0-20%	20.7	157	75
20-30%	5.6	560(5.7)	63

Organic solvent fractionation was carried out at  $-15^{\circ}\text{C}$ . The precipitate obtained in each step was dissolved in 2.0 ml. 0.1M phosphate buffer, pH 7.4, and all fractions including crude extract were assayed under similar conditions (10  $\mu\text{l}$  for 10 min. at  $25^{\circ}\text{C}$ ). The purification factor over crude extract is included in brackets in the column of specific activity.

UNIVERSITY OF GUELPH  
GUELPH, ONTARIO, CANADA

It was therefore thought that n-propanol or isopropanol (or higher alcohol) might cause the extraneous proteins to precipitate in the region of 0-15%, whereas the ATCase activity would precipitate in the 15-20% region. Subsequently it was observed that n-propanol caused complete inactivation of the enzyme, but isopropanol gave results as anticipated with about 8-fold purification and approximately 60-70% recovery. Table 9A illustrates an example.

However, isopropanol treatment of the clear supernatant resulting from treatment of the crude extracts with protamine sulphate to 1% instead of 0.1% as was the case above, caused precipitation of the ATCase activity in the 0-10% fraction. The precipitate obtained, though soluble only with difficulty in buffer, went into solution quite readily in the presence of buffer and 30% ammonium sulphate. Consequently, an ammonium sulphate fractionation step follows alcohol fractionation (step 3, Table 9B ).

This procedure resulted in 10-15-fold purification relative to crude extract with 60-83% recovery and Table 9B shows a typical example in which 62% of the ATCase activity was recovered. This protamine sulphate treat-

RESEARCH CHEMISTRY  
MONTREAL, CANADA

Table 9

Purification of ATCase by Isopropanol Fractionation

Treatment	Protein (mg/ml)	Optical Density (560 mμ)	Specific Activity	Inhibition (%)
A.				
1. none	29.9	806	109.2	76
2. protamine sulphate to 0.1%	26.6	762	118.9	78
3. isopropanol 0-15% (v/v)	13.7	481	145.7	70
4. isopropanol 15-20% (v/v)	5.9	1227	863 (8x)	71
B.				
1. none	30.6	698	94.2	77
2. protamine sulphate 1% solid	25.4	593	97.7	81
3. isopropanol 0-10% + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 33-45%	3.6	859	998 (10.6)	70

Crude extract was treated with protamine sulphate to 0.1% (v/v) with a 2% (w/v) protamine sulphate solution (A, step 2) and to 1% (B, step 2) with solid protamine sulphate before isopropanol fractionation (A,B steps 3,4) Fractionation procedure was described in Methods. Purification factors are in brackets.

UNIVERSITY OF TORONTO  
 LIBRARY CONTINUOUS SERVICE

ment must have removed some of the proteins usually precipitated during early methanol or ethanol fractionation. Attempts to carry out this isopropanol fractionation procedure at 0°C resulted in loss of the regulatory properties of the enzyme.

#### 4. Adsorption and Elution Studies

##### (a) Gels and other materials

A variety of adsorbents was tried in the attempt to purify the ATCase. These included charcoal,  $AlO_3$ , silica gel, calcium phosphate gel, MN-cellulose, and manganese pyrophosphate gel (Sundararajan and Sarma, 1959). The enzyme was adsorbed only to calcium phosphate gel, i.e. there was a good adsorption and subsequent elution by 0.20M phosphate buffer pH 7.4. The alkali treated form of calcium phosphate gel (hydroxylapatite) did not give a clear cut elution pattern as compared with the gel form, nor was the recovery as good. The batch treatment was used preferentially to column chromatography since the regulatory property was more stable during the former manipulations. Typically, the procedure gave up to a 2-fold increase in specific activity over that obtained in step 3, Table 9B.

(b) Column Chromatography

Ion exchange chromatography and gel filtration: further purification by column chromatography of the  $\text{CaPO}_4$  gel eluate by ammonium sulphate fractionation resulted in either a complete loss of activity (e.g. cellulose phosphate), or in a decrease both in specific activity and the sensitivity of the enzyme to UTP (e.g. CM cellulose, Sephadex G-200, CM-Sephadex A-50); some agents (e.g. DEAE cellulose, DEAE-Sephadex, hydroxylapatite) caused an increase in specific activity with a marked decrease in the regulatory function. The enzyme was not adsorbed to CM-cellulose from 0.05M phosphate buffer; further attempts to accomplish adsorption to this cation-exchanger by decreasing ionic strength and/or pH resulted in inactivation. However, the enzyme was adsorbed to DEAE cellulose and could be eluted with 0.25M phosphate buffer. Table 10 lists the specific activity of the enzyme and its capacity to be inhibited by  $2 \times 10^{-3}$  M UTP before and after column chromatography. The eluted fraction containing the largest amount of activity is represented here. A dialysis step which usually precedes column chromatography was omitted altogether since the treatment resulted in almost complete loss of the feedback

RESEARCH INSTITUTE OF CHEMISTRY  
UNIVERSITY OF TORONTO  
TORONTO, ONTARIO, CANADA

Table 10

The Effect of Dialysis and Column Chromatography on  
ATCase Specific Activity and Feedback Inhibition

Treatment or Chroma- tographic Material	Buffer System	Specific Activity	
		Before	After
Dialysis	0.01 imidazole-HCl buffer pH 7.0 con- taining $2 \times 10^{-3}M$ $\beta$ - mercaptoethanol + $2 \times 10^{-4}M$ EDTA	2088 (79.5%)	1307 (13.1%)
CM-Sephadex*	0.05M phosphate buffer pH 6.8 containing $2 \times 10^{-3}M$ $\beta$ -mercaptoethanol	2027.4 (62%)	1542.2 (39%)
Sephadex G-200	0.05M phosphate buffer pH 6.8 containing $2 \times 10^{-3}M$ $\beta$ -mercaptoethanol	1987 (65%)	980 (8%)
DEAE-cellulose*	gradient elution 0.2M to 0.25M phos- phate buffer pH 6.8 containing $2 \times 10^{-3}M$ $\beta$ -mercaptoethanol	772 (52%)	1530 (20%)
calcium phos- phate gel	stepwise elution: 0.1M, 0.15M phos- phate buffer pH 7.4	2024 (66%)	3671 (9.5%)

\*The cellulose and dextran (Sephadex) forms of ionic exchanger gave similar results. Figures in brackets are percentage inhibition by UTP.

UNIVERSITY OF GUELPH  
GUELPH, ONTARIO CANADA

property (Table 10 ).

It was obvious from these results that if further purification was to be accomplished by column chromatography, conditions in which both the enzyme activity and its 'regulatory function' were stable had to be sought.

#### 5. Stability Studies

In general, the ATCase activity was very unstable, but its 'feedback property' was even more labile. Earlier studies indicated that storage (by freezing at  $-20^{\circ}\text{C}$ ) during any steps beyond the crude extract resulted in at least 50% loss in activity after thawing.

These stability studies were carried out by leaving the enzyme preparation overnight to several days at  $0^{\circ}\text{C}$  or by heating at  $37^{\circ}\text{C}$  for one hour.

(a) Effect of pH - The activity was found to be more stable than the 'feedback property' of the enzyme. The activity and 'feedback property' behaved in similar fashion with respect to pH. They were found to be most stable in 0.1M phosphate buffer pH 6.8, where 88% of the original activity remained and over 70% of the 'regulatory function' was lost after 24 hours at  $0^{\circ}\text{C}$ .

Under similar conditions, but at pH 6.0, 50% of the original activity remained and at pH 7.4 72% remained. The ATCase activity was extremely sensitive to pH's below 5.5.

(b) Sulfhydryl reagents - Cleland's reagent (dithiothreitol), B-mercaptoethanol, cysteine and glutathione, all at a concentration of  $1 \times 10^{-2} M$  in 0.1M phosphate buffer pH 6.8, were not able to stabilize either the activity of the enzyme or the 'feedback property'.

(c) Effect of high salt concentration - High concentrations of ammonium sulphate have been found to stabilize certain enzymes. Therefore, the effects of various ammonium sulphate concentrations on this regulatory enzyme were studied. Concentrations ranging from 1% to 20% were used and it was found that the activity became more labile with increasing concentration of ammonium sulphate. Complete loss of the activity resulted after 48 hours at 0°C at the 20% level. However, at this same concentration (20%), the 'regulatory function' was more stable; after 24 hours at 0°C the activity which was only 13% of the original could still be inhibited up to 46%, whereas in 1% ammonium sulphate, where 45% of the original activity remained, only 30%

STANLEY CANTARIC CANVAS

inhibition was observed.

Higher concentrations of phosphate buffer pH 6.8 had no effect on the general pattern of decreased activity and greater loss of the 'regulatory function'. After 24 hours at 0°C, 91% of the original activity was retained but inhibition by  $2 \times 10^{-3}$  M UTP was only 30% compared to 75% for the fresh enzyme.

(d) Effect of substrates, products and inhibitors: Another common practice which stabilizes certain enzymes is their storage in the presence of their substrate, cofactors and/or inhibitors (Satyanarayana et al., 1968). The substances used in this study included the substrate of the CPSase activity, i.e., glutamine,  $Mg^{++}$ ,  $HCO_3^-$ , carbamyl phosphate and aspartate, and the inhibitor UTP. In these studies, it was observed that only the presence of UTP served to stabilize the 'feedback property' of the enzyme against heating at 37°C for one hour; however, the stabilizing effect was not complete. In the absence of UTP only 4% inhibition remained as compared to 45% in its presence; the original untreated material was inhibited up to 62%. The activity was retained (100%) after this heat treatment.

(e) The effect of glycerol: Fig. 11 shows the

ENTRADA GIBRALTAR CANADIA

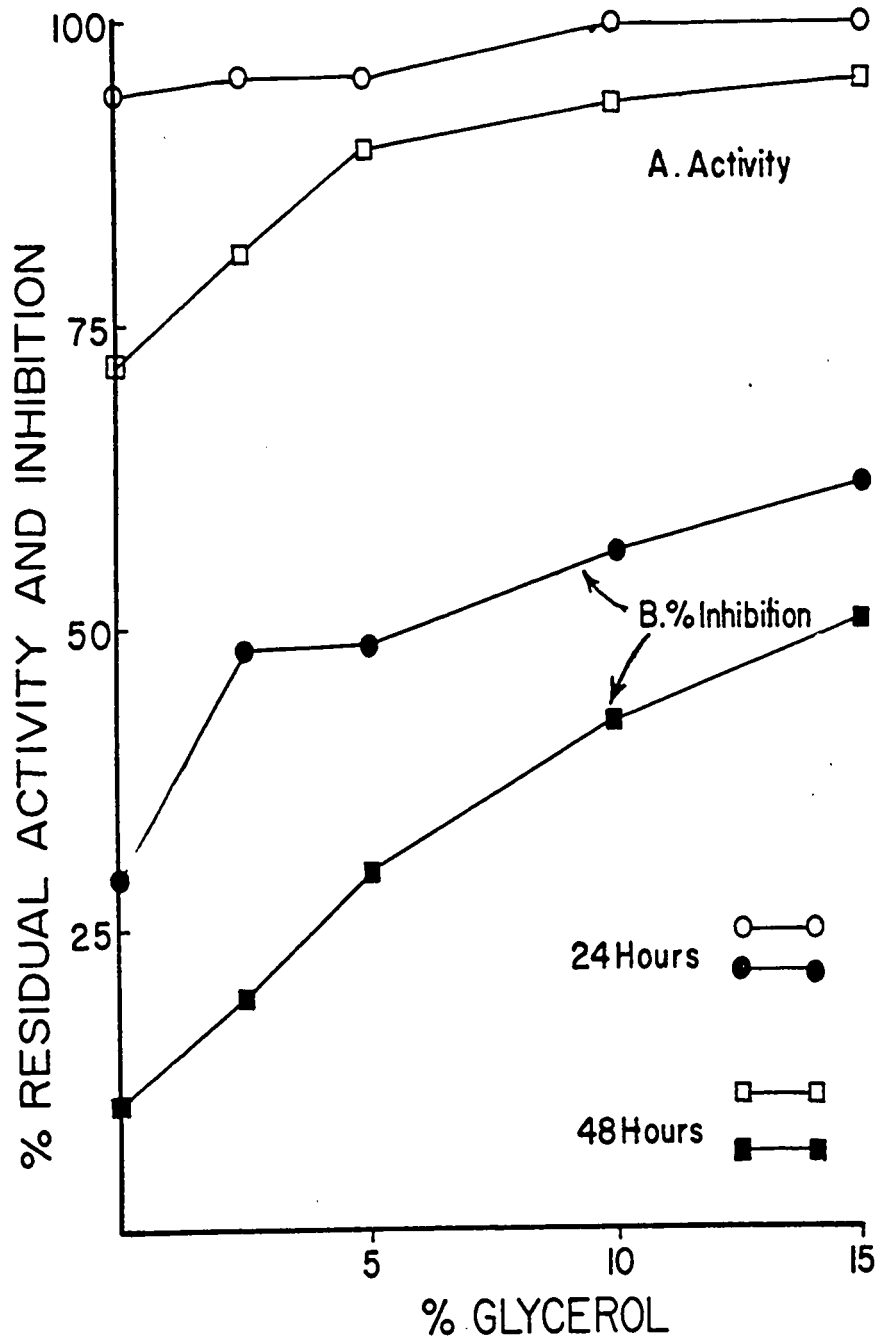


Fig. 11 . Stabilizing effect of various glycerol concentrations on the ATCase activity and feedback inhibition during storage for 48 hr. at 0°C. The activity and % inhibition at time of preparation were taken as 100%.

stabilizing effect of various concentrations of glycerol after 24 and 48 hours at 0°C. It can be seen that 10-15% glycerol stabilized both the activity which was completely retained and inhibition (which was 50-60% of the original) by  $2 \times 10^{-3}$  M UTP. The original enzyme activity was inhibited 75% by the same concentration of UTP. This inhibition was taken as 100% in Fig. 11. It was also found that 10% glycerol and  $5 \times 10^{-3}$  M UTP served to increase the stability of the 'regulatory property'; after 24 hours at 0°C almost 90% of the original inhibition was retained. The use of glycerol for stabilizing enzymes has been frequently practised (Guthohrlein and Knappe, 1968; Henrikson and Goldwasser, 1964). Similar results were obtained with 0.5% bovine serum albumin.

## 6. Conclusion

The results of the studies described here led to the purification scheme which is outlined under "Enzyme Purification".

Jambak et al.  
J Biol Chem. (1962)  
237, 345

## II. Enzyme Purification

### 1. Cultivation of MD169<sub>(c)</sub> and MD170-7X

Cells were grown at 25°C with vigorous shaking.

Growth was initiated by inoculating 100 ml. of complete medium, YPG + 250 ug. uracil/ml. with a loopful of MD169<sub>(c)</sub> stock culture. After 24 hours, 5.0 ml. of this preculture was used to inoculate each of 7 litres of G<sub>0</sub> plus 250 ug uracil/ml. Cells were allowed to grow for 40-42 hours after which time they were collected by centrifugation and washed with distilled water. The cell paste was stored at -20°C in volumes of 30.0 ml. each in 250 ml. Erlenmeyer flasks. Seven litres yielded approx. 40.0 ml. of cell paste. Growth of MD170-7X was carried out in G<sub>0</sub> supplemented with 225 ug. uracil/ml.

The preparation described here is based upon the use of 30.0 ml. of cell paste. Where larger amounts were used, volumes were adjusted accordingly.

### 2. Preparation of Crude Extracts

All centrifugation was carried out at 0°C and at 20,000 g. unless otherwise stated.

Crude extracts were prepared by the method of Kaplan

et al. (1967) scaled up. The 30 ml. cell paste was re-suspended in 50 ml. cold 0.2M phosphate buffer pH 7.4. Portions of 9 ml. each were then broken in bottles containing 10 g. glass beads (size 0.05-0.45 mm.) in a Braun disintegrator. The bottles were cooled with CO<sub>2</sub> during three 45 second periods of homogenization. After homogenization, the suspension was centrifuged for 30 min. and the resulting pellet then washed with 10.0 ml. 0.1M phosphate buffer pH 7.4. The washings were then combined with the first supernatant to give the crude extract (80.0 ml.) (step 1).

### 3. Protamine Sulphate Treatment

To the crude extract was added 800 mg. finely ground protamine sulphate. The addition was slow, with vigorous stirring, over a period of 8 min., and carried out in an ice bath. After 30 min. of stirring, the white solution was centrifuged for 20 min. Usually about 75 ml. supernatant was recovered (step 2).

### 4. Isopropanol-Ammonium Sulphate Fractionation

To the above supernatant, in a -15°C ice-salt bath, precooled isopropanol was added dropwise to a concen-

LIBRARY  
UNIVERSITY OF  
TORONTO

centration of 12% (10.2 ml. + 75.0 ml. supernatant) with rapid mixing. When the temperature of the mixture reached  $-8^{\circ}\text{C}$ , it was transferred to two cold polyethylene tubes and centrifuged in a pre-cooled rotor at  $-15^{\circ}\text{C}$  for 15 min. The resultant precipitate was dissolved in a solution of 20 ml. 0.1M phosphate buffer pH 6.8, containing 8.5 ml. saturated  $(\text{NH}_4)_2\text{SO}_4$  (i.e. 30% saturated). After 15 min. at  $0^{\circ}\text{C}$ , the solution was centrifuged for 10 min. and the supernatant brought to 45% saturation with  $(\text{NH}_4)_2\text{SO}_4$  (i.e. an additional 8.0 ml. saturated  $(\text{NH}_4)_2\text{SO}_4$  was added). The precipitate obtained after 20 min. at  $0^{\circ}\text{C}$  and 20 min. of centrifugation was dissolved in 10.0 ml. 0.1M phosphate buffer (step 3).

5. Calcium Phosphate Gel Adsorption and Elution and Ammonium Sulphate Fractionation

To the 10.0 ml. of enzyme solution was added 2.0 ml. of calcium phosphate gel (37.0 mg. dry wt/ml) and the resultant solution thoroughly mixed. After 10 min. at  $0^{\circ}\text{C}$ , the solution was centrifuged for 10 min. and an additional 4.0 ml.  $\text{Ca}_3(\text{PO}_4)_2$  gel was mixed with the supernatant. After 30 min. at  $0^{\circ}\text{C}$ , the gel was washed once with 10.0 ml. 0.1M phosphate buffer pH 6.8, and then

LIBRARY OF THE NATIONAL ARCHIVES CANADA

twice with 12.5 ml. 0.2M phosphate buffer pH 7.4 to yield the enzyme eluate. To this combined eluate (25.0 ml.) was added 20.5 ml. saturated  $(\text{NH}_4)_2\text{SO}_4$ ; after 20 min. at  $0^\circ\text{C}$  the precipitate was collected and redissolved in buffer immediately before use (step 4). All the experiments carried out in this study employed the enzyme in this state of purity unless otherwise stated.

#### 6. Gel Filtration on Sepharose 6B

The precipitate obtained above was dissolved in 2.0 ml. 0.5M phosphate buffer pH 7.6, containing 10% glycerol,  $2 \times 10^{-3}\text{M}$   $\beta$ -mercaptoethanol,  $2 \times 10^{-3}\text{M}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $5 \times 10^{-4}\text{M}$  glutamine and  $3 \times 10^{-3}\text{M}$  UTP, and applied to a Sepharose 6B column (2.5x88 cm.) which had been equilibrated overnight with the same buffer. The enzyme was eluted with this buffer mixture and the effluent was collected in 5.0 ml. fractions at a flow rate of 10 ml/hr. ATCase and CPSase activities were eluted between the 43rd and 52nd fraction tubes. These fractions were pooled and the enzyme was precipitated with 50% saturated ammonium sulphate. The precipitate obtained was dissolved in 0.5 ml. .05M phosphate buffer pH 7.4, containing the above additives with the exception of

glycerol. In some experiments, this material was subjected to analysis in a Model E analytical ultracentrifuge. Table 11 illustrates one such purification using 21 litres of the diploid strain MD169<sub>(c)</sub>. Table 11A shows that the specific ATCase activity of the crude extract of this derepressed strain was 181, approximately 4x that of the wild type strains. After purification, the specific activity rose by a factor of 26x after step 4, and up to 78x after Sepharose 6B. It should also be noted that the capacity to be inhibited by UTP increased with purification. Table 11B shows the effect of the purification steps which have been worked out for the ATCase activity on the CPSase activity from step 3 onwards. It can be seen that the CPSase activity was also purified by this procedure. Assay of the CPSase activity during the first 2 steps of the purification was omitted for the following reasons: the presence of the arginine-specific CPSase would give an incorrect estimate of the pyrimidine-specific CPSase in crude extracts; furthermore, the high blanks of the crude extracts due to interfering substances were a problem in CPSase assay even after passage through G-25 Sephadex (Lue and Kaplan, 1969), because of the resultant dilution and low activity

FORNIA  
CORPORA  
MILITIA

Table 11

## Purification of Yeast ATCase and CPSase from Strain MD169(C)

Purification Steps	Volume (ml.)	Total Protein	Total Activity Units	Specific Activity	Purification	Recovery (%)	Inhibition by $2 \times 10^{-3}M$ UTP (%)
A. ATCase							
1. crude extract	280	5393	976836	181	1.0	100	55
2. protamine sulphate	4139	4139	759058	183	1.0	78	58
3. isopropanol ppt. (0-10%) ammonium sulfate (30-45%)	31	187	373265	1996	11.0	38	58
4. calcium phosphate gel wash. ammonium sulphate (30-45%)	2.0	53.2	245582	4616	25.5 (2.3)	25	72
5. Sepharose 6B eluate ammonium sulphate (30-45%)	0.5	12.2	173050	14397	78.3 (7.2)	18	74

-124-

Table 11 (contd)

Purification Steps	Volume (ml.)	Total Protein	Total Activity Units	Specific Activity	Purifi- cation	Recovery (%)	Inhibition by 2x10 <sup>-5</sup> M UTP (%)	Ratio ATCase CPSase
B. CPSase								
3. isopropanol ppt (0-10%) ammon. sulf- phate (30-45%)	31	187	137039	733			84	3.7
4. calcium phos- phate gel wash. ammon. sulf- phate (30-45%)	2.0	53.2	103385	1943	(2.65)		80	2.4
5. Sepharose 6B eluate ammon. sulf- phate (30-45%)	0.5	12.2	83525	6846	(9.3)		70	2.1

Numbers in brackets represent the purification factor using step 3 as unity.

All other calculations are based on crude extract (step 1).

of the eluted enzyme preparations. If the purification obtained at step 3 is taken as unity, the specific activity of ATCase increased by 2.3 and 7.2x after steps 4 and 5. On the other hand, CPSase increased by 2.65 and 9.3, a roughly parallel co-purification. In all experiments, purification of the CPSase activity was consistently found to be equal to or greater than that of ATCase (Lue and Kaplan, 1969).

Table 12A shows the purification of the enzymes extracted from 28 litres of strain MD170-7X, a derepressed strain lacking the CPSase activity of the arginine pathway. The ATCase activity was purified by a factor of 23x, and again the sensitivity of the ATCase activity to inhibition by UTP increased with purification. The CPSase activity was co-purified as shown by the ratio of ATCase:CPSase specific activity, which was 3.1 in this strain, compared with 2.1 in the strain possessing the CPSase of the arginine pathway. Hence it is clear that, during purification of the ATCase, it is the CPSase of the pyrimidine pathway which is being co-purified.

Purification of the ATCase and CPSase of the haploid strains FL233-3C and FL80-2A, which lack the arginine-specific CPSase, gave very similar results which have

Table 12  
Purification of Yeast ATCase and CPSase from Strain MDL70-7X  
Which Lacks the CPSase of the Arginine Pathway

Purification Steps	Volume (ml.)	Total Protein	Total Activity Units	Specific Activity	Purification	Recovery (%)	Inhibition by $2 \times 10^{-3}M$ UTP (%)
A. ATCase							
1. crude extract	465	12235	1534500	125		100	40
2. protamine sulphate supernatant	430	8170	936450	114.6		61	64
3. isopropanol ppt. (0-10%) ammonium sulphate (30-45%)	36.5	486.5	831678	1709.5	13.7	54	85
4. calcium phosphate gel wash. ammonium sulphate (30-45%)	6.1	219.6	621264	2829.1	22.6 (1.7)	41	83

Table 12 (contd.)

Purification Steps	Volume (ml.)	Total Protein	Total Activity Units	Specific Activity	Purifi- cation	Recovery (%)	Inhibition by $2 \times 10^{-3}M$ UTP (%)	Ratio ATCase / CPSase
B. CPSase								
3. isopropanol ppt (0-10% ammonium sulfate (30-45%))	36.5	486.5	280546	577.2			81	3.0
4. calcium phosphate gel wash. ammonium sulfate (30-45%)	6.1	219.6	203189	925.3			85	3.1

Numbers in brackets represent the purification factor using step 3 as unity.

All other calculations are based on crude extract (step 1).

already been published (Lue and Kaplan, 1969). In that paper, the CPSase activities were measured approximately in crude extracts to permit estimation of the degree of purification. The ATCase activity purified through step 4 showed a purification of 23x and the CPSase approximately 65x in the former strain; the purification was 13x and 89x for the latter strain.

In connection with the apparently greater co-purification of CPSase than ATCase, it was of interest (for reasons which will become clear below) to examine the extent to which the ATCase activity of the fractions discarded during purification was sensitive to inhibition by UTP. Table 13 shows that, with each step of purification, the ATCase activity of the discarded fractions became progressively less sensitive to inhibition by UTP as opposed to the increased sensitivity of the retained fractions (compare Table 12). This indicated that there was present initially a fraction possessing ATCase activity which was insensitive to feedback inhibition, but which was eliminated during the purification procedure.

Table 13  
Sensitivity to Feedback Inhibition of the  
Fractions Discarded During Purification

Purification Step	Specific Activity	% of Total Activity	Inhibition by $2 \times 10^{-3} M$ UTP (%)
2(A) isopropanol supernatant	3.4	1.3	80
3(A) ammonium sulphate supernatant after 45%	49.7	1.4	69
4(A) supernatant after the enzyme was adsorbed to $Ca_3(PO_4)_2$ gel	51.7	0.5	34
4(B) ammonium sulphate fraction supernatant after 45%	85.6	1.6	8

ENTANA GRIFFIN CANADA

### III. Molecular Weight Estimation

#### 1. Gel Filtration

Fig.12 shows the elution profile from Sepharose 6B in the presence of UTP. It can be seen that ATCase and CPSase are co-eluted with the peak of activities at fraction #46. This was slightly ahead of thyroglobulin (molecular weight 670,000), whose peak was at fraction #49. The ATCase activity was highly sensitive to inhibition by UTP (about 80% inhibition by  $2 \times 10^{-3} \text{M}$  UTP). As expected, the CPSase was also highly sensitive to UTP. Also indicated in Fig.12 is the elution profile for catalase, blue dextran and bovine serum albumin; the elution profiles for ovalbumin and ribonuclease were also determined but are not indicated in Fig.12. In a single experiment UTP was omitted both from the enzyme preparation and from the mixture used to equilibrate the column. In this experiment, the ATCase activity was distributed over a very wide range, from fractions 43-64. The ATCase maximum was observed at fraction 54, whereas the CPSase was at 56. This activity was also spread out like the ATCase.

Fig.13 shows the elution profile of the ATCase activity along with several standard proteins of known mole-

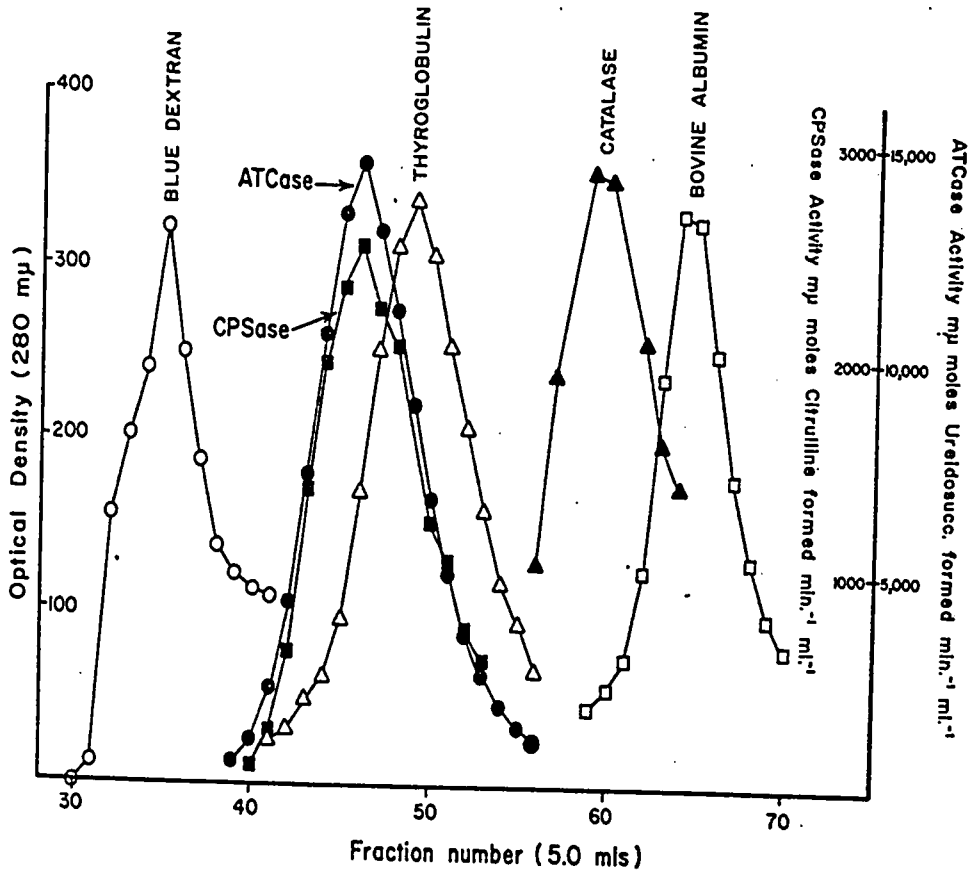


Fig.12 . Elution curves of several marker proteins, ATCase and CPSase activities from a 2.5x94 cm. Sepharose 6B column at 5°C. The sample size was 2.0 ml. containing 20 mg/ml. thyroglobulin ( $\Delta$ - $\Delta$ ), bovine serum albumin ( $\square$ - $\square$ ), ovalbumin and ribonuclease (not shown), catalase (15 mg/ml.,  $\blacktriangle$ - $\blacktriangle$ ) and blue dextran (0.25%,  $\circ$ - $\circ$ ). These were estimated by their absorbance at 280 m $\mu$ . The elution rate was 10 ml/hr. and 5.0 ml. fractions were collected. Further details are given under Methods.

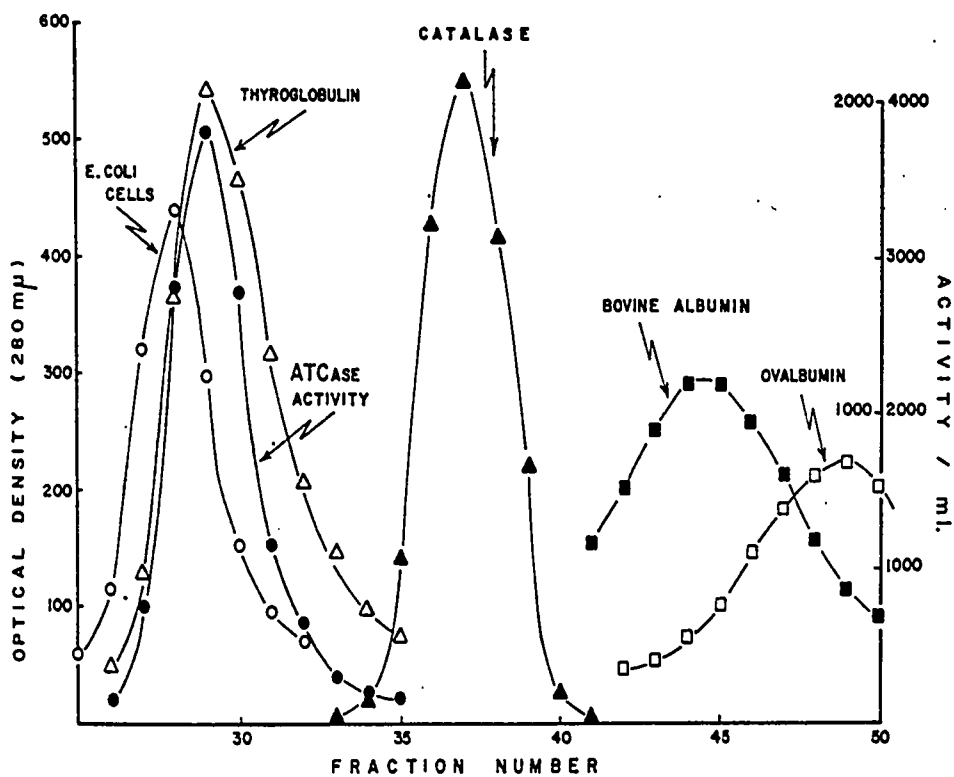


Fig. 13 . Elution curves of several proteins and the ATCase activity from a 1.5x88.5 cm. Sephadex G-200 column at 5°C. The sample size was 0.6 ml. containing 20 mg/ml. thyroglobulin (Δ-Δ), bovine serum albumin (■-■), ovalbumin (□-□), catalase (15 mg/ml., ▲-▲), and *E. coli* cells (1 mg/ml., O-O). These were estimated by their absorbance at 280 mμ. The elution rate was 9 ml/hr. and 2.1 ml. fractions were collected. Further details are given under Methods.

cular weight from Sephadex G-200 in the absence of UTP. Unlike the spreading out which occurred with Sepharose 6B, the ATCase activity was eluted in a pronounced symmetrical peak despite the absence of UTP, slightly after E. coli cells, but again slightly ahead of thyroglubulin. The CPSase activity was greatly reduced after elution but could be detected in the most active ATCase fractions. The ATCase activity could be inhibited to 65% by  $2 \times 10^{-3} M$  UTP before gel filtration; however, after elution, the activity could be inhibited only to 8% by the same concentration of UTP.

When the Sepharose 6B data were plotted by the method of Whitaker (1963), in which  $K_{av}$  is the ordinate and log molecular weight the abscissa, the results shown in Fig.14 were obtained. All the standard proteins fit the curve drawn except for catalase, which lies slightly above. It may be noted in this regard, that in the calibration data furnished by the manufacturer in their brochure introducing the gel, catalase was omitted. From this plot, a value for ATCase of  $1 \times 10^6$  daltons was obtained when the column was pre-equilibrated with UTP. When UTP was omitted, the extrapolated molecular weight of ATCase fell off to  $3.6 \times 10^5$  daltons; CPSase was estimated to

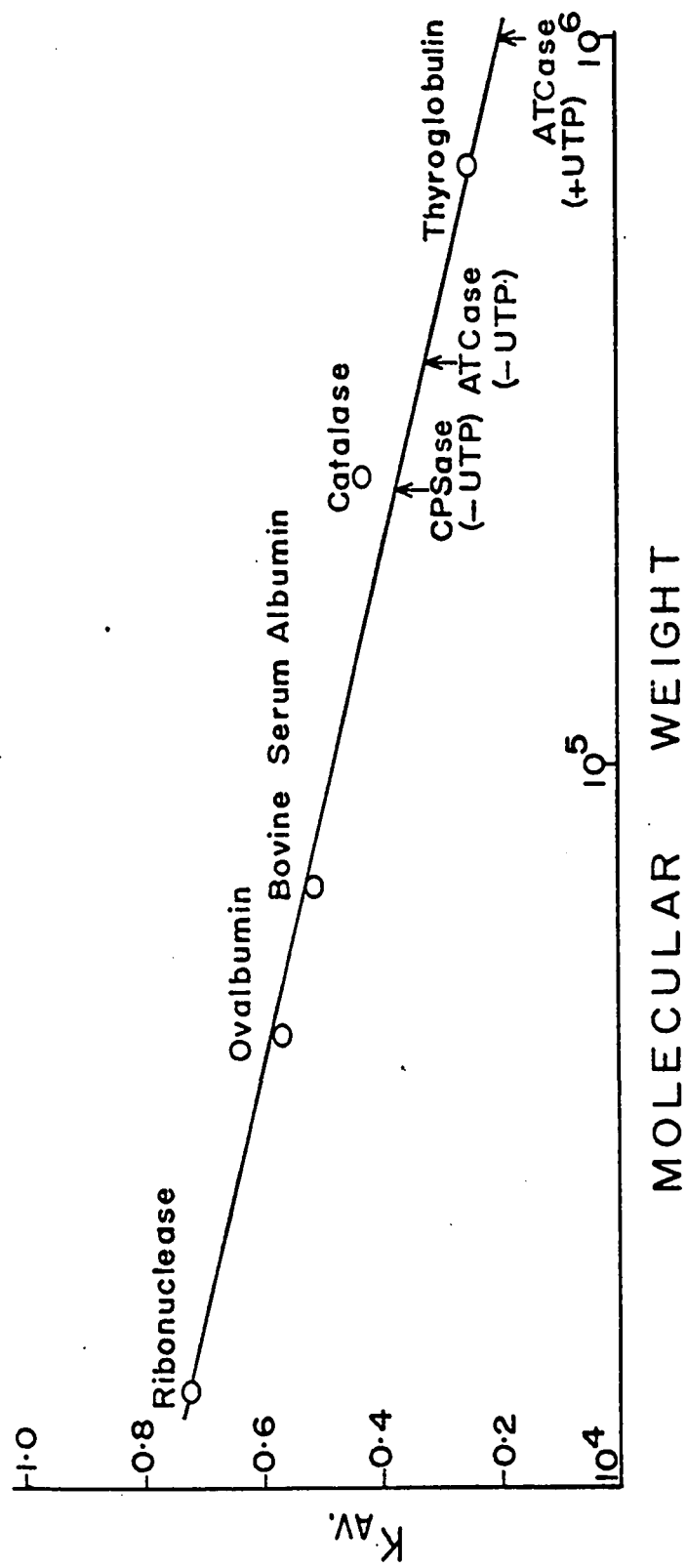
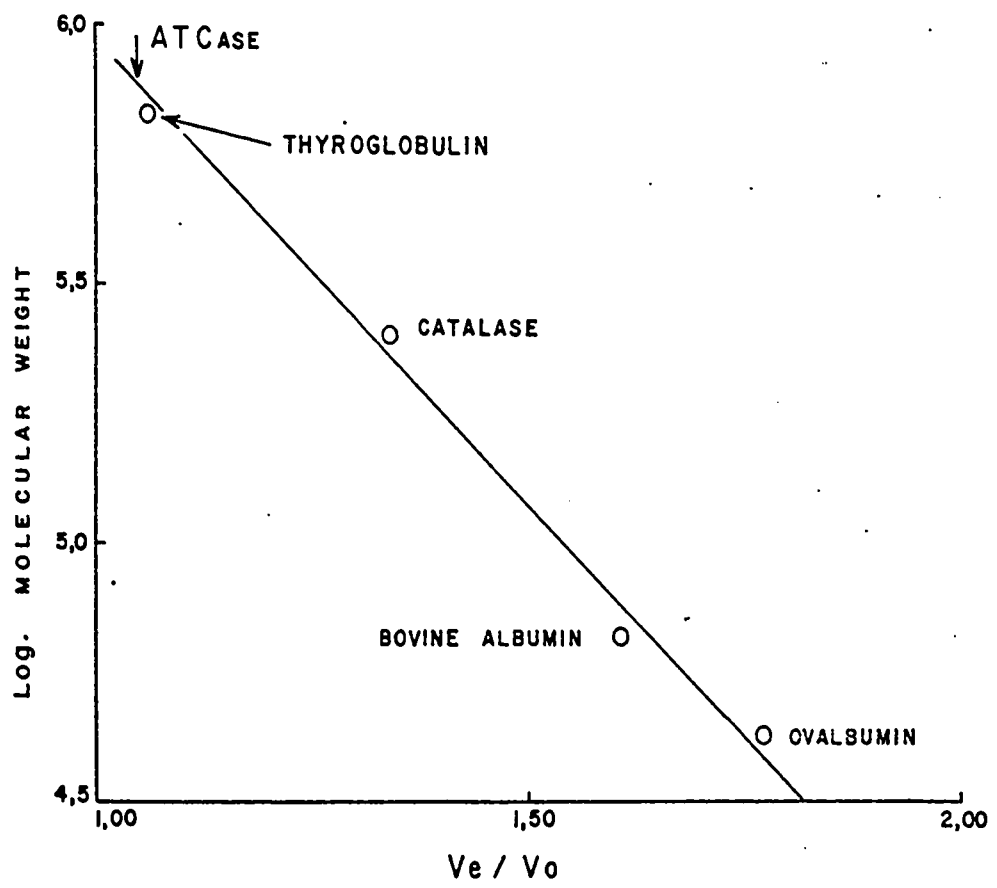


Fig. 14. Correlation of the elution data from Sepharose 6B with molecular weight.

correspond to a molecular weight of  $2.5 \times 10^5$  daltons under these conditions. Both of these latter estimates are highly approximate due to the great flattening of the elution profiles of the two activities when UTP was omitted.

Fig.15 shows the elution data from Sephadex G-200 plotted according to the recommendations of Whitaker (1963). A molecular weight of 7-800,000 was estimated from Fig.15 .

The molecular weight of the marker proteins together with the molecular weight of the enzyme as estimated by gel filtration are given in Table 14.



OTTAWA, ONTARIO, CANADA

Fig. 15 . Correlation of elution data from Sephadex G-200, with molecular weight. The elution data are plotted according to Whitaker (1963), i.e.  $\frac{V_e}{V_o}$  vs log (MW).

Note legend under Fig. 13 for details.

Table 14

Molecular Parameters of Standard Proteins and ATCase  
Determined by Gel Filtration

Proteins	Molecular Weight	References
Ovalbumin	$4.25 \times 10^4$	Light & Hager (1968)
Bovine Serum Albumin	$6.6 \times 10^4$	Siegel & Monty (1966)
Catalase	$2.5 \times 10^5$	" " "
Thyroglobulin	$6.7 \times 10^5$	Light & Hager (1968)
ATCase per Sephadex G-200 (-UTP)	$7-8.0 \times 10^5$ *	experimental per G-200
ATCase per Sepharose 6B (+ UTP)	$1 \times 10^6$ *	experimental per Sepharose 6B
ATCase (-UTP)	$3.6 \times 10^5$ *	" "
CPSase (-UTP)	$2.4 \times 10^5$ *	" "

\* Molecular weight estimation according to the methods of Whitaker (1963) and Andrews (1965).

2. Centrifugation in a Model E Analytical Ultracentrifuge

Fig. 16 shows a schlieren sedimentation pattern of a sample purified to step 5, Table 11, which was dissolved in 0.5 ml. 0.05M phosphate buffer containing  $2 \times 10^{-3}M$   $\beta$  mercaptoethanol,  $2 \times 10^{-3}M$   $MgSO_4$ ,  $5 \times 10^{-4}M$  glutamine and  $3 \times 10^{-3}M$  UTP. Two major sedimentation peaks and another minor peak are seen, indicating that the sample was not homogenous.

A low estimate of the M.W. can be calculated on the basis of assuming a partial specific volume of  $0.725 \text{ cm}^3/\text{g}$ , diffusion coefficient of 4.02F (Guthohrlein and Knappe, 1968), and density of 1.0. The greatest discrepancy doubtless occurs because of the assumption that density was 1, since the enzyme was precipitated by  $(NH_4)_2SO_4$  fractionation from a 10% glycerol solution and no steps were taken to remove these contaminants.

On these assumptions the heavier major peak had a sedimentation coefficient of 22.3S (MW 600,000) and the second (but smaller) major peak was 11.6S (MW 300,000). The sedimentation value (and MW) of the minor peak could not be calculated. The schlieren pattern also showed that the enzyme sample was at best 60% pure, on the assumption that the largest peak is our enzyme complex.

OTTAWA CENTRAL CANADA

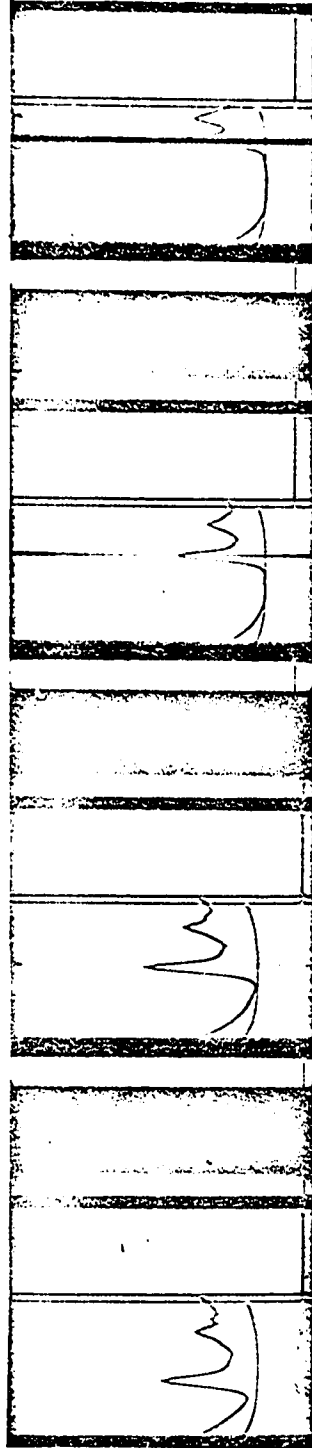


Fig. 16 Sedimentation pattern of a preparation after step 5, Table 11  
The sedimentation is from right to left and the pictures were taken 24, 28, 32 and  
36 minutes after the centrifuge had reached 48,000 rpm; the bar angle was 70°.

3. Ultracentrifugation in Sucrose Density Gradients

Partially purified enzyme from strains FL233-3C and MD170-7X together with beef liver catalase (MW 250,000) as an internal marker was subjected to sucrose density gradient centrifugation;  $3 \times 10^{-3} \text{M}$  UTP was included both in the mixture layered on the gradient and in the sucrose solution. Fig.17 shows that ATCase and CPSase co-sedimented in a single peak. This peak corresponds to a sedimentation coefficient of 26.3S and to a molecular weight of 810,000. The sedimentation coefficient above was calculated according to Bishop (1966) and by assuming a partial specific volume of  $0.725 \text{ cm}^3$ . The sedimentation coefficient for catalase calculated by this method was 12S.

Sensitivity of the ATCase activity to  $2 \times 10^{-3} \text{M}$  UTP is indicated on the graph for fraction 5, the peak at which ATCase and CPSase co-sedimented and tube 13, where there was a minor peak of activity; the percent inhibition was 86 at the former and 33 at the latter. This minor peak corresponds to a sedimentation coefficient of 15.1S and the molecular weight of 355,000 daltons. The scatter between the two peaks noted for both activities seems of questionable significance but

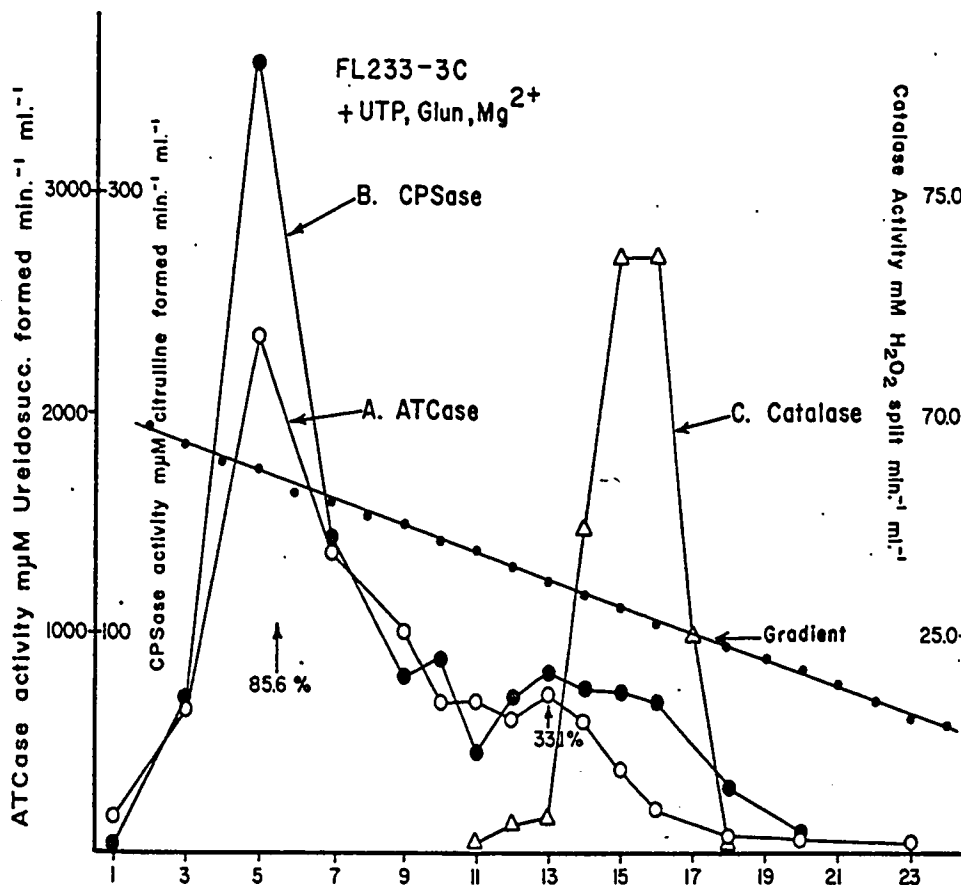


Fig. 17. Sucrose density gradient centrifugation of a purified preparation from FL233-3C, spun at 60,000 rpm for 3 hr. at 5°C. Linear gradients (4.6 ml.) using 5 and 20% (w/v) sucrose in 0.02M phosphate buffer pH 7.6, containing 0.01M MgSO<sub>4</sub>·7H<sub>2</sub>O, 4x10<sup>-4</sup>M glutamine, 3x10<sup>-3</sup>M UTP, and 10<sup>-3</sup>M B-mercaptoethanol were prepared as described by Martin and Ames (1961). Sample (0.1 ml.) was layered on the gradient with catalase as an internal marker. After centrifugation in the SW-65 rotor of the Beckman model L2-65 ultracentrifuge, fractions containing 13 drops (0.22 ml.) were collected. Catalase activity was estimated by a modification of the method of Chantrenne (1955). The activities are expressed as mM H<sub>2</sub>O<sub>2</sub> split/min/ml. aliquots and ATCase 10 ul.

may indicate the existence of intermediate states of aggregation of subunits.

Fig.18 shows the results of a sucrose density gradient ultracentrifugation of another preparation of enzyme from strain FL233-3C under conditions identical to those of Fig.17 except that UTP was omitted. It will be seen that the two activities co-sedimented in a sharp peak corresponding to 15.6S and to a molecular weight of 376,000. The ATCase had a considerably reduced sensitivity to UTP (approx. 20-30% inhibition by  $2 \times 10^{-3} \text{M}$  UTP, as opposed to 75-85% after centrifugation in the presence of UTP). Again minor peaks and shoulders were noticed on either side of the main peak. However, it will be noticed that there was essentially no activity peak sedimenting in tube 5, where both activities would have co-sedimented had UTP been present during the ultracentrifugation.

It is therefore possible to conclude that CPSase and ATCase exist within the cell as a high molecular weight aggregate in which form they are co-extracted, co-purified, co-eluted and co-sedimented. UTP is essential for the maintenance of the aggregate in its high molecular weight form; omission of UTP

ETHAN, GUYARD, CHAMBERLAIN

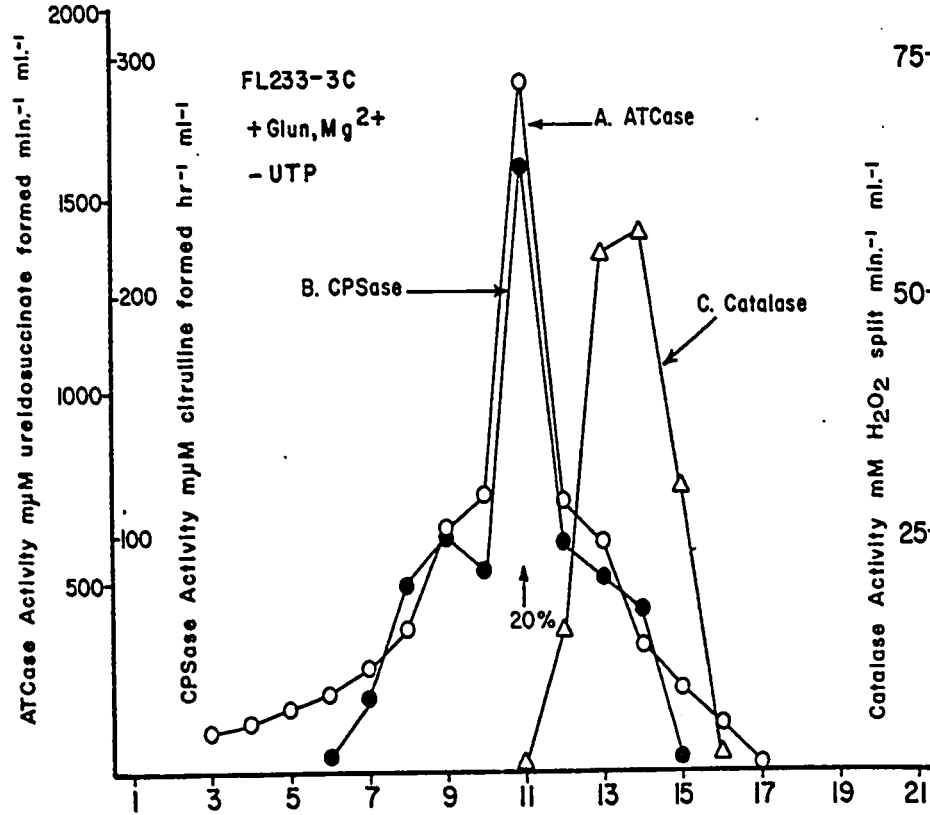


Fig. 18. Sucrose density gradient centrifugation of another preparation from FL233-3C. Conditions are similar to those of Fig. 17, except that UTP was omitted from the gradient and the sample solution.

from either gel filtration or sucrose gradient results in disaggregation to a size approximately one half the original, with both activities still associated, but with the ATCase of greatly reduced sensitivity to inhibition by UTP.

In an attempt to dissociate the ATCase and CPSase peaks in partially disaggregated material, a number of different experimental approaches were attempted. Under most conditions of centrifugation in the absence of UTP, the two activities co-sedimented in a single peak similar to the experiment shown in Fig.18 . However, when the glutamine and  $Mg^{++}$  were omitted from the gradient, there was a tendency for the CPSase peak to trail behind the ATCase peak. One such experiment is illustrated in Fig.19 for a preparation from strain FL233-3C. The peak of the ATCase activity occurred in fraction 12 (curve A), whereas the CPSase peak occurred in fraction 13 (curve B) as did catalase (curve C). Also plotted is the ATCase activity in the presence of  $2 \times 10^{-3}$  M UTP (curve A'). It is interesting to note that immediately after the peak of ATCase activity, the amount of inhibition caused by UTP falls drastically and at the same position the CPSase activity is increased. There are several possible

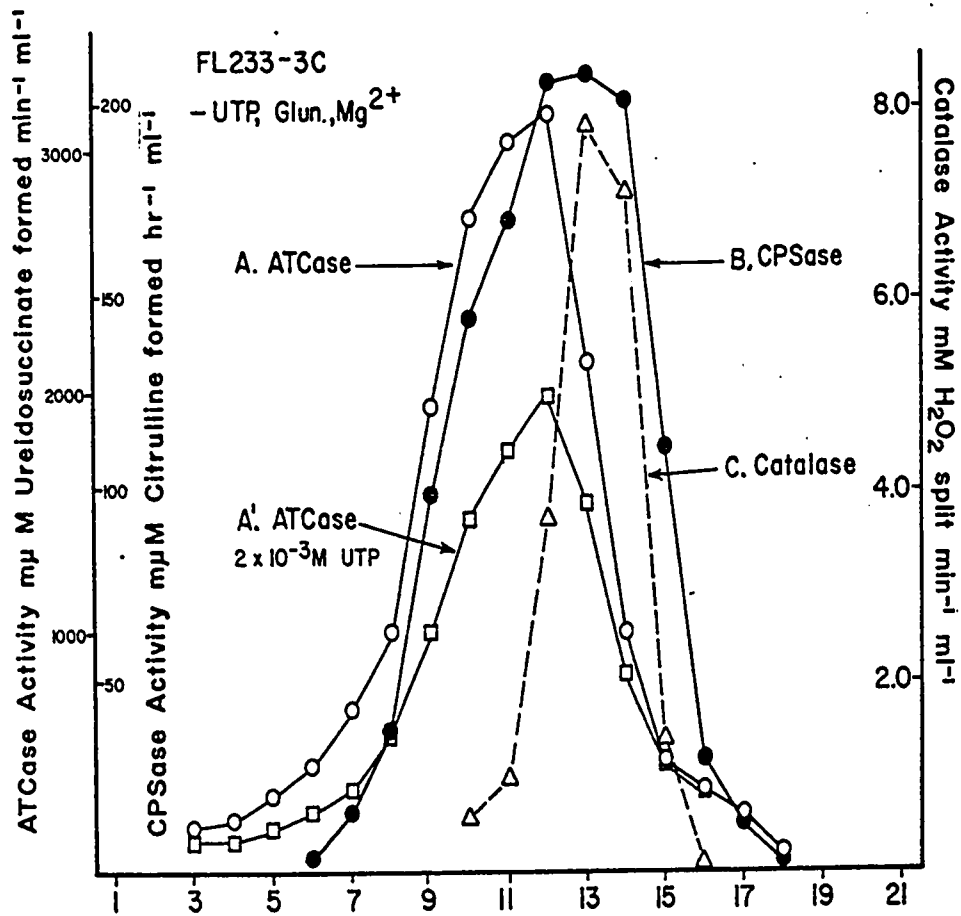


Fig. 19. Sucrose density gradient centrifugation of another preparation of FL233-3C. Conditions are similar to those in the previous 2 figures, except that Mg<sup>++</sup>, glutamine and UTP were omitted from the gradient and the sample solution.

The fractions are the same as those shown in curve A. UTP was present only in the ATCase assay mixture of curve A', not during the centrifugation.

explanations of this apparent dissociation of CPSase from ATCase. One conceivable explanation would be the presence of some arginine specific CPSase of lower molecular weight. To exclude this unlikely possibility the experiment was repeated with MD170-7X, a strain which lacks the arginine-specific CPSase. The results are shown in Fig. 20 . Curve A shows the sedimentation of the ATCase activity in the sucrose gradient. The major peak of ATCase activity occurs in tube 12, as in the previous experiment. However, another somewhat heavier peak was noted in tube 8, which was absent in the results of the other strain. This peak had low but detectable CPSase associated with it as shown in curve B. Curve B also shows a single peak at tube 13, corresponding to catalase as in the previous experiment. The possibility that the trailing of the ATCase by the CPSase was due to an extraneous arginine-specific enzyme was therefore eliminated. It will be noted in curve A in tube 17 another peak of ATCase occurred, this one with only traces of CPSase activity and the ATCase activity was totally insensitive to inhibition by UTP.

Table 15 summarizes the molecular weight of the various fractions as determined by sucrose gradient cen-

ATPase, GTPase, Cytosol

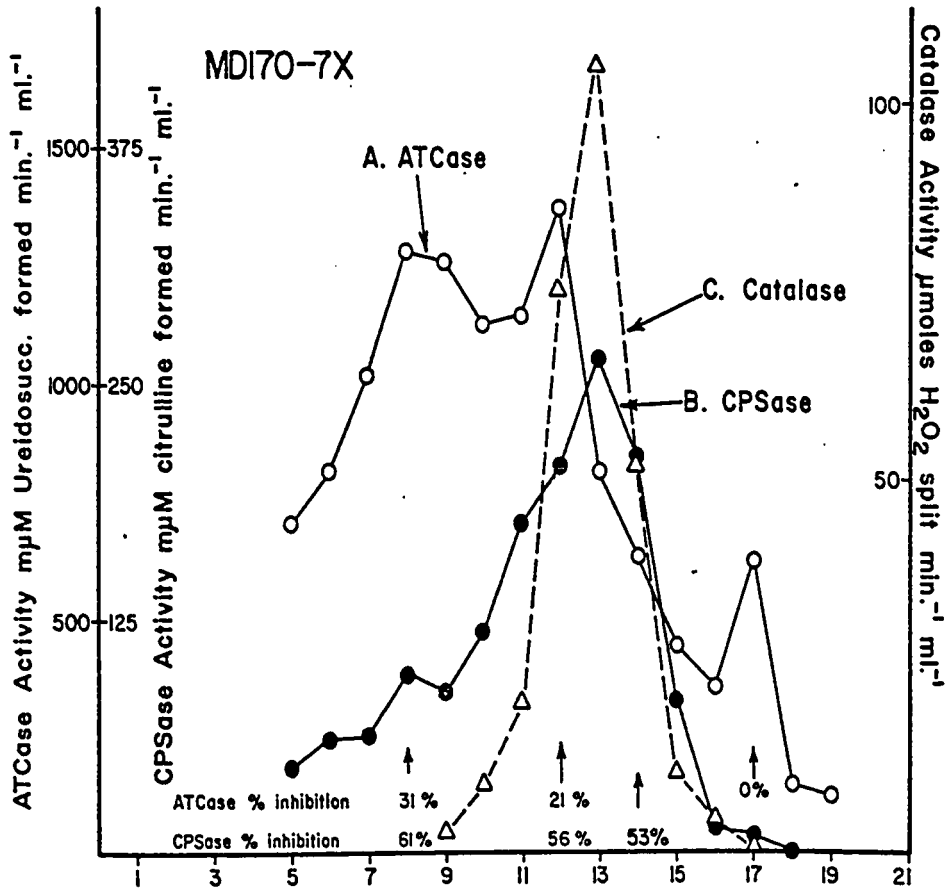


Fig. 20. Sucrose density gradient centrifugation of a preparation from MD170-7X. Conditions are identical to those of Fig. 19.

Table 15

Molecular Weights from Sedimentation Data

Conditions	Strain	ATCase M.W. (daltons)	ATCase % inh. $2 \times 10^{-3} \text{M UTP}$	CPSase M.W. (daltons)
1. UTP present + $\text{Mg}^{++}$ + Glun	FL233-3C	$8.1 \times 10^5$	85	$8.1 \times 10^5$
2. UTP absent + $\text{Mg}^{++}$ + Glun	FL233-3C	$3.8 \times 10^5$	20-33%	$3.8 \times 10^5$
3. UTP, Glun & $\text{Mg}^{++}$ absent	FL233-3C	$3.8 \times 10^5$	20-30%	$2.5 \times 10^5$
	170-7X	$3.8 \times 10^5$		$2.5 \times 10^5$

ETTING OFFICIAL CORNER

trifugation.

These data lead to certain conclusions. Where UTP was present during the ultracentrifugation, the ATCase is associated with the CPSase in an aggregate of very high molecular weight ( $7.8 - 9.6 \times 10^5$  daltons), and in this state the ATCase is highly sensitive to feedback inhibition by UTP. But, when the centrifugation is carried out in the absence of UTP, the ATCase activity recovered was of low sensitivity to feedback inhibition. With glutamine and  $Mg^{++}$  present, the two activities co-sedimented in a single peak of molecular weight of approx. one-half. If glutamine and  $Mg^{++}$  were omitted as well as UTP there was a tendency of the CPSase activity to sediment in a peak slightly lighter than that of the ATCase, but in which some ATCase activity was always present. On the other hand, the CPSase activity was always sensitive to feedback inhibition.

#### IV. Experiments Showing Further Disaggregation

It was desirable to find a treatment which would cause further disaggregation of the enzyme complex into fragments of lower molecular weight than those obtained by ultracentrifugation in the absence of UTP,  $Mg^{++}$  and

glutamine.

1. DEAE Sephadex

Fig.21 shows an elution profile of the ATCase activity from columns of DEAE-Sephadex at room temperature. This treatment yielded an eluate which showed but a single band upon electrophoresis on cellulose acetate strips; its specific ATCase activity increased by a factor of 5-10x over that of the calcium phosphate eluates (Table 11 , step 4). However, 2/3 of the activity placed on the column was lost. Such preparations had lost all of their CPSase activity and their ATCase was completely insensitive to feedback inhibition. Attempts to find the CPSase activity by elution with higher buffer concentrations, as well as mixing the active with certain inactive fractions, were without success. Fig.22 shows a sucrose density gradient run on the combined contents of tubes 6 & 7 of Fig.22 together with catalase as an internal marker. A single peak of ATCase activity was recovered which was of considerably lower molecular weight than the catalase marker. The molecular weight was calculated to be 130,000.

ENTRADA ANTARCTICO CANADA

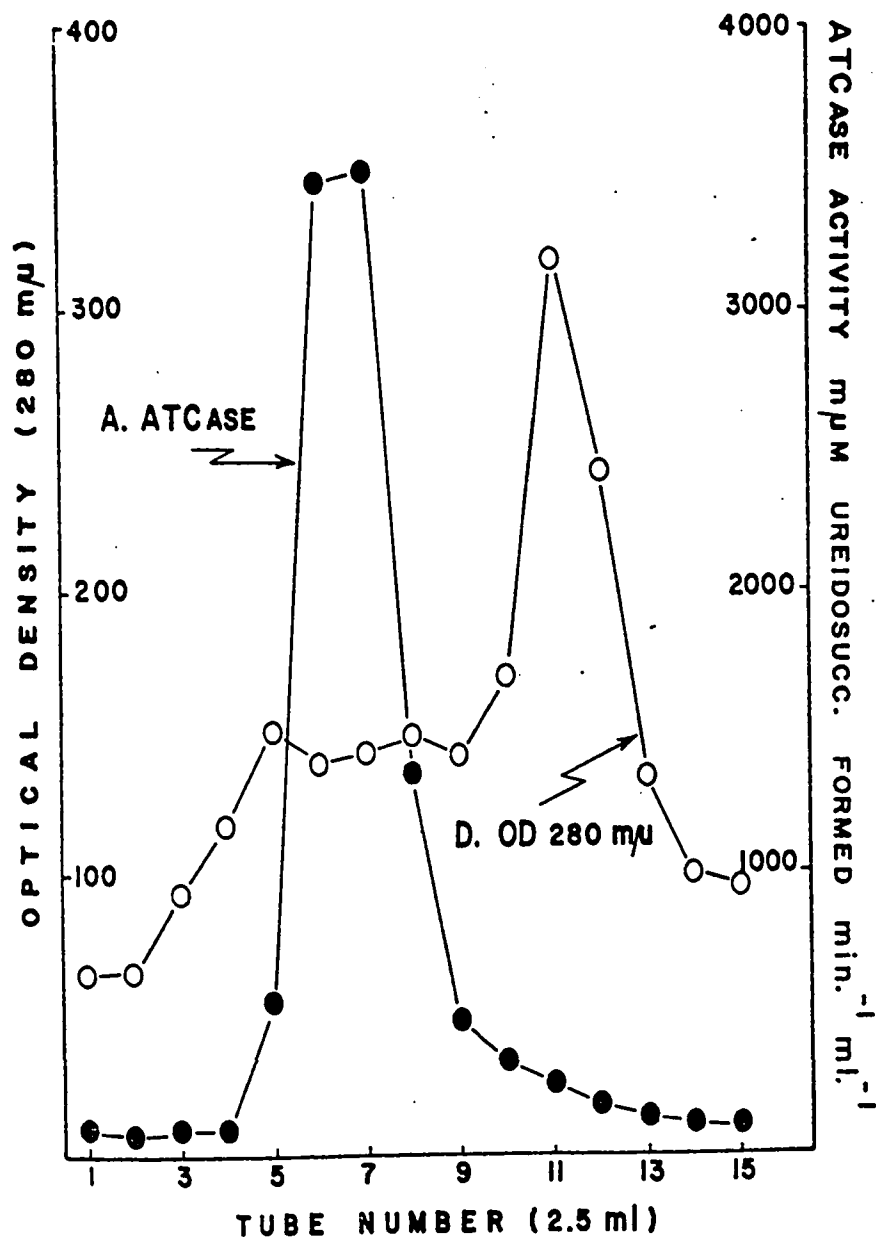


Fig. 21. DEAE-Sephadex column chromatogram. The purified preparation was dissolved in 0.5 ml. of 0.2M phosphate buffer, pH 6.85, containing  $10^{-3}$ M B-mercaptoethanol and  $2 \times 10^{-4}$ M  $MgSO_4$ . This sample was applied to a 1.5x27 cm. column of DEAE-Sephadex and eluted with the same buffer at room temperature. Each fraction contained 2.5 ml.

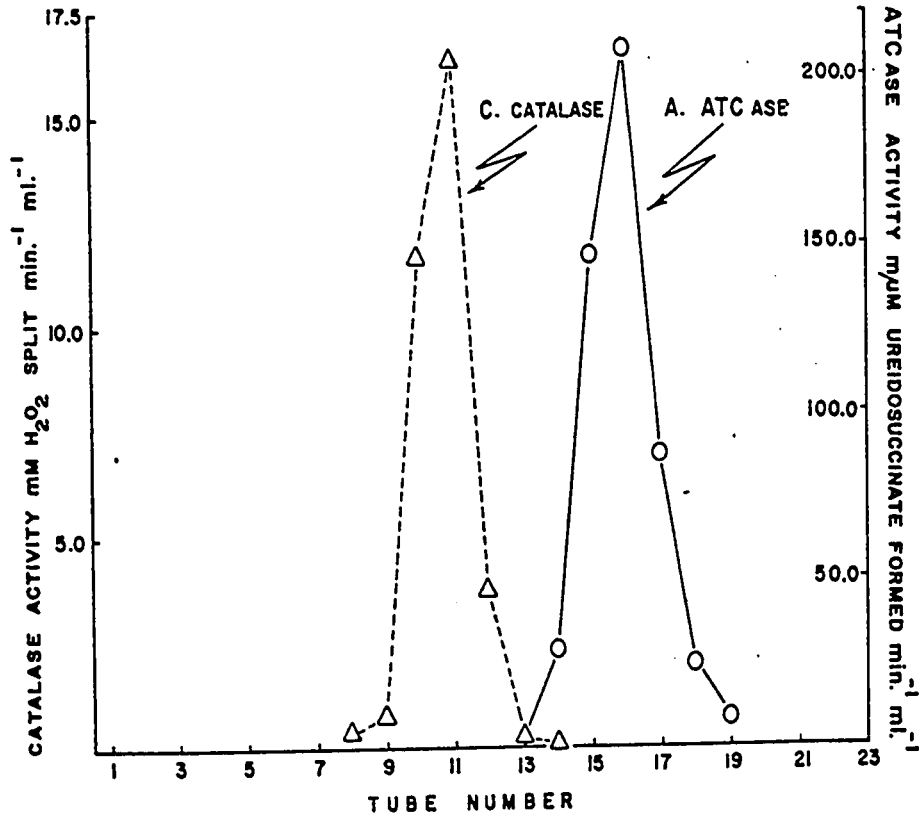


Fig. 22. Sucrose density gradient centrifugation of  $(\text{NH}_4)_2\text{SO}_4$  preparation of tubes 6 and 7 (Fig. 21) spun at 60,000 rpm for 5 hr. at 5°C. The procedure was the same as that of Fig. 20.

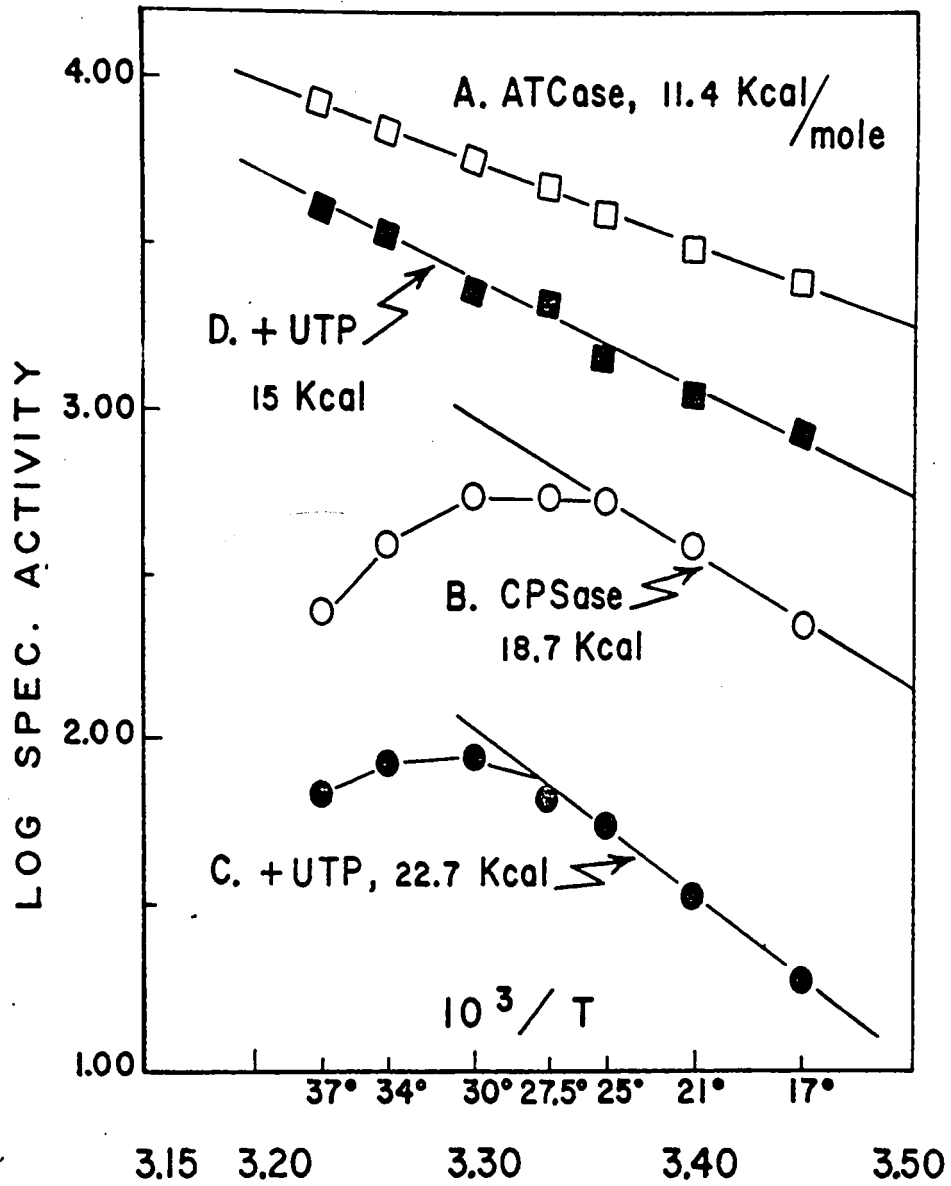
BRITISH CHEMICAL COUNCIL

## 2. Effects of Heat

Heat has been shown to cause disaggregation of the ATCase of E. coli (Gerhart and Pardee, 1962), and it was therefore tried on this system.

Fig. 23, an Arrhenius plot, illustrates the effect of temperature on the enzyme-substrate system for both enzyme activities. Activation energies for ATCase, in absence and presence of UTP, were 11.4 and 15 Kcal/mole respectively; in another experiment they were 11.2 and 14.2 respectively, values quite close to those previously reported (Kaplan and Messmer, 1969; Kaplan et al., 1967). The values for CPSase were considerably higher (18.7 and 22.7 Kcal/mole). The curves (B,D) for ATCase were linear up to 37°C and higher; in another experiment, the ATCase activity was found to fall off slightly at 35°C. CPSase activity in all experiments was optimal between 25 and 30°C and fell off at higher temperatures of assay (curves A and C).

Another experiment was performed in which an enzyme preparation was heated at 50°C, aliquots were removed at various periods of the incubation and subsequently assayed for ATCase and CPSase activities in the presence and absence of UTP. Fig. 24 illustrates the effect of



ELIANA ORTIZINO CANALES

Fig. 23: Arrhenius plot of ATCase and CPSase activities in presence and absence of  $2 \times 10^{-3} M$  UTP.

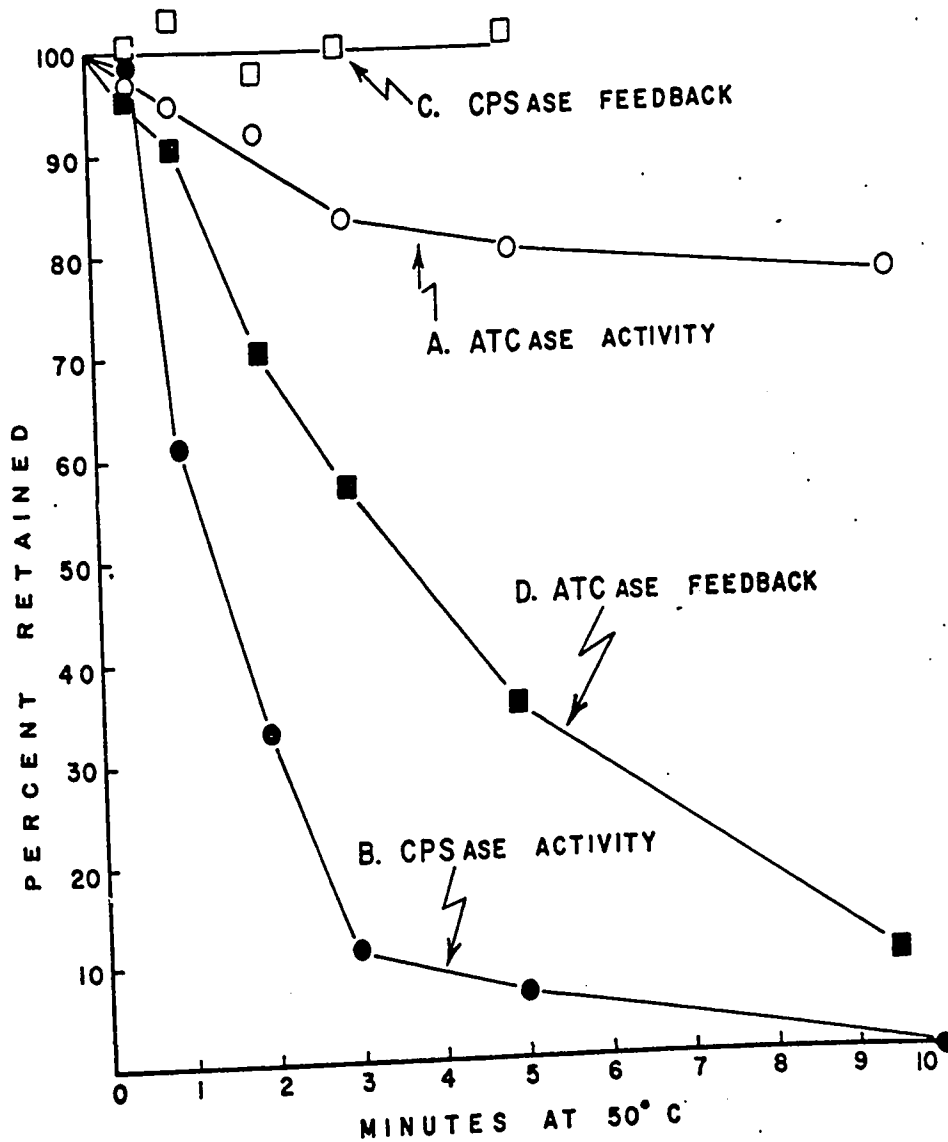


Fig. 24. The effect of heating at 50°C on the activities and feedback inhibitions of ATCase and CPSase. At zero time ATCase was inhibited to 54% and CPSase to 79% by  $2 \times 10^{-3} M$  UTP. These percentages were considered as 100% inhibition.

WILSON, GILBERT, CANADON

incubation at 50°C on the two enzyme activities and on their sensitivity to UTP. Curve A shows that the ATCase activity fell off slightly but significantly in a somewhat regular way, exactly as previously reported (Kaplan et al., 1967). Curve D shows that the sensitivity of the residual ATCase activity to UTP diminished sharply during heating, as previously described (Kaplan et al., 1967). Curve B indicated that CPSase activity was highly thermo-labile, falling off rapidly and more or less parallel to the decline in sensitivity of the ATCase to inhibition by UTP. Curve C illustrates the remarkable thermal stability of the sensitivity to feedback inhibition of the residual CPSase activity; so long as there was detectable residual CPSase activity, this remained fully sensitive to UTP (Lue and Kaplan, 1970).

In other experiments, a purified preparation was pretreated for 5 min. at 50°C and subsequently subjected to sucrose density gradient ultracentrifugation. One such experiment is illustrated in Fig. 25. As in the case of DEAE Sephadex, but one peak of the ATCase activity (MW 140,000) occurred devoid of sensitivity to UTP and considerably lighter than the catalase marker. No trace of the CPSase activity remained after this treatment.

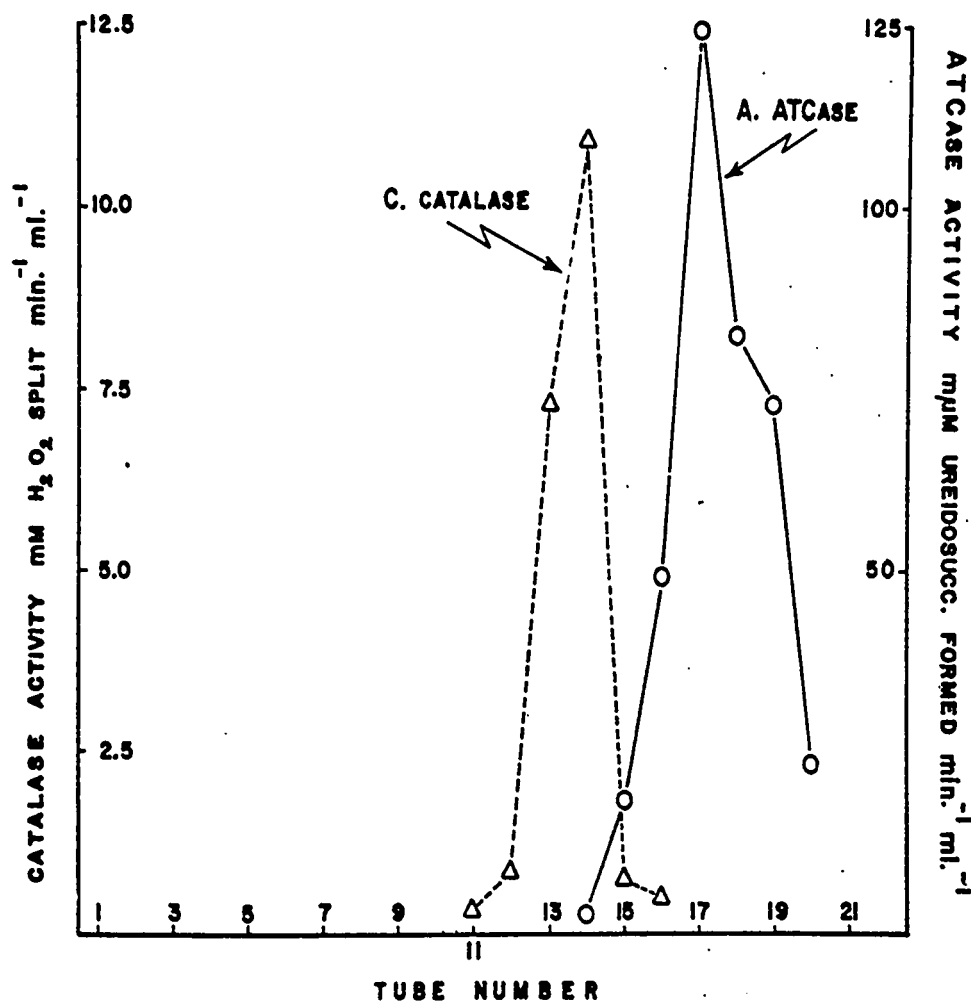


Fig. 25. Sucrose density gradient centrifugation of a purified preparation which has been heated at 50°C for 5 minutes. Catalase was used as an internal standard. Fractions were collected after centrifugation at 60,000 rpm. for 3 hr. Catalase was assayed by a modification of the method by Chantrenne (1955). In unheated preparations centrifuged under identical conditions, the ATCase and CPSase co-sedimented in a single peak in tube #5 in the presence of UTP, Mg<sup>++</sup> and glu'n, and tube #11 in the presence of Mg<sup>++</sup> and glu'n.

After 5 min. of heating at 50°C, measureable inhibition remained (Fig.24 ); however, all traces of inhibition disappeared after centrifugation in sucrose density gradient, as stated above, presumably due to the subsequent dilution and/or the ultracentrifugation itself.

Fig.24 shows a sucrose density gradient profile of two standard proteins, catalase and yeast alcohol dehydrogenase for the purpose of comparison. Catalase has a molecular weight of 250,000 and the dehydrogenase a molecular weight of 150,000. By comparison with the elution profiles of heated material as well as the material from DEAE Sephadex, when the centrifugation was carried out under the same conditions, ATCase and yeast alcohol dehydrogenase sedimented in the same tube. Since the M.W. of ADH is known to be 150,000, it is safe to assume that the subunit that has been recovered with ATCase activity has a M.W. of close to 150,000.

Table 16 summarizes the M.W. data obtained by sucrose density gradient studies of the disaggregated material obtained by chromatography on DEAE Sephadex and by heat treatment.

RESEARCH REPORTS

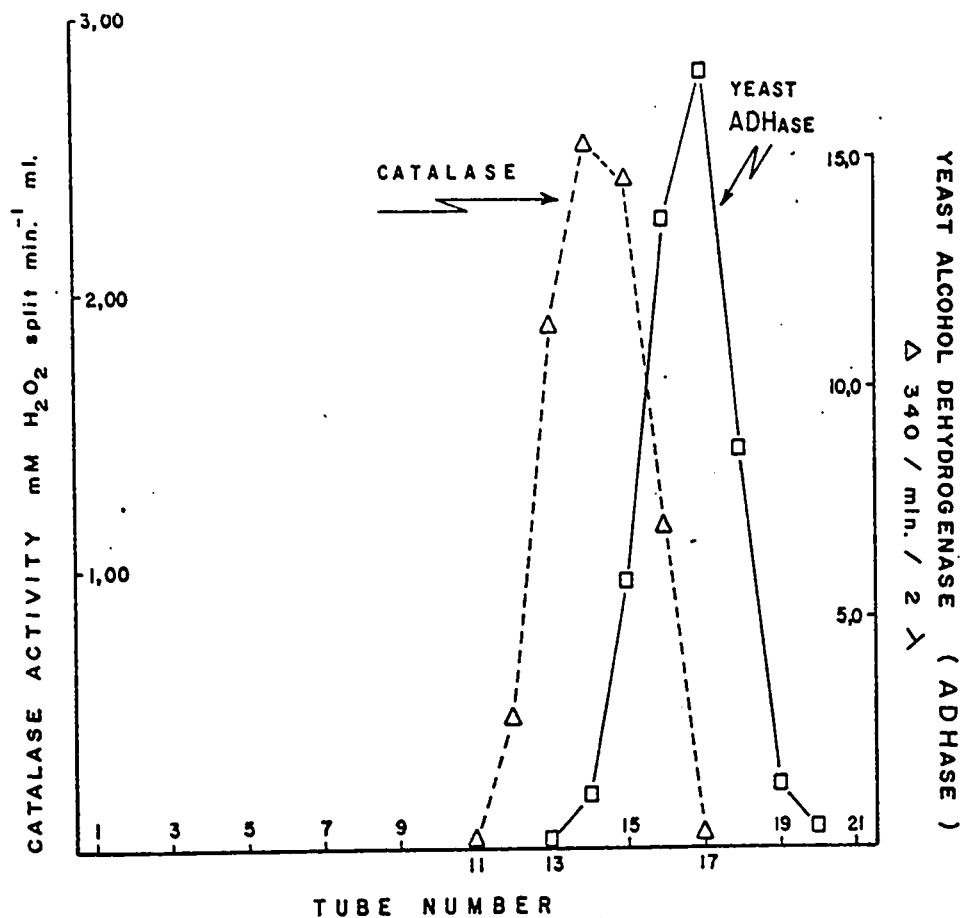


Fig. 26. Sucrose density gradient centrifugation of two standard proteins, yeast alcohol dehydrogenase and catalase. Conditions were identical as in previous studies, where gradients of 4.6 ml. were used with 0.1 ml. sample, which were then spun at 60,000 rpm for 3 hr. at 5°C. Fractions of 0.22 ml. were collected and yeast alcohol dehydrogenase was estimated by the method of Vallee and Hoch (1955), and catalase by a modification of the method of Chantrenne (1955).

ROBERTO CHANTRENNE

Table 16  
Molecular Weight of Non-inhibited Material

Treatment	Molecular Weight	Internal Marker
DEAE Sephadex	130,000	catalase
heat	140,000	catalase, yeast alcohol dehydrogenase

Molecular weight was calculated by the method of Martin and Ames (1961).

3. Effect of Heat in the Presence of Bovine Serum Albumin

In earlier studies it was observed that 0.4% bovine serum albumin (BSA) served to stabilize the enzyme. Furthermore it was observed that this stabilizing effect could be extended to the non-inhibited material from DEAE Sephadex (Fig.27 ). Curve A shows that the ATCase activity of the disaggregated material was extremely sensitive to heat. Curve B shows that the activity was greatly stabilized by BSA. It should be borne in mind that elution from DEAE Sephadex results in a great dilution of the protein recovered. The protein concentration in these experiments was approximately (0.5 mg/ml), whereas in the experiment described previously (Fig.24 ), the ATCase was at a much higher concentration (5.3 mg/ml).

Recently, it was shown that the combined effects of temperature and dilution resulted in a considerable activation of the ATCase activity depending on the temperature of assay (Kaplan and Messmer, 1969). When a greatly diluted preparation (1000-fold) was assayed at 40°C a four-fold activation by comparison with the highly concentrated material was observed. At 25°C a two-fold activation was observed. Since heat has been

Material from DEAE Sephadex

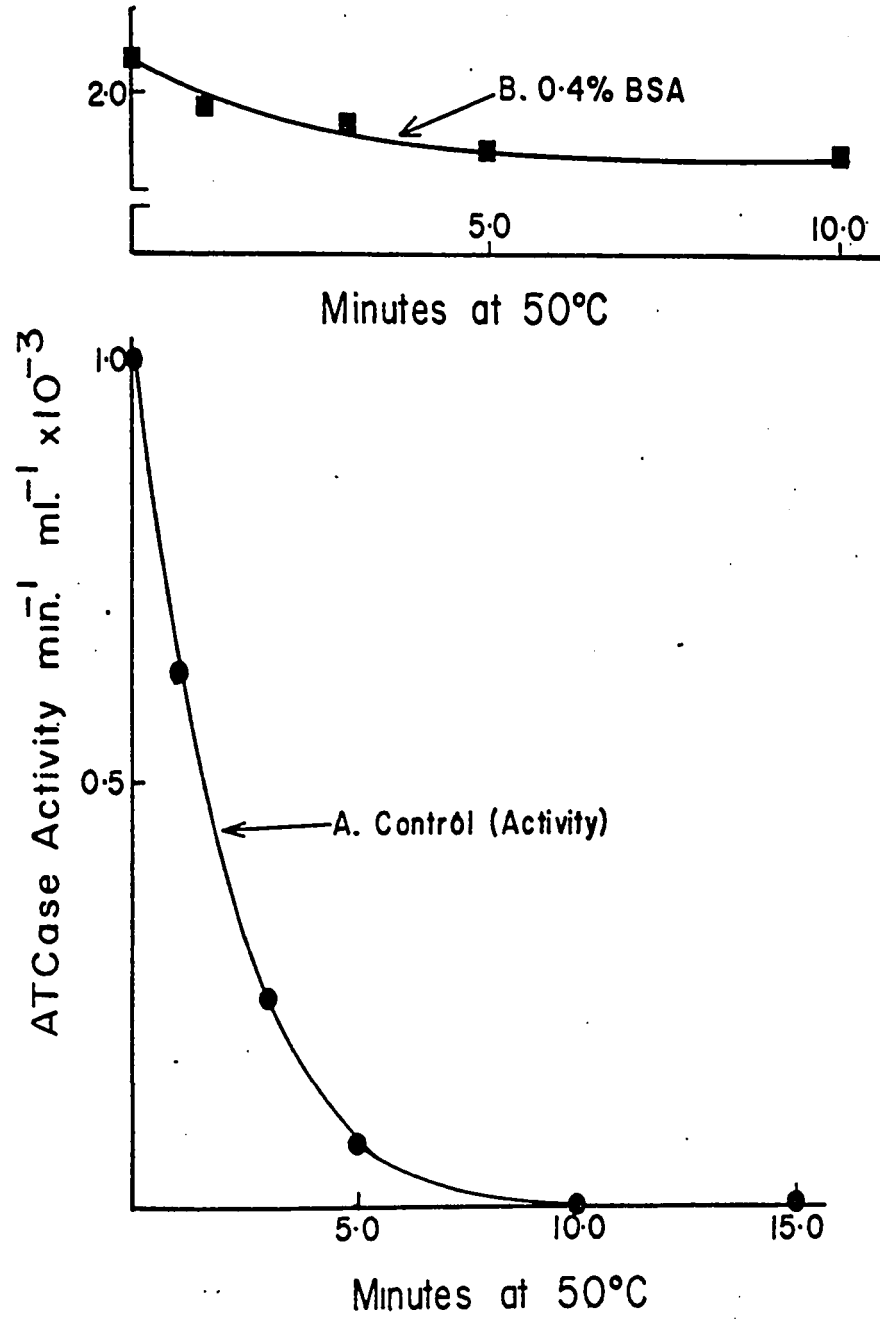


Fig. 27. The effect of heating on DEAE-Sephadex prepared material in the presence and absence of 4 mg/ml. BSA.

shown to cause extensive disaggregation, one may interpret the results (Fig. 24 ) as being the results of two competing effects: 1) heat induced disaggregation with activation of the ATCase activity; 2) rapid heat inactivation of the dissociated subunits. If this interpretation were correct, one might expect the presence of BSA in the medium might stabilize the dissociated subunit and thus permit detection of the heat induced activation. Fig. 28 shows the results of such an experiment, identical in all respects as shown in Fig.24 except for the presence of 0.4% BSA. Here it can be noted from curve A that no noteworthy increase of the ATCase activity occurred during the heating. The results are similar to that obtained in Fig.24 except the CPSase activity and ATCase inhibition are more stable to heating as a result of the presence of BSA.

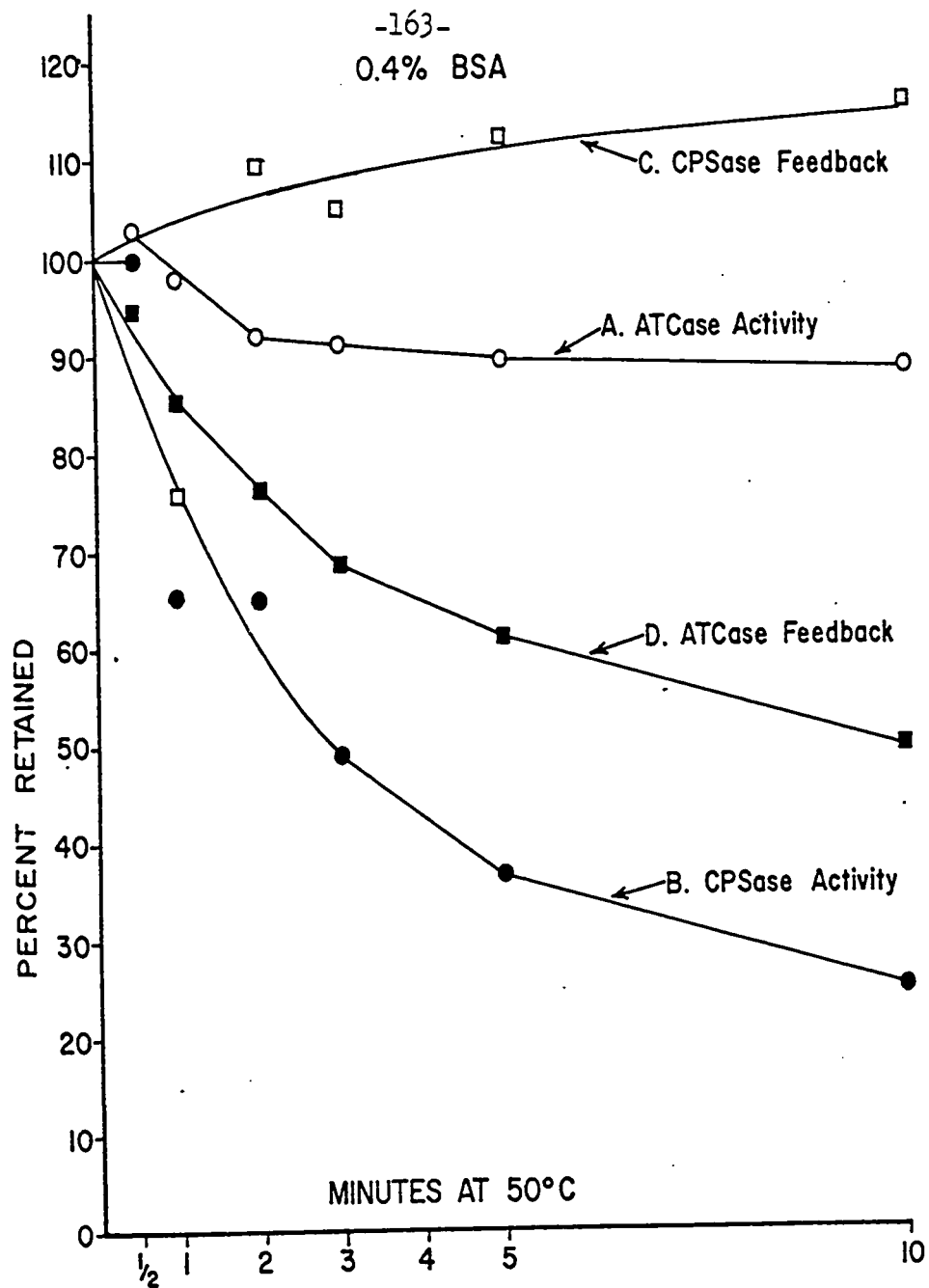


Fig. 28. Effect of heating at 50°C on the CPSase and ATCase activities and feedback inhibitions in the presence of 0.4% BSA. Plot is similar to that of Fig. 24. At zero time ATCase was inhibited to 70% and CPSase to 75% by  $2 \times 10^{-3} M$  UTP. These percentages were considered as 100% inhibition.

V. Characterization of the 20-fold Purified ATCase

1. Effect of pH

Figure 29 shows the effect of pH on the partially purified ATCase with and without  $2 \times 10^{-3}$  M UTP. As shown in curve A, a <sup>NCI</sup> plateau of activity was observed at pH's above 8; however, as shown in curve C, the peak of inhibition occurred at pH 7.1-7.6. Because of our interest in the regulatory aspects of this enzyme, all further studies were carried out at pH 7.4 unless otherwise stated. At this pH the activity was approx. 66% of that observed at pH 8.5. Similar results were obtained by Kaplan et al. (1967) in their studies of the semipurified wild-type enzyme. It was also observed that the activities were 10-20% higher in Tris buffers than in phosphate buffers (between pH 7-8); the inhibition by UTP was somewhat less marked in Tris buffer.

2. Effect of Pyrophosphate

Fig.30 shows the effect of varying concentrations of pyrophosphate in the presence and absence of UTP on the ATCase activities. It can be seen that pyrophosphate is a good inhibitor of ATCase activity (curve A). The

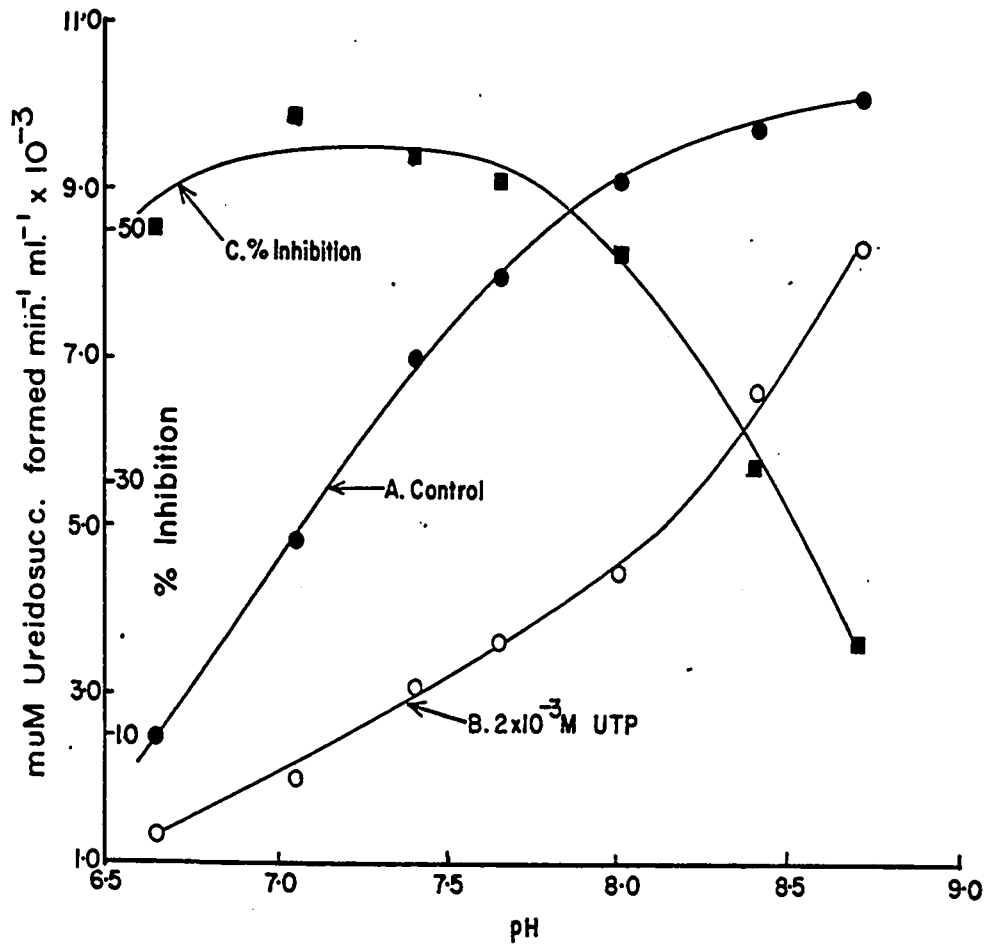


Fig. 29. Dependence of ATCase activity on pH. The colorimetric method of assay was used (Methods). Reaction mixtures contained: enzyme; carbamyl phosphate (10  $\mu$  moles); aspartate (50  $\mu$  moles); and K phosphate buffer (pH 6.5-7.5) and Tris-phosphate (to pH 7.6, 50  $\mu$  moles).

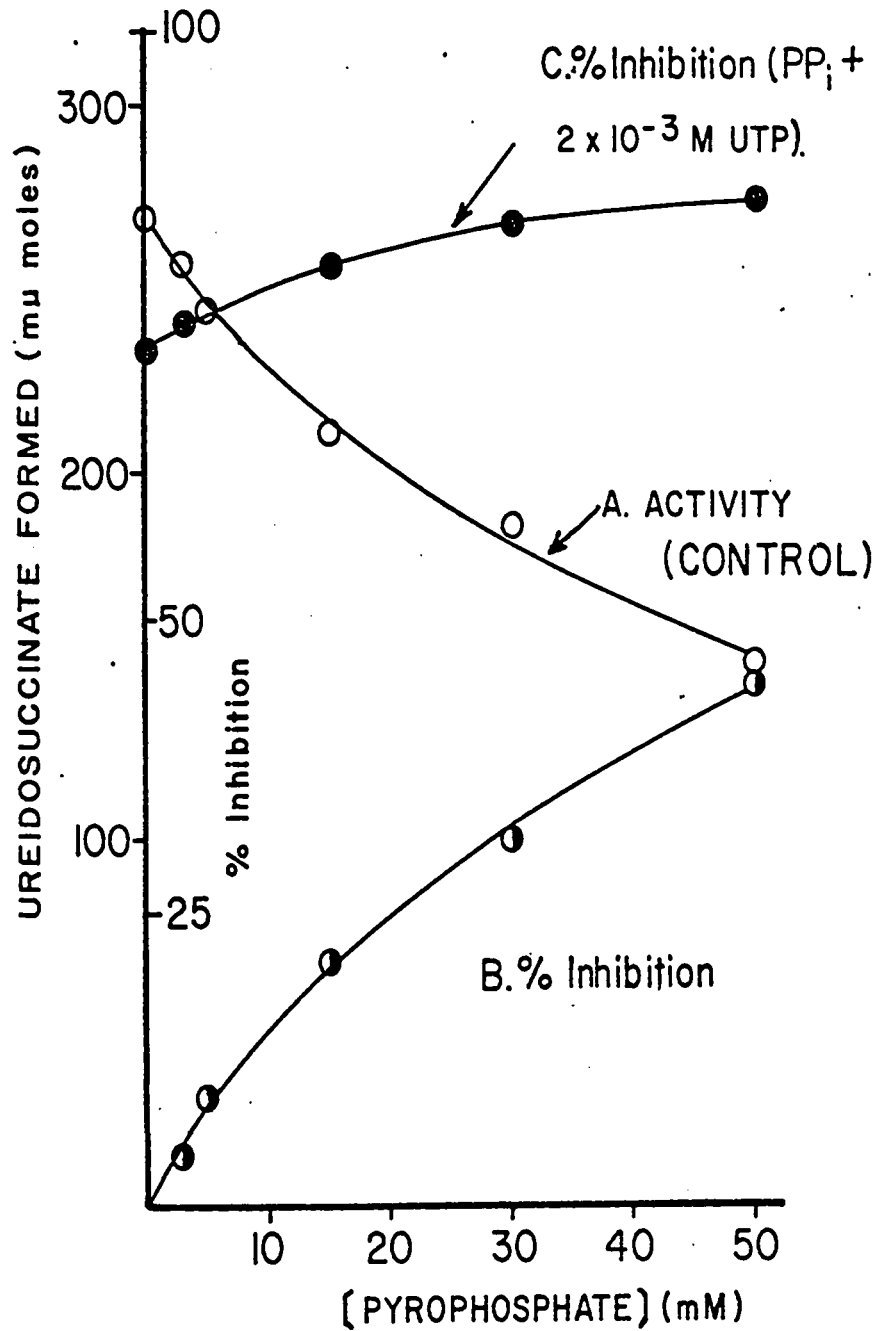


Fig. 30. The effect of pyrophosphate on the ATCase activity and inhibition by UTP. A shows the activity in the presence of various concentrations of PPi; B, % inhibition by these concentrations of PPi; C, the total % inhibition caused by these concentrations of PPi plus  $2 \times 10^{-3} \text{ M UTP}$ . The ATCase activity was colorimetrically assayed as described in Methods.

inhibition caused by the different concentrations of pyrophosphate is shown in curve B; these inhibitions were found to be additive (by calculation) to the inhibition exerted by UTP (curve C and Table 17). Table 17 compares the actual observed inhibition of ATCase activity caused by the simultaneous presence of P<sub>P</sub>i and  $2 \times 10^{-3}$  M UTP and the inhibition calculated on the assumption that both inhibitors act independently of one another. The agreement is so close that it may be concluded that the assumption is correct. The fact that inhibition is separate indicates that two separate sites are involved.

### 3. Effect of Various Concentrations of Carbamyl Phosphate

Fig.31 shows the substrate saturation curve of ATCase for carbamyl phosphate in the presence and absence of pyrophosphate and UTP. As shown in curve A, the saturation curve for carbamyl phosphate was hyperbolic and the presence of UTP caused a change in the maximum velocity attained (curve B). However, addition of  $5 \times 10^{-3}$  M pyrophosphate converted the carbamyl phosphate saturation curve from hyperbolic to a sigmoidal form (curve C). Curves B' and C' show the

Table 17

Additive Inhibition of ATCase Caused by PPi and UTP

Conc. of PPi	Units of Activity	% Inhibition	Observed Inhibition (PPi + UTP)	Expected if Inhibitions are Additive
0	270		73 (UTP alone)	73
2.5	258	4.4	76	74
5.0	244	9.6	78	76
15.0	211	21.9	80	79
30.0	188	30.4	84	81
50.0	150	44.4	87	85

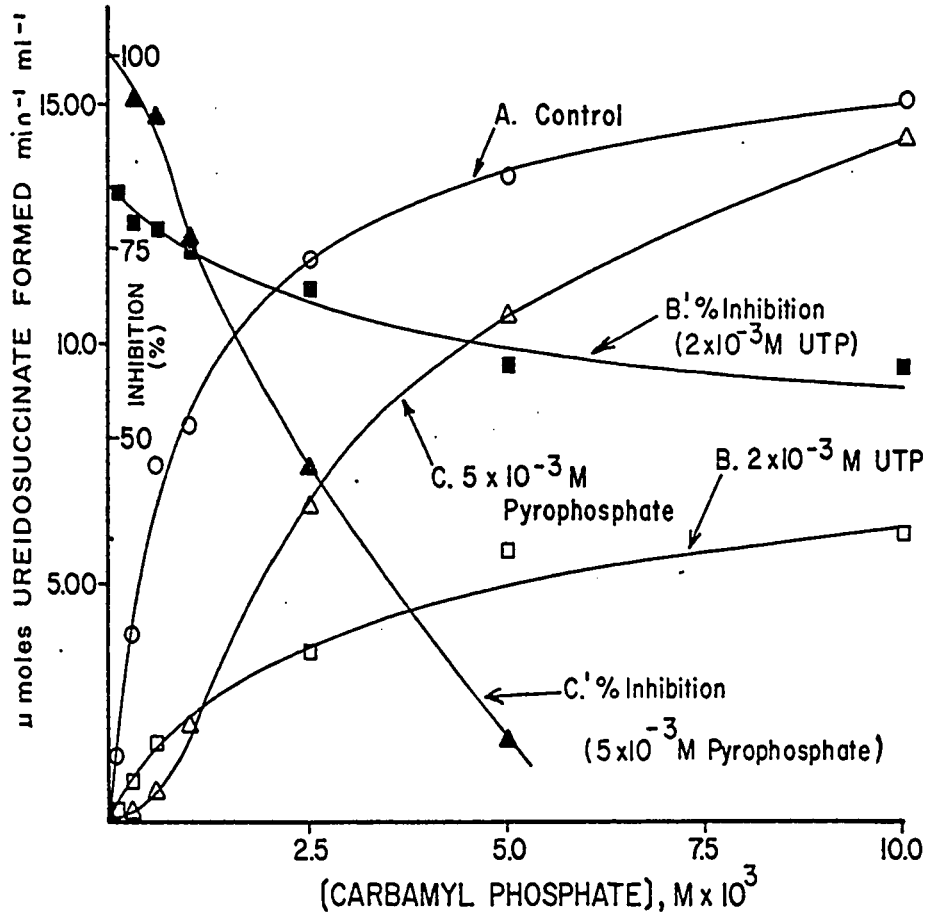


Fig. 31. The effect of carbamyl phosphate concentrations on the activity of ATCase at pH 7.4. The standard reaction mixture was used containing either  $5 \times 10^{-3} \text{ M}$  pyrophosphate or  $2 \times 10^{-3} \text{ M}$  UTP and various concentrations of carbamyl phosphate, as described under Methods. The ATCase activity was colorimetrically assayed either in the absence (A, O-O) or in the presence of  $5 \times 10^{-3} \text{ M}$  pyrophosphate (C, Δ-Δ) or in the presence of  $2 \times 10^{-3} \text{ M}$  UTP (B, □-□). The % inhibitions caused by the presence of  $2 \times 10^{-3} \text{ M}$  UTP (B', ■-■) and in the presence of  $5 \times 10^{-3} \text{ M}$  PPI (C', ▲-▲) at various concentrations of carbamyl phosphate are also indicated.

inhibition caused by UTP and pyrophosphate respectively at various concentrations of carbamyl phosphate (CP). It can be seen that the inhibition by pyrophosphate was most effective at low concentrations of CP (curve C and C'). At the lowest concentration of CP ( $10^{-4}M$ ) there was no detectable activity in the presence of pyrophosphate (curve C). Thus the increased concentration of CP resulted in a sharp decrease in the inhibition by pyrophosphate; at a concentration of  $5 \times 10^{-3}M$  only 10% inhibition was observed. On the other hand, increased CP concentration caused UTP inhibition to decrease only slightly (curve B'). At higher substrate levels ( $5 \times 10^{-3}M$ ) there was no tendency for the inhibition by UTP to be reversed (curve B'). The inhibition is clearly non-competitive. However, at very low CP concentration, the inhibition tends to be more pronounced (curves B & B'). In the Lineweaver-Burk plot (Fig. 32), the  $K_m$  for CP, which was Michaelian, was  $2 \times 10^{-3}M$  (A). As shown by curve C, the kinetics were non-Michaelian, but approached the same  $V_{max}$  control, indicating competitive inhibition. A similar  $K_m$  value ( $4 \times 10^{-3}M$ ) for CP was obtained by Kaplan et al. (1967).

When these data are rearranged according to the

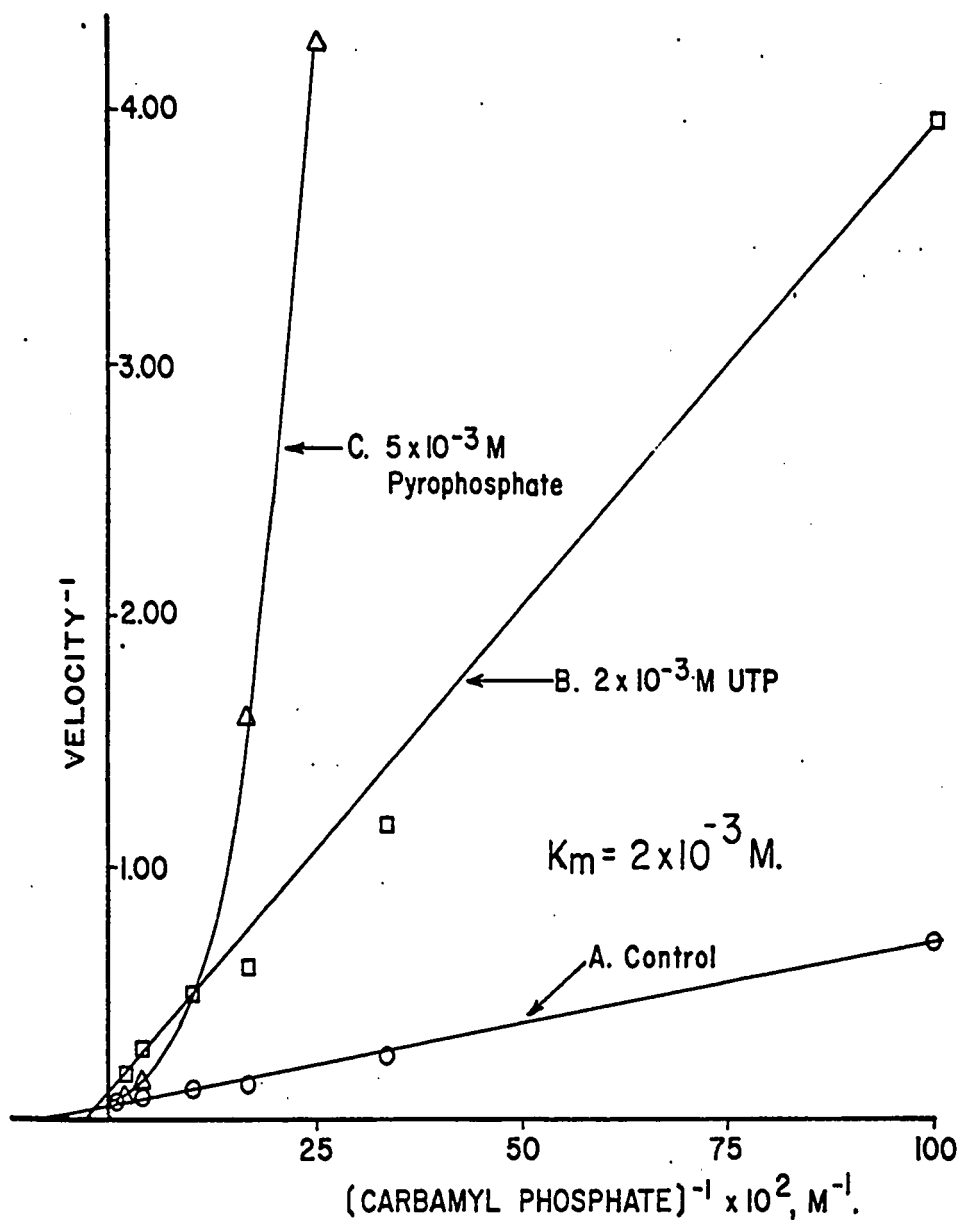


Fig. 32. Double reciprocal plots of velocity versus carbamyl phosphate concentrations in the presence of pyrophosphate ( $\Delta$ - $\Delta$ ) or UTP ( $\square$ - $\square$ ) or in their absence ( $\circ$ - $\circ$ ). The plots were made from the values shown in Fig. 31.

equation of A.V. Hill (1910), as shown in Fig. 33, the slopes obtained in the presence and absence of UTP were approximately unity (A,B) as previously shown (Kaplan et al., 1967). However, in the presence of pyrophosphate, the slope was approximately 2 (curve C). This indicates some cooperative interaction of CP sites in the presence of pyrophosphate.

Similar studies were carried out with pyrophosphate using enzyme preparations lacking feedback properties. Fig. 34 shows the substrate saturation curve for carbamyl phosphate using a preparation which had been subjected to chromatography on DEAE Sephadex and which had therefore lost its feedback inhibition (see above). By comparison to Fig. 33, it can be seen that the saturation curve in the presence of pyrophosphate had been converted from a sigmoidal to a hyperbolic form (curve B). Curves A' and B' show these data rearranged in the form of a Lineweaver-Burk plot. Again the inhibition by pyrophosphate is shown to be competitive.

Curve A' shows that disinhibition and disaggregation (DEAE Sephadex) did not cause modification of the  $K_m$  for carbamyl phosphate. Curve B' shows that the inhibition by pyrophosphate remains competitive and the  $K_m$  has

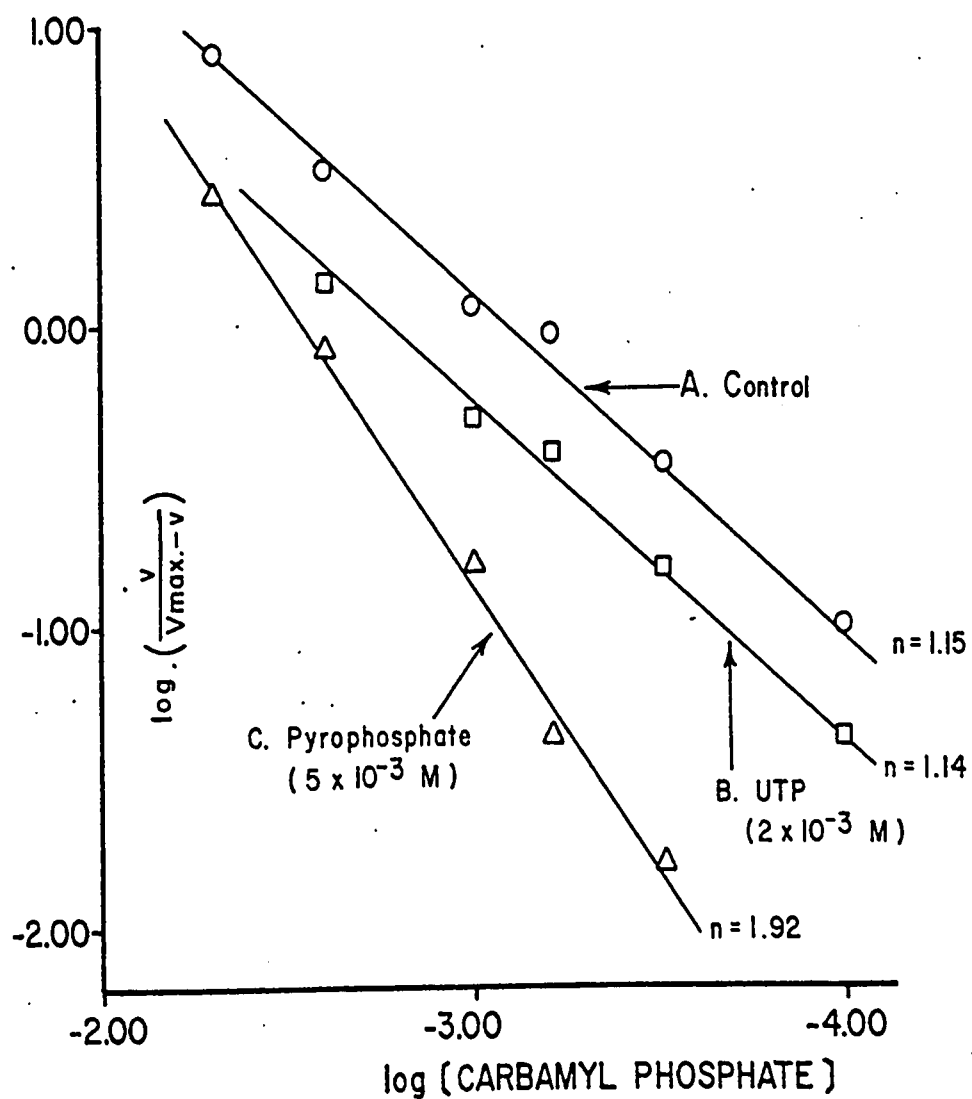


Fig. 33. Hill plot of the values shown in Fig. 31.

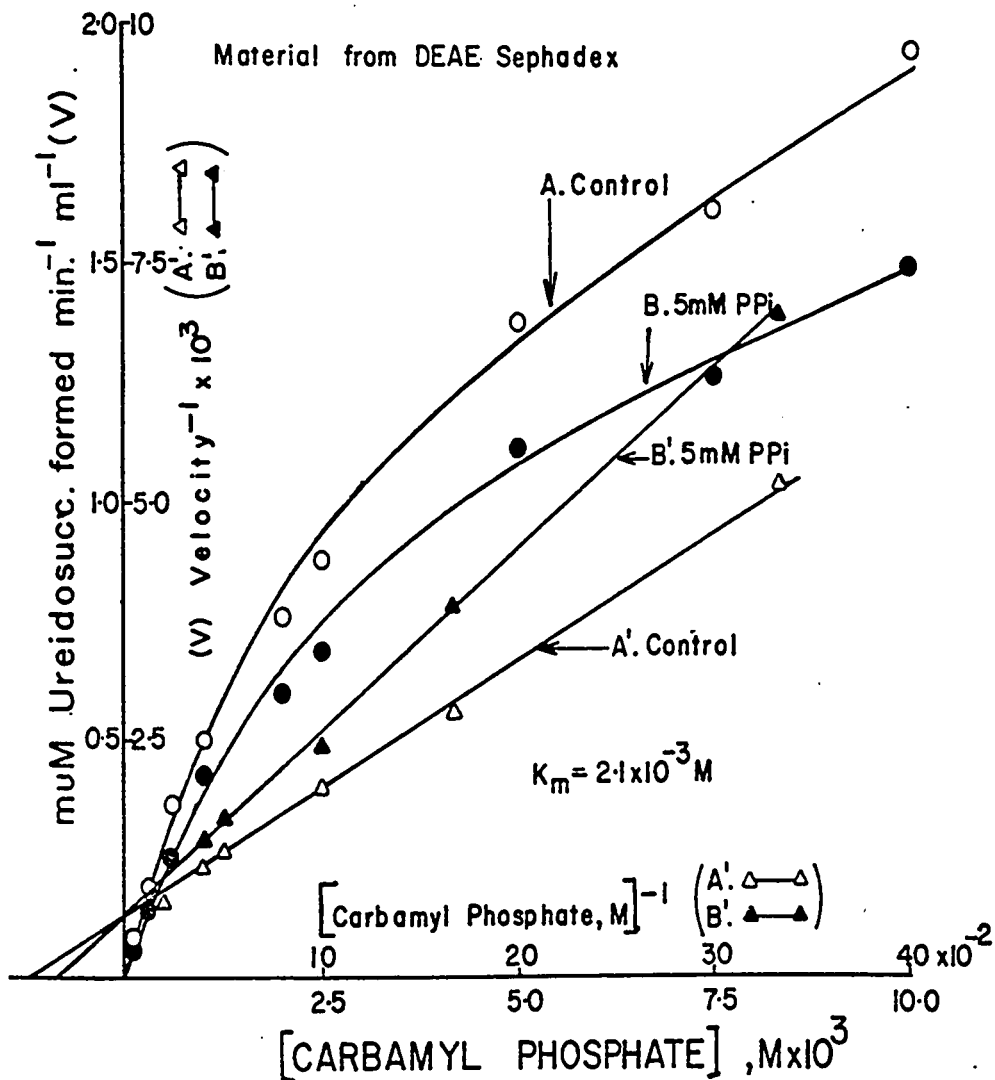


Fig. 34. Effect of carbamyl phosphate concentration on the disinhibited ATCase activity of a preparation eluted from DEAE-Sephadex at pH 7.4. The ATCase activity was colorimetrically assayed using the standard reaction mixture containing various concentrations of carbamyl phosphate in the absence (A, O-O) and presence (B, ●-●) of  $5 \times 10^{-3} \text{ M}$  pyrophosphate. The double reciprocal plot of these data is also indicated (A',  $\Delta$ - $\Delta$ ) in the absence and (B',  $\blacktriangle$ - $\blacktriangle$ ) in the presence of  $5 \times 10^{-3} \text{ M}$  pyrophosphate.

increased to  $3 \times 10^{-3} \text{M}$ .

Fig. 35 shows the results of similar studies carried out on heated enzyme ( $60^{\circ}\text{C}$  for 3 min.) in the form of a Lineweaver-Burk plot. The results are similar to those obtained in Fig. 34. Michaelis-Menten kinetics were obtained in the presence of pyrophosphate, which again is seen to be a competitive inhibitor of carbamyl phosphate in the ATCase reaction.

The  $K_m$  values in presence and absence of pyrophosphate were  $3.7 \times 10^{-3}$  and  $2.9 \times 10^{-3} \text{M}$  respectively.

#### 4. Effect of Various Concentrations of Aspartate

Fig. 36 shows the saturation curve for aspartate with and without  $2 \times 10^{-3} \text{M}$  UTP. The aspartate saturation curve in the absence (curve A) and in the presence (curve B) were hyperbolic with UTP affecting the  $V_{\text{max}}$  of the ATCase reaction. It can also be seen that the inhibition by UTP was approx. constant at all concentrations of aspartate (curve C). The  $K_m$  for aspartate as shown by the double reciprocal plot of Fig. 37 was  $3.3 \times 10^{-2} \text{M}$  and the inhibition of the ATCase reaction by UTP was noncompetitive. These results are in agreement with those obtained with semipurified enzyme

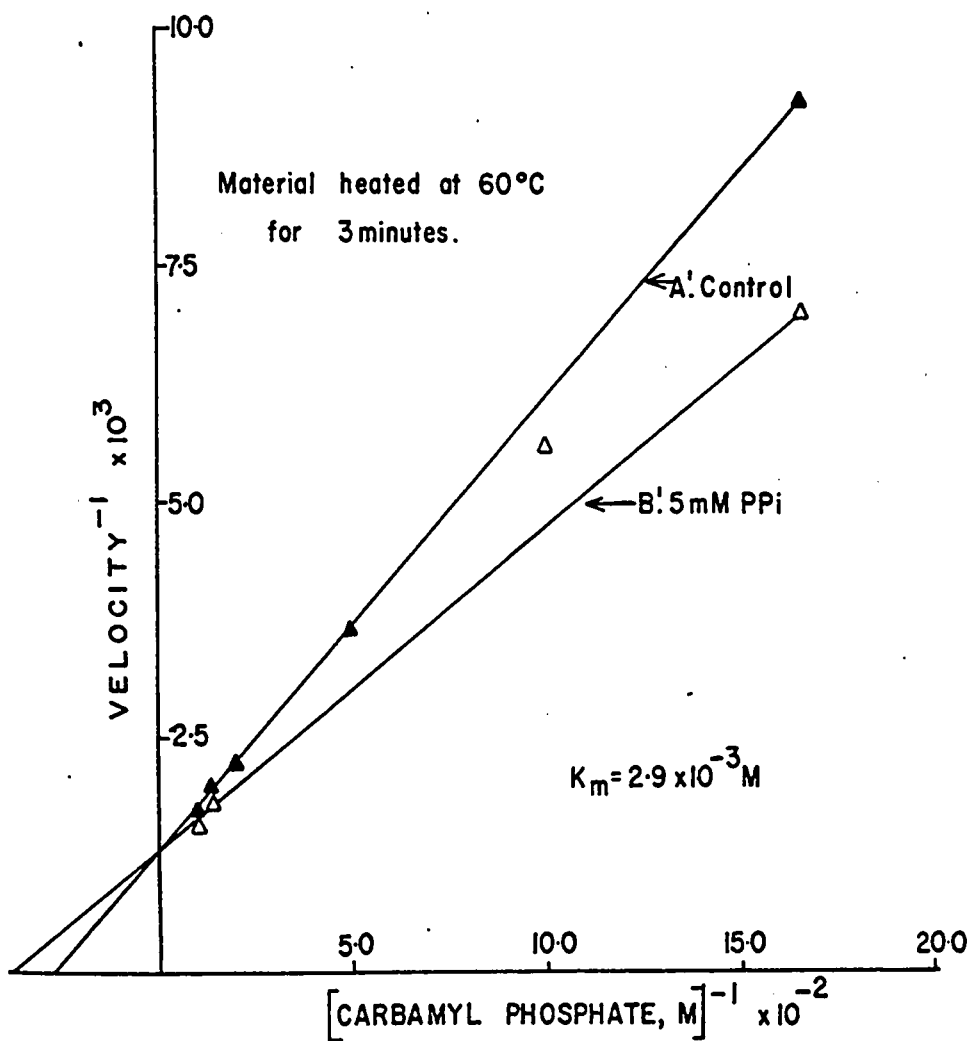


Fig. 35. Effect of carbamyl phosphate concentration on the purified material which had been heated at 60°C for 3 min. at pH 7.4. The data (which were hyperbolic) are expressed as a double reciprocal plot in the absence (A, ▲-▲) and presence of 5x10<sup>-5</sup>M pyrophosphate (B, △-△) at various concentrations of carbamyl phosphate. The standard colorimetric method of assay was used.

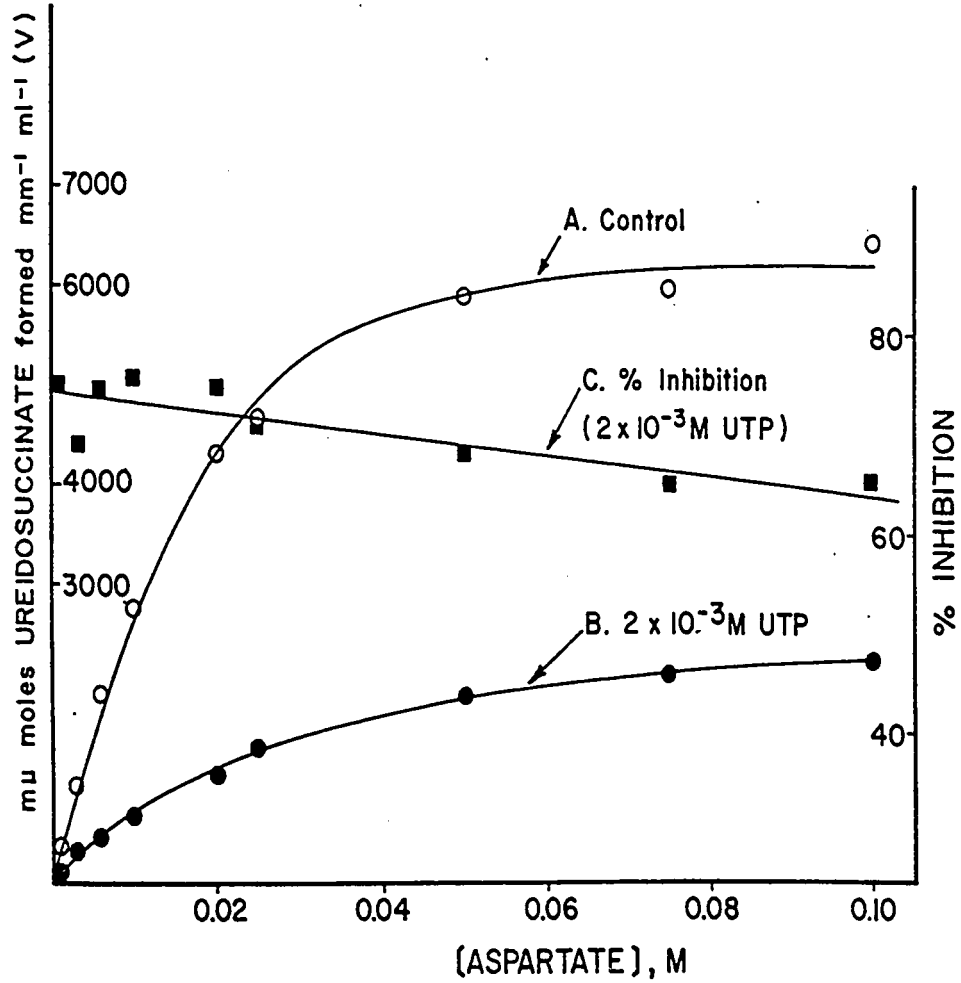


Fig. 36. Effect of aspartate concentrations on the ATCase activity at pH 7.4 in the absence (A,  $\circ-\circ$ ) and presence (B,  $\bullet-\bullet$ ) of  $2 \times 10^{-3} \text{M UTP}$ . The % inhibition caused by  $2 \times 10^{-3} \text{M UTP}$  at various concentrations of aspartate is also indicated (C,  $\blacksquare-\blacksquare$ ). The standard colorimetric assay, as described under Methods, was used.

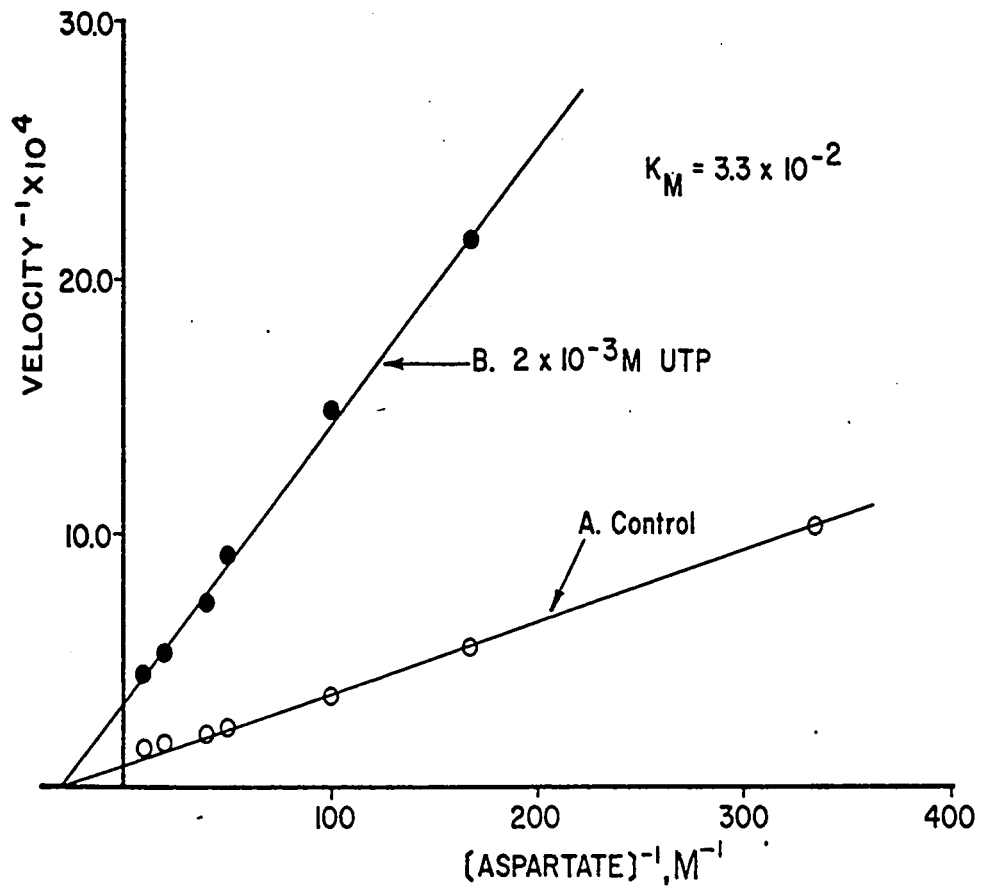


Fig. 37. Double reciprocal plot of velocity versus aspartate concentration in the absence (A,  $\circ$ - $\circ$ ) and presence of  $2 \times 10^{-3} \text{M UTP}$  (B,  $\bullet$ - $\bullet$ ). The plots were made from the values shown in Fig. 36.

(Kaplan et al., 1967).

### 5. Inhibition by UTP

Fig. 38 shows the effect of varying concentration of UTP on the ATCase activity. The plateau of inhibition occurred at  $2 \times 10^{-3} \text{M}$  UTP (curve A); also presented is the inhibition by UTP of the CPSase reaction (curve B). It can be seen that the CPSase activity was more susceptible than the ATCase activity to inhibition by UTP, and it will be shown below that UTP at higher concentrations ( $5-7 \times 10^{-3} \text{M}$ ) can almost completely inhibit the CPSase. The  $K_i$  was  $8.5 \times 10^{-4} \text{M}$  for the inhibition of ATCase by UTP as calculated from the  $1/V$  versus UTP plot for a non-competitive inhibitor (Fig. 39 ). It should be noted that the inhibition of ATCase by UTP was found to vary from one preparation to another. In preparations using MD170-7X and FL233-3C, the inhibition by  $2 \times 10^{-3} \text{M}$  UTP was found to vary between 40-85% in the crude extract.

## VI. Carbamyl Phosphate Synthetase - Properties

### 1. Requirements for the Reaction

Table 18 shows the requirements for the CPSase

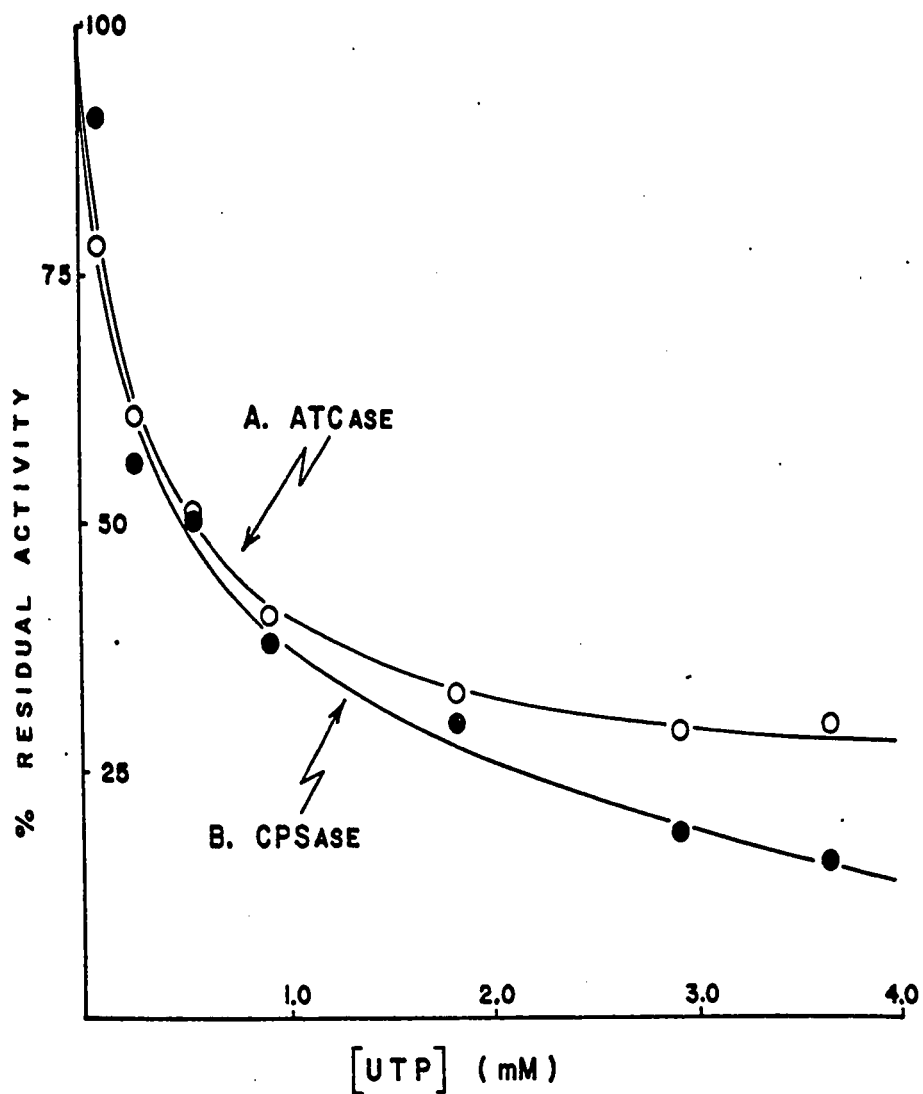


Fig. 38. Effect of various concentrations of UTP on the ATCase (curve A) and on the CPSase (curve B) activities. Standard assay conditions were used for ATCase and assay system I was used for CPSase, as described under Methods.

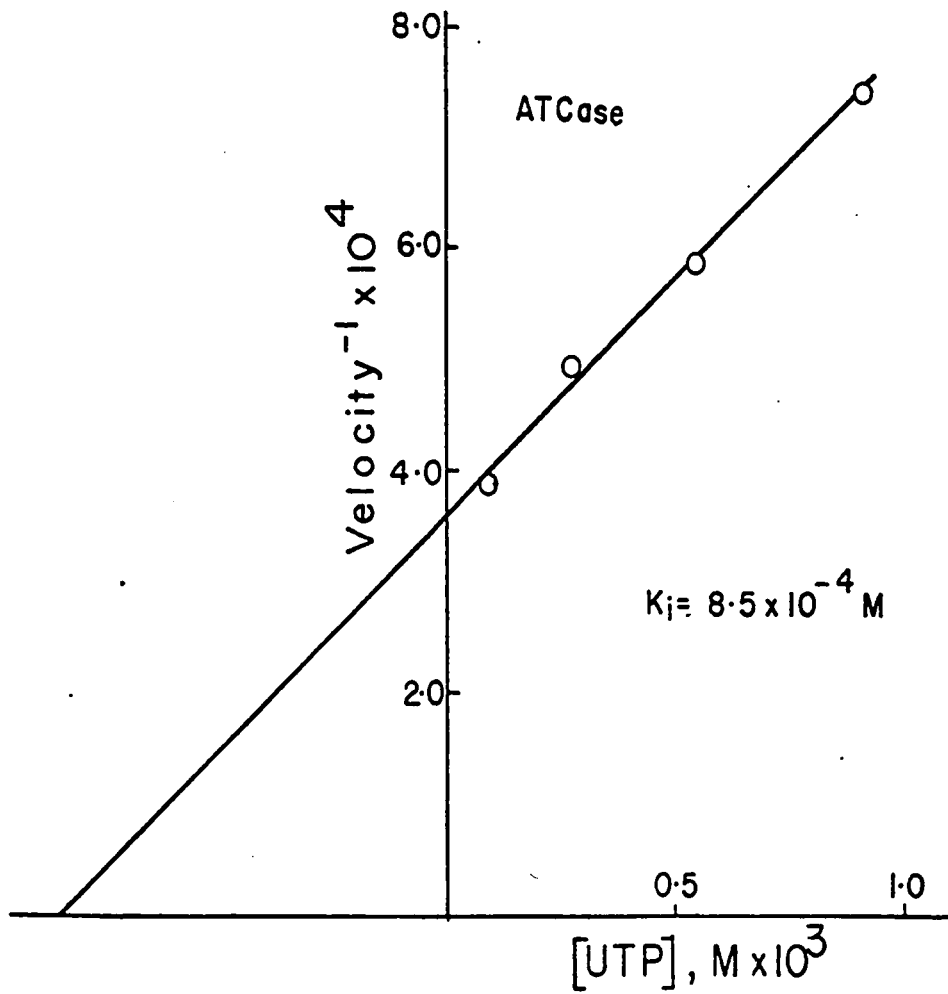


Fig. 39. Plot of  $1/V$  versus (UTP) for a non-competitive inhibitor to give the  $K_i$  for UTP inhibition of ATCase. The plot was made from values obtained from Fig. 38.

Table 18

Requirements for the Enzymatic Synthesis of  
Citrulline by Yeast CPSase

Incubation System	Citrulline Formed (mu moles)
Complete	103.7
" minus ATP	0.9
" " Mg <sup>++</sup>	2.2
" " Glutamine	0
" " K <sup>+</sup>	1.9
" " HCO <sub>3</sub> <sup>-</sup>	3.4
" " Ornithine	1.3
" " OTCase	0.6
" " Enzyme (blank)	0

The complete assay mixture contained: ATP (20 u moles), MgSO<sub>4</sub> (20 u moles), Glutamine (6 u moles), KCl (100 u moles), NaHCO<sub>3</sub> (30 u moles), ornithine (6 u moles), OTCase (excess), Tris-SO<sub>4</sub> (100 u moles pH 7.6) and enzyme (0.64 mg. protein) in a final volume of 1.0 ml. Incubation was for 20 min. at 25°C (page 81). The enzyme preparation was passed through G-25 using 0.1M Tris-SO<sub>4</sub> buffer pH 7.6, before it was used in the experiment.

reaction and its coupled system of assay. In the absence of  $Mg^{++}$ , ATP,  $K^+$  or glutamine, the amount of citrulline produced was reduced to very low levels. Ornithine and OTCase were also required; however, the small activity observed in the absence of either ornithine or OTCase could be due to the carbamyl phosphate produced (Kaplan et al., 1967). On the other hand, the activity observed in the absence of bicarbonate may be due to atmospheric carbon dioxide in the reaction mixture. In this study no attempt was made to remove completely this source of interference.

## 2. Stoichiometry of the CPSase Reaction

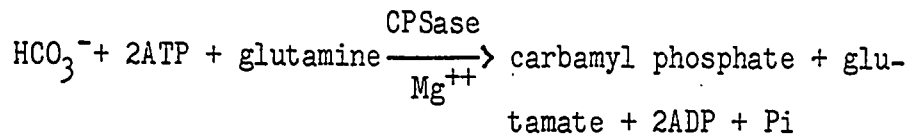
The stoichiometry of the CPSase reaction is given in Table 19, in which the products of the CPSase reaction are compared on a molar basis. The CPSase activity was assayed colorimetrically both as citrulline and ureidosuccinate. The results were in close agreement but it was consistently found that citrulline estimation gave slightly lower values than the ureidosuccinate method; this observation is discussed below. One mole of citrulline or ureidosuccinate was produced per mole of glutamine and per 2 moles of ATP utilized. The reaction catalyzed by yeast CPSase can be written as

Table 19

Stoichiometry of the Carbamyl Phosphate Synthetase Reaction

Incubation Time (min.)	ADP (mu moles)	Glutamate (mu moles)	Citrulline (mu moles)	Ureido- succinate (mu moles)
15	254.4	93.6	96.8	98.7
30	432.0	213.6	190.6	201.3

The assay system used is described in Table  
Citrulline and ureidosuccinate were estimated as des-  
cribed in Methods. After the incubation period, 0.1  
ml. 0.4N HCl was added to stop the reaction. After  
10 min. at 0°C, 0.1 ml. 0.4N NaOH was added to the  
acidified sample and then 0.1 ml. each was used for the  
estimation of ADP and glutamate.



The reversibility of this reaction was not investigated.

### 3. Effect of Avidin and Biotin

It has been found that biotin plays an important role in systems involving the activation of  $\text{CO}_2$  (e.g. carboxylase) and that avidin, an extract from egg white, specifically inhibits the action of biotin (Kaziro et al., 1960). Table 20 shows the effect of biotin and avidin, separately and together, after various periods of incubation. It can be seen that both biotin and avidin served to inhibit the CPSase activity and that the inhibitions were additive (Expt. I). Biotin had no stimulatory effect, nor did it reverse the inhibition by avidin, as would be expected if it were a cofactor of CPSase activity; however, it appeared to have some stabilizing effect during incubation (Expt. II). These results suggest that yeast CPSase is not a biotin enzyme and that activation of  $\text{CO}_2$  may occur by a different mechanism.

The method used is described by Peng and Jones (1969), except for the concentrations of biotin and avidin used. Twelve milligrams avidin were dissolved in 0.5 ml. of 0.1M Tris-SO<sub>4</sub> buffer pH 7.6. Sixteen mg. of biotin was dissolved in 1.0 ml. 0.1M Tris-SO<sub>4</sub> buffer pH 7.6. To four vessels the following were added: vessel 1, 0.4 ml. Tris buffer;

vessel 2, 0.2 ml. buffer, 0.2 ml. avidin sol.

vessel 3, 0.2 ml. buffer, 0.2 ml. biotin sol.

vessel 4, 0.2 ml. avidin, 0.2 ml. biotin sol.

These tubes were then incubated at 25°C for 15 min., after which time 0.1 ml. enzyme solution was added to each tube and incubation continued. At 10, 20, 40 and 60 minute intervals after addition of enzyme, 0.1 ml. of each mixture was assayed for 15 min. at 25°C. The assay system used is described under Table For Expt. II, 6 mg. avidin was dissolved in 0.7 ml. Tris-SO<sub>4</sub> buffer pH 7.6, and 4 mg. biotin in 2.0 ml. buffer.

WALTER LIBRARY  
UNIVERSITY OF OTTAWA  
414 KING ST. W.  
OTTAWA, ONTARIO

Table 20

Effect of Avidin and Biotin on CPSase Activity

Additions	% Residual Activity ( $\mu$ moles citrulline) after various incubation intervals			
	10 min.	20 min.	40 min.	60 min.
Expt. I				
1. none	108.7	101.9	89.1	74.7
2. Avidin (4.8 mg.) 10.9 units	31.1	24.4	18.3	18.3
3. Biotin (3.2 mg.)	25.6	24.0	20.2	16.7
4. Avidin + Biotin (4.8 mg + 3.2 mg)	10.9	8.0	5.8	5.1
Expt. II				
1. none	184.8	161.7	137.0	127.7
2. Avidin (1.7 mg.) (3.9 units)	111.9	99.0	88.1	79.5
3. Biotin (0.4 mg.)	178.2	160.4	145.2	139.6
4. Avidin + Biotin (1.7 mg + 0.4 mg)	100.3	90.1	80.9	73.6

#### 4. Effect of pH

Fig. 40 shows the activities in the presence and absence of  $5 \times 10^{-4} \text{M}$  UTP when the enzyme was assayed at varying pH. Curve A shows that the optimum pH for CPSase activity was around 7.4 when glutamine was the substrate. In the presence of UTP, the optimum was a rather short plateau between 7.2 and 7.9. It can also be seen that the inhibition was almost constant up to pH 7.4, beyond which the inhibition decreased.

Fig. 40, curve D shows that glutamine can be replaced by ammonium ion; the activity versus pH curve was considerably flatter than with glutamine. At pH 7.4,  $\text{NH}_4^+$  (50  $\mu$  moles) gave only one-fifth the activity obtained with glutamine; however, at pH 8.0, the optimum for the use of  $\text{NH}_4^+$  as the nitrogen donor, the activity was one-half of that obtained with glutamine.

#### 5. Effect of Divalent Ions

$\text{Mn}^{++}$ ,  $\text{Co}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Zn}^{++}$ , and  $\text{Ca}^{++}$  were investigated with respect to their capacity to replace  $\text{Mg}^{++}$  at various pH values.  $\text{Ca}^{++}$  and  $\text{Zn}^{++}$  were completely inactive

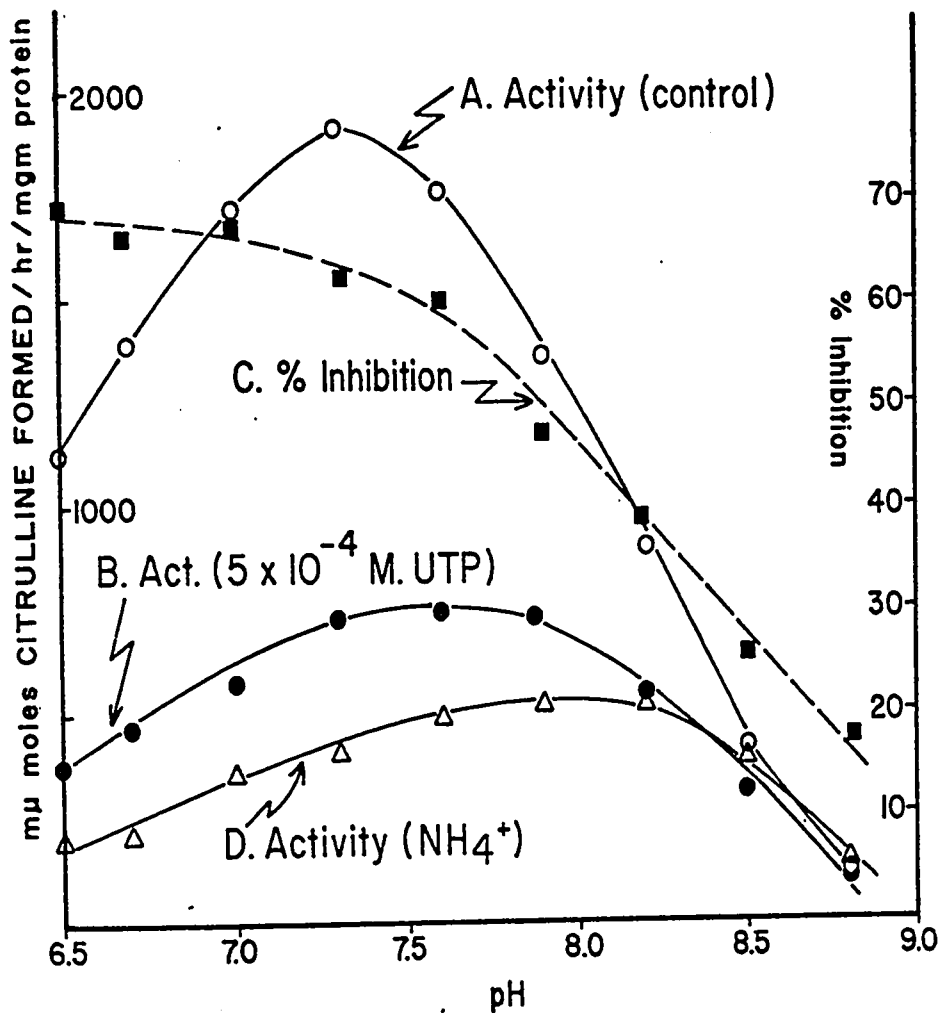


Fig. 40. The effect of pH on the CPSase in the absence (curve A) and presence of  $5 \times 10^{-4}$  M UTP (curve B). % inhibition is shown in curve C. The complete reaction mixture contained: ornithine, glutamine (6  $\mu$  moles each); ATP,  $\text{MgSO}_4$  (20  $\mu$  moles each); KCl (100  $\mu$  moles); OTCase (excess); protein (0.4 mg.) and Tris- $\text{SO}_4$  buffer (100  $\mu$  moles, pH as required) in a final volume of 1.0 ml. 50  $\mu$  moles  $\text{NH}_4\text{Cl}$  was substituted for glutamine in curve D.

whereas  $\text{Fe}^{++}$  and  $\text{Co}^{++}$  interfered with the development of color.  $\text{Mn}^{++}$  not only replaced  $\text{Mg}^{++}$  but considerably enhanced the rate of the CPSase reaction, as shown in Fig. 41, curves A and B. Curve B shows that in presence of  $\text{Mn}^{++}$ , the optimum pH was shifted to the acid side (approx. pH 7.0), with what appears to be a shoulder between pH 7.8-8.0. Other  $\text{Mg}^{++}$  requiring enzymes also have been found to exhibit 2 pH optima with  $\text{Mn}^{++}$  (Switzer, 1969). Curve C shows that  $\text{Ca}^{++}$  could not replace  $\text{Mg}^{++}$ . Neither  $\text{Mn}^{++}$  nor  $\text{Mg}^{++}$  affect the bacterial OTCase reaction at the concentration used (Rogers and Novelli, 1962).

## 6. Effect of Substrate Concentrations

### (a) ATP and Magnesium ( $\text{Mg}^{++}$ )

Fig. 42 shows the activity with varying ATP concentrations in the presence and absence of UTP. The saturation curve showed Michaelis-Menten type kinetics with an apparent  $K_m$  constant of  $3.5 \times 10^{-3} \text{M}$  (curve A). Optimum activity was at 10-13 mM ATP; at higher concentrations substrate inhibition was noted (curve A). Curve B shows that inhibition by UTP was non-competitive and seemed somewhat more effective at low concentrations of ATP.

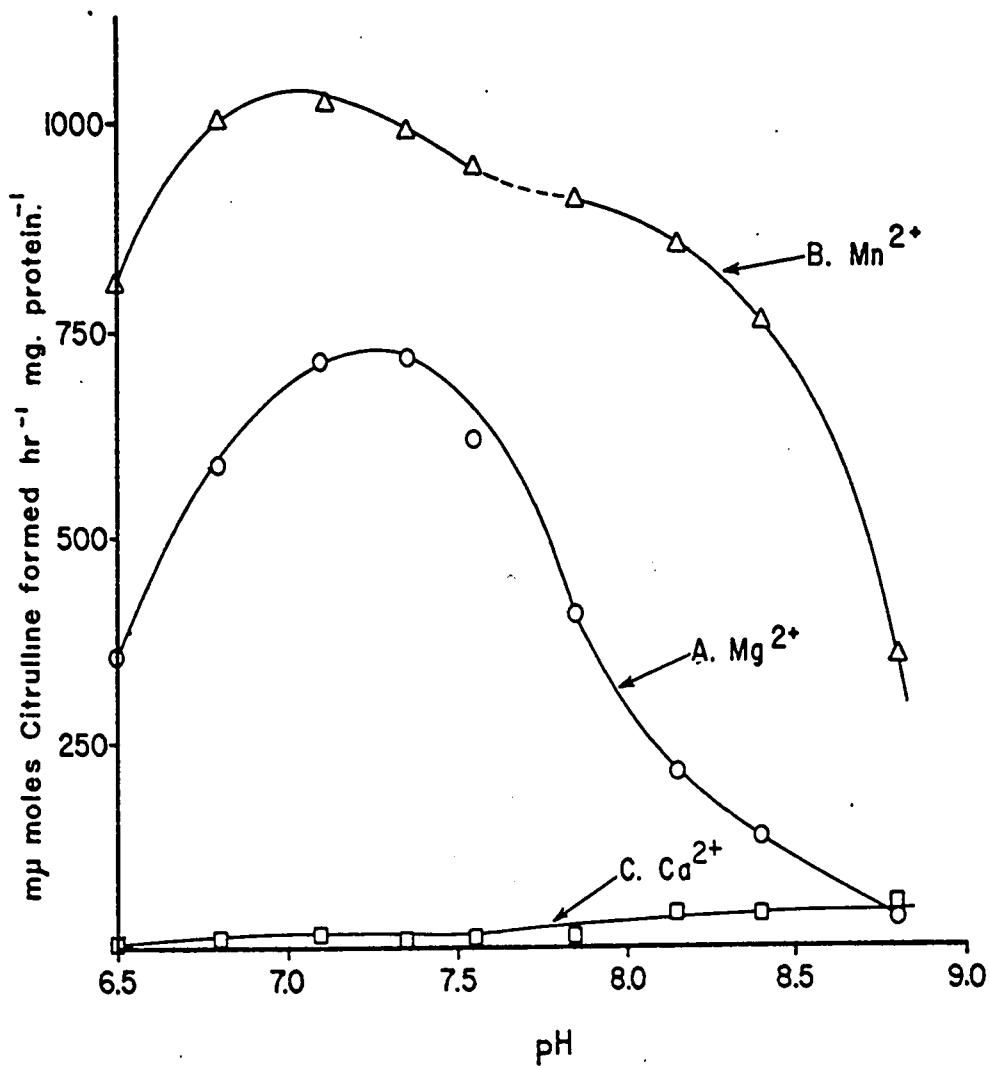


Fig. 41. Capacity of certain divalent ions to replace  $Mg^{++}$  studied as a function of pH. The reaction mixture contained: ornithine (6 u moles); glutamine (6 u moles); ATP (20 u moles); divalent ions (20 u moles; curve A,  $Mg^{++}$ ; curve B,  $Mn^{++}$ ; curve C,  $Ca^{++}$ ); KCl (100 u moles); OTCase (excess); protein (0.35 mg.) and Tris-Cl buffer (100 u moles pH as required) in a final volume of 1.0 ml.

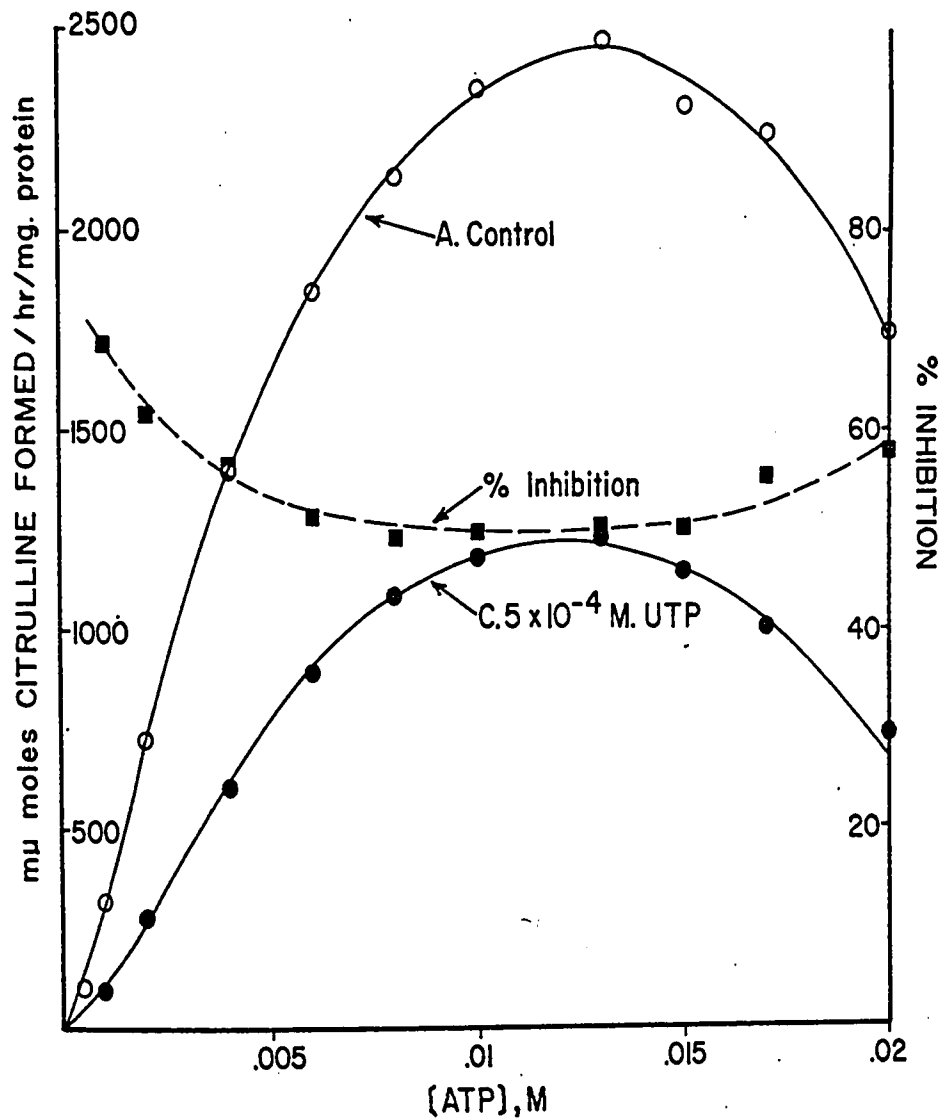


Fig. 42. Effect of varying concentrations of ATP in the absence (curve A) and presence of  $5 \times 10^{-4}$  M UTP (curve B). % inhibition is given in curve C. The complete reaction mixture contained:  $\text{NaHCO}_3$  (20 u moles);  $\text{MgSO}_4$  (20 u moles); glutamine (6 u moles);  $\text{KCl}$  (100 u moles); ornithine (6 u moles), OTCase (excess); protein (0.28 mg.);  $\text{Tris-SO}_4$  buffer (100 u moles, pH 7.6) and varying concentrations of ATP to a final volume of 1.0 ml.

It has been demonstrated that ATP in the presence of  $Mg^{++}$  ions results in the formation of Mg-ATP up to 99.5% (Keech and Barritt, 1967). Thus the possibility exists that the CPSase reaction utilizes the Mg-ATP complex as substrate and not their free forms.

Fig. 43 shows an experiment in which CPSase activity is assayed as a function of ATP in the presence of varying amounts of  $Mg^{++}$ . As the concentration of  $Mg^{++}$  increases, the activity increases sharply and at the same time there is a shift to a higher concentration of ATP, e.g. at 5, 10 and 20 mM  $Mg^{++}$  ions, the optimum ATP concentration was approx. 3mM, 7mM, and 12mM respectively. Thus, optimum activity occurs at a point where the molar ratio of  $Mg^{++}$  to ATP is of the order of 2:1. When the ratio of  $Mg^{++}$  to ATP falls below 1:1 (arrows on Fig. 43 ), the free ATP causes a sharp decline in activity. This observation suggests that the substrate is in fact the Mg-ATP complex and that the reaction is inhibited by free ATP. Similar effects in other systems will be compared in the discussion below. In Fig. 44 the data in Fig. 43 are rearranged in the form of a double reciprocal plot: it will be apparent that  $Mg^{++}$  has not

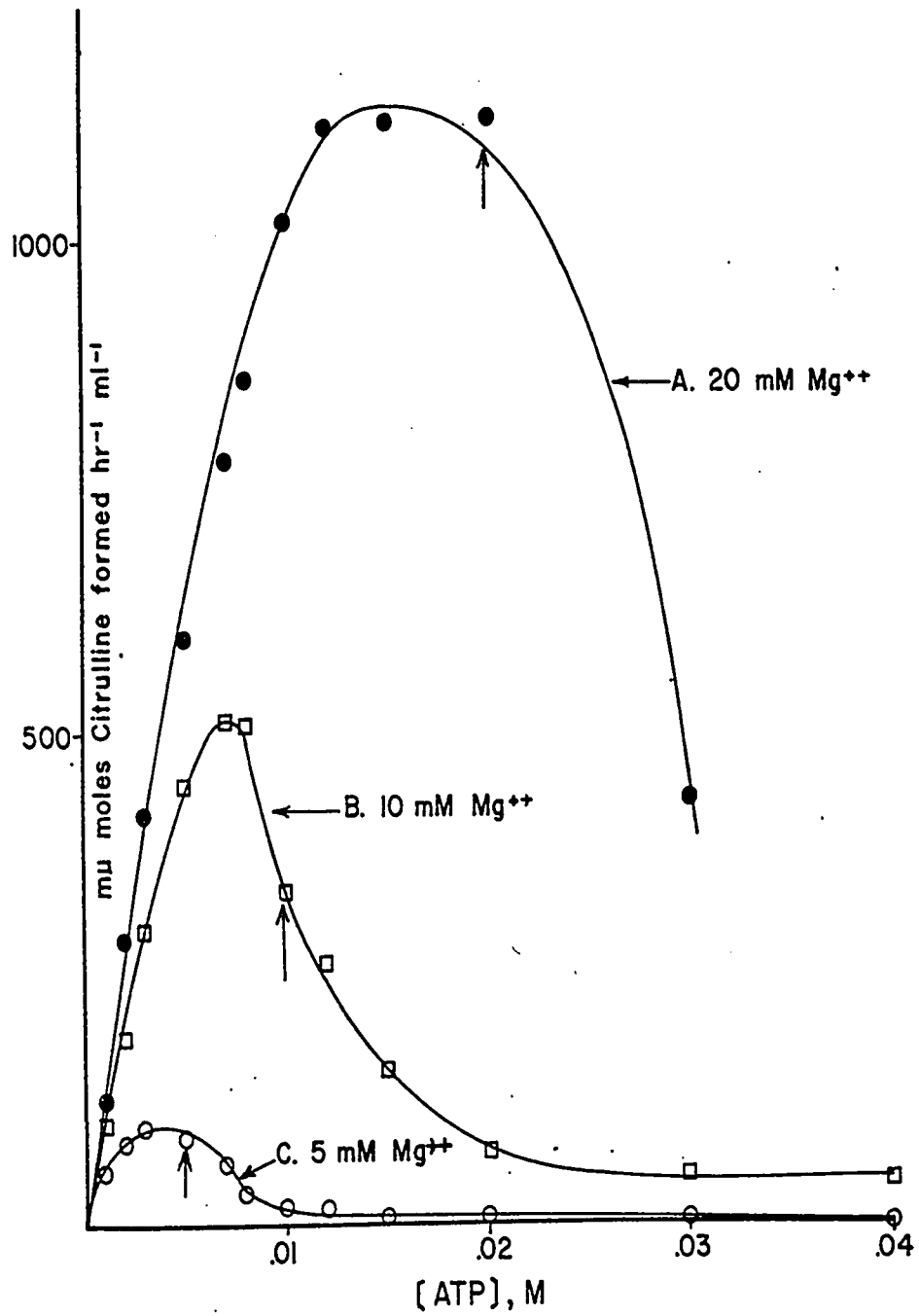


Fig. 43. The effect of varying concentrations of ATP at three concentrations of Mg<sup>++</sup> (20 mM Mg<sup>++</sup>, curve A; 10 mM Mg<sup>++</sup>, curve B; 5 mM Mg<sup>++</sup>, curve C). The reaction mixture was as previously described.

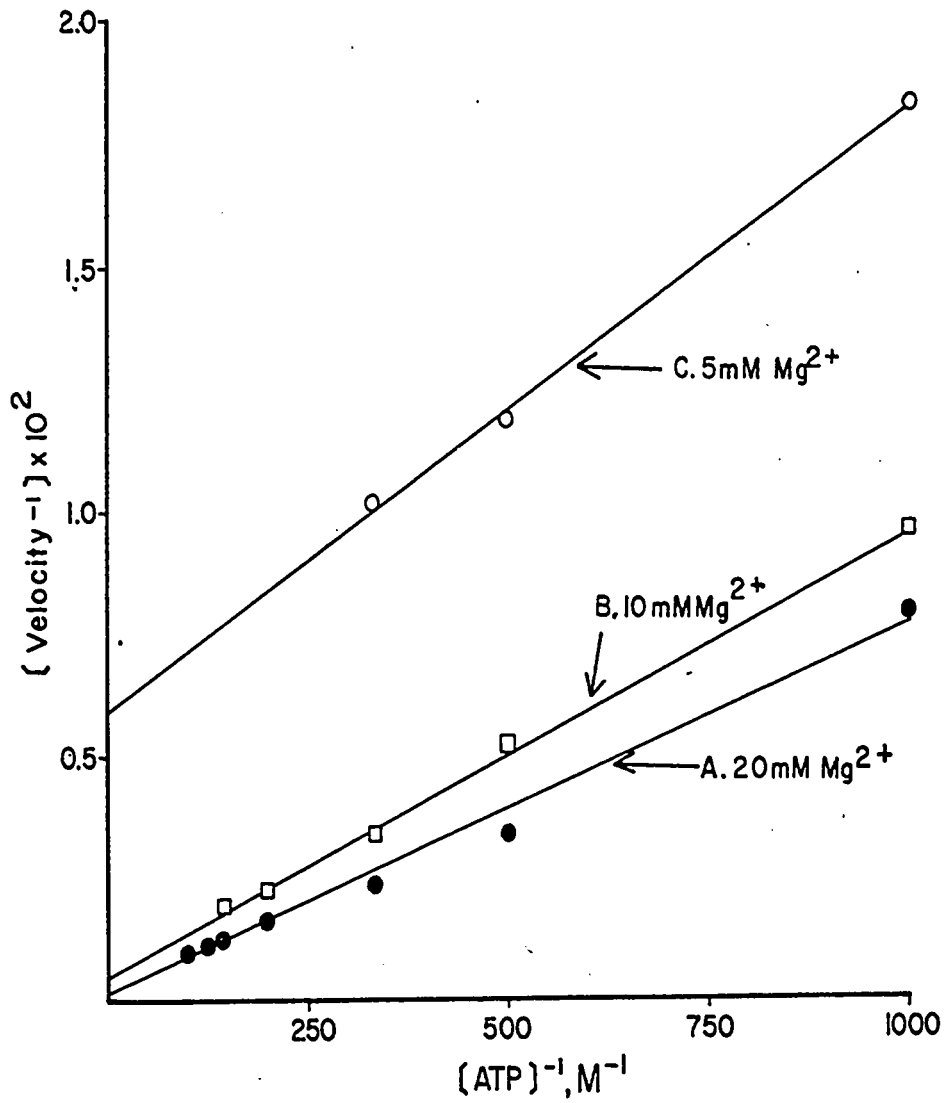


Fig. 44. Double reciprocal plot of velocity versus ATP concentrations at three concentrations of  $\text{Mg}^{++}$  (20 mM  $\text{Mg}^{++}$ , curve A; 10 mM  $\text{Mg}^{++}$ , curve B; 5 mM  $\text{Mg}^{++}$ , curve C). The plot was made from values obtained from Fig. 43.

only increased the  $V_{\max}$ , but also decreased the  $K_m$ . This suggests the possibility that, at low ATP concentrations, where free  $Mg^{++}$  is in excess, this divalent cation has produced a conformational change in the enzyme such that there is decreased affinity for the substrates. Fig. 45 shows the effect of varying  $Mg^{++}$  concentration in the presence of different concentrations of ATP. For 5, 10 and 20 mM ATP, optimal activity occurred at approximately 20, 24 and 36 mM  $Mg^{++}$ . Again the molar ratios of  $Mg^{++}$  to ATP were approx. 2:1. It will be seen that, comparing curve A, B and C, the higher the concentration of ATP used in the assay, the greater is the concentration of  $Mg^{++}$  required to observe measureable activity. It is interesting that the activity became observable (on the abscissa) when the ratio of  $Mg^{++}$  to ATP concentration was 1:2. These observations suggest again that free ATP is an inhibitor of this reaction and not the substrate. The arrows indicate the activity at equimolar  $Mg^{++}$  to ATP; presumably at this point, almost all of the ATP and  $Mg^{++}$  are in the form of the complex, as shown by the equilibrium constant of 18,000 for this reaction (O'Sullivan and Perrin, 1964). Increasing the  $Mg^{++}$  beyond this point results in

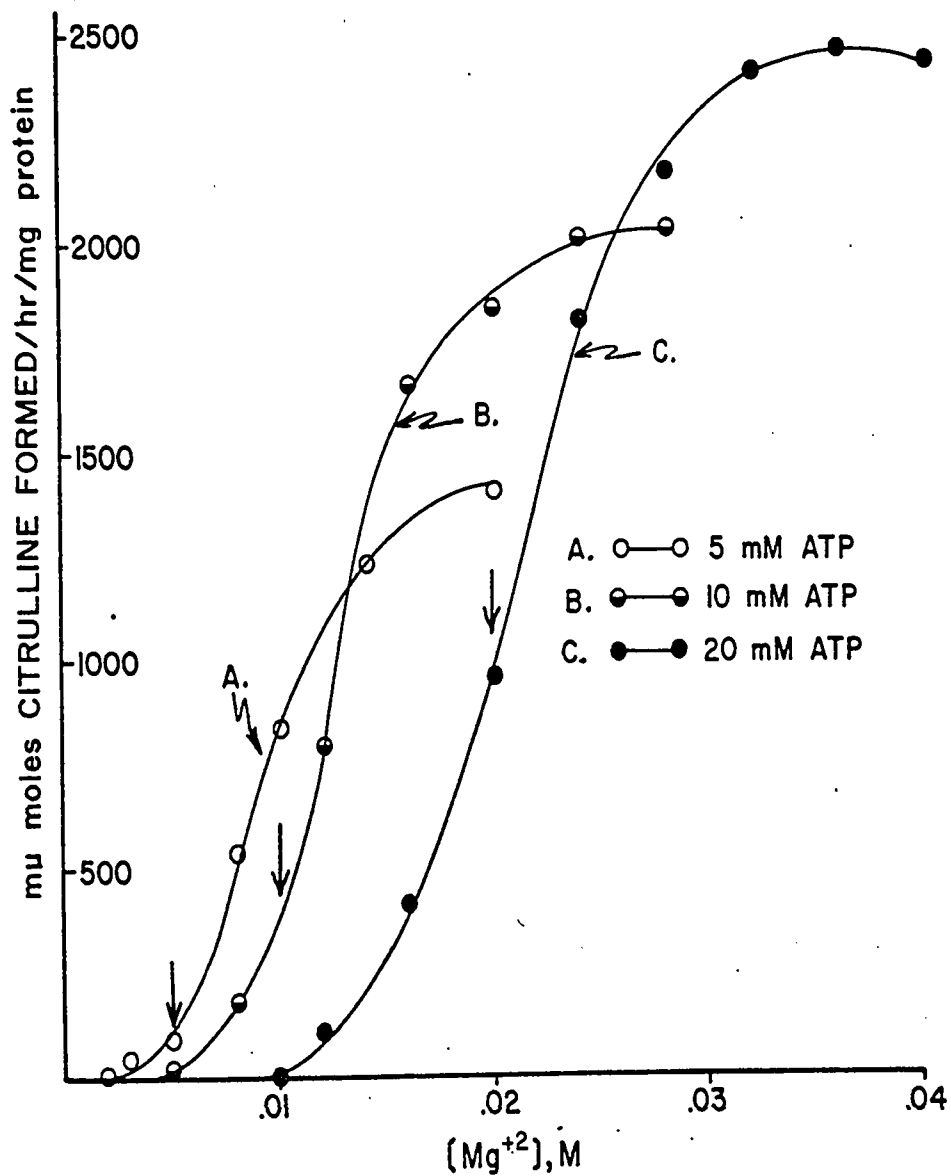


Fig. 45. Effect of various Mg<sup>++</sup> concentrations at three concentrations of ATP (curve A, 5mM; curve B, 10 mM; curve C, 20 mM). The complete reaction mixture contained: glutamine (6 u moles); KCl (100 u moles); NaHCO<sub>3</sub> (20 u moles); ornithine (6 u moles); OTCase (excess); protein (0.3 mg.); Tris-SO<sub>4</sub> buffer (100 u moles, pH 7.6); ATP (u moles as indicated) and varying concentrations of Mg<sup>++</sup> in a final volume of 1.0 ml.

What enzyme?

a sharp and considerable increase in CPSase activity, confirming the conclusions drawn from Fig. 43 and 44 . It will be noted that the curves relating  $Mg^{++}$  to ATP concentration are sigmoidal in all cases. This suggests cooperative or homotropic interaction among the  $Mg^{++}$  binding sites. Fig. 46 shows these data arranged in the form of a Hill plot, the  $n$  values at three different concentrations of ATP were 3,5 and 6 respectively, which indicated a high degree of cooperativity among the  $Mg^{++}$  binding sites and that the cooperativity increased as the ATP concentration increased.

In Fig. 47 , curve A shows the effect on the specific activity of varying levels of Mg-ATP substrate; throughout this experiment equimolar amounts of  $Mg^{++}$  and ATP were mixed to give the concentrations shown along the abscissa. Curve B shows the effect of adding to each of the tubes free ATP to a concentration of  $5 \times 10^{-3}M$ ; again strong inhibition is noted. Curves C and D show the effect of adding  $Mg^{++}$  ions to a concentration of 5 &  $10 \times 10^{-3}M$  respectively; strong activation was observed. It should be noted that with Mg-ATP as substrates, all curves are sigmoidal. The effect of ATP is to increase the sigmoidicity and the

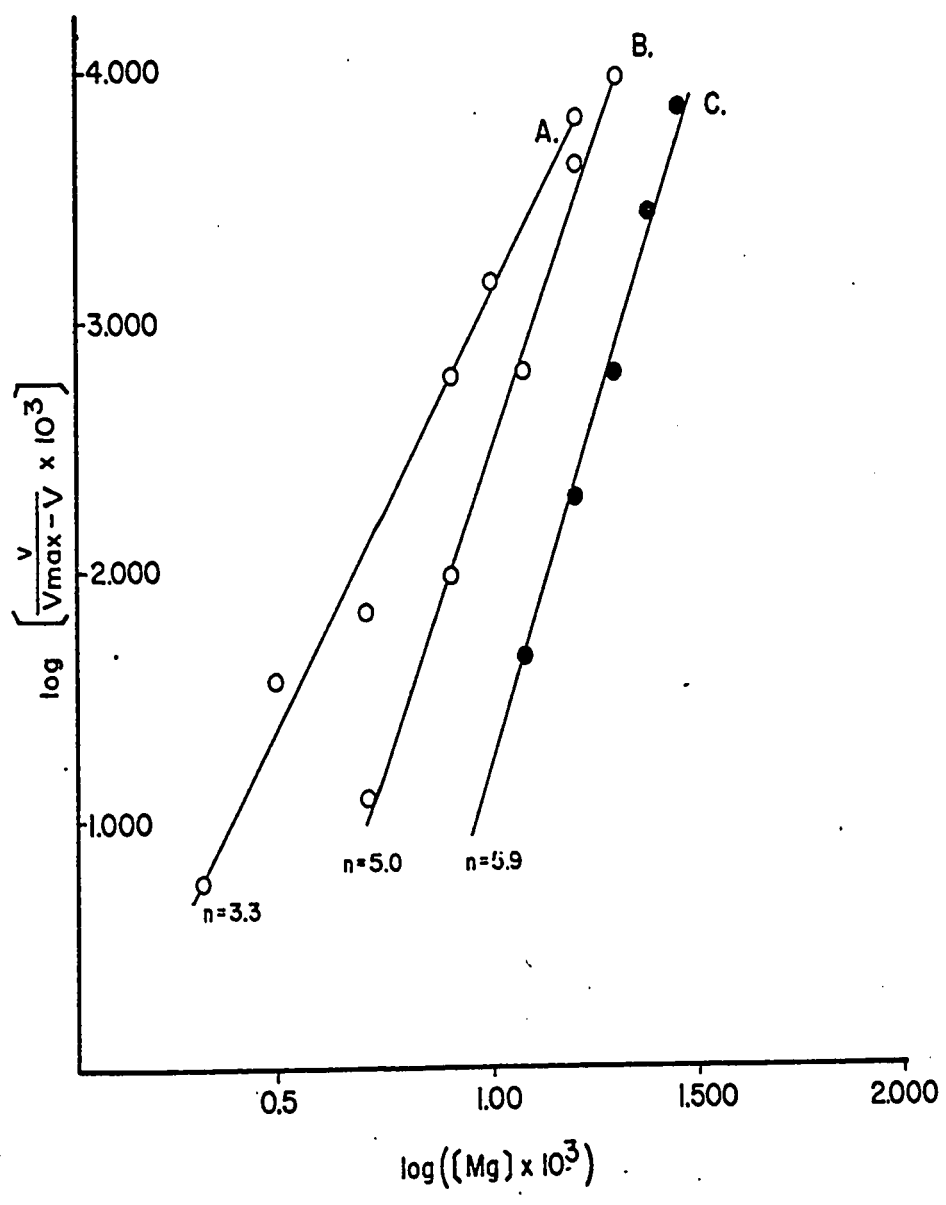


Fig. 46. Hill plot of the data obtained in Fig. 45 (curve A, 5mM ATP; curve B, 10 mM ATP; curve C, 20 mM ATP).

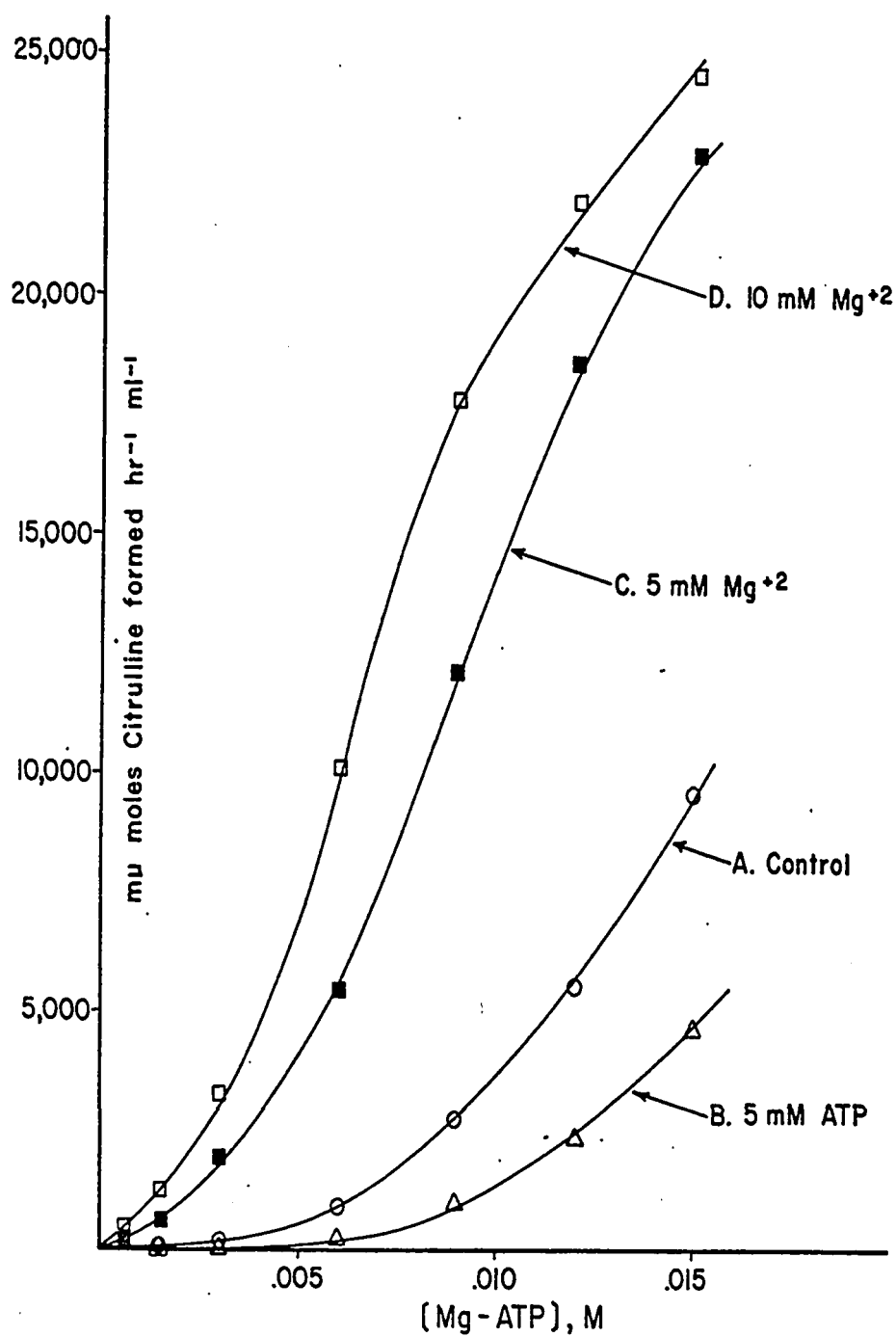


Fig. 47. Effect of various Mg-ATP concentrations on the CPSase activity in the absence (curve A) and presence of 5 mM ADP (curve B), and the presence of 5mM and 10mM Mg<sup>++</sup> (curves C and D respectively).

effect of  $Mg^{++}$  ions is to decrease this phenomenon. These results will be discussed below.

(b) Glutamine

Fig. 48 shows the effect of various concentrations of glutamine with and without  $5 \times 10^{-4}M$  UTP. Curve A indicates that the kinetics are Michaelian with respect to glutamine concentration. The  $K_m$  was  $5 \times 10^{-4}M$  as shown by the plot of  $S/V$  against  $S$  in Fig. 49. UTP inhibition of the reaction was non-competitive and constant at saturated levels of glutamine. However, at low concentration of glutamine the inhibition was reduced, suggesting a requirement for glutamine-binding before full expression of the inhibition by UTP.

(c) Bicarbonate

Fig. 50 shows the activity of CPSase with varying concentrations of bicarbonate with and without  $5 \times 10^{-4}M$  UTP. As shown in the case of ATP, the inhibition was non-competitive and increased at low concentrations of bicarbonate. The substrate saturation curve (A) was hyperbolic with an apparent  $K_m$  of  $3 \times 10^{-3}M$  as shown in the plot of  $S/V$  against  $S$  in Fig. 51.

(d) Potassium

Fig. 52 shows the effect of  $K^+$  ions on the CPSase

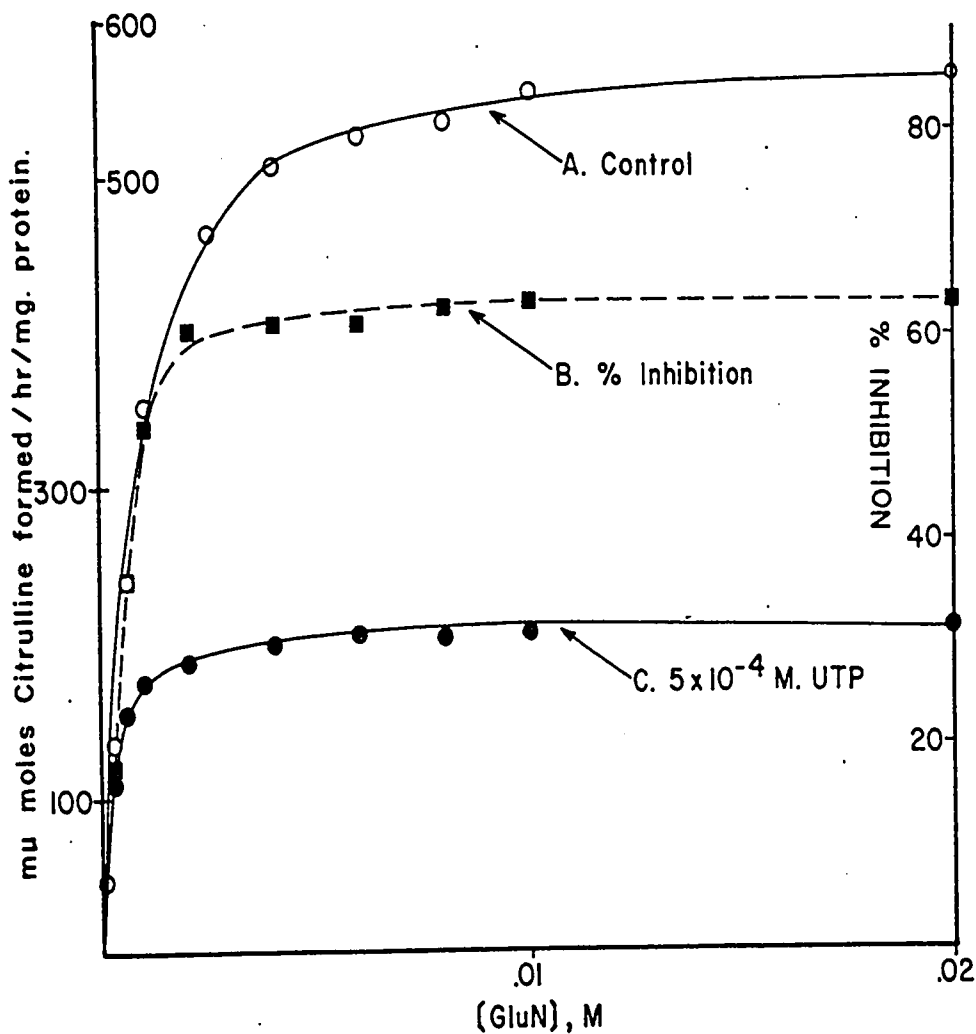


Fig. 48. Effect of various concentrations of glutamine in the absence (curve A) and presence of  $5 \times 10^{-4} M$  UTP (curve B). % inhibition is given in curve C. The reaction mixture contained:  $MgSO_4$  (20 u moles);  $Na_2ATP$  (20 u moles),  $NaHCO_3$  (20 u moles),  $KCl$  (100 u moles), ornithine (6 u moles), OTCase (excess), protein (0.34 mg.),  $Tris-SO_4$  buffer (100 u moles, pH 7.6) and varying concentrations of glutamine in a final volume of 1.0 ml.

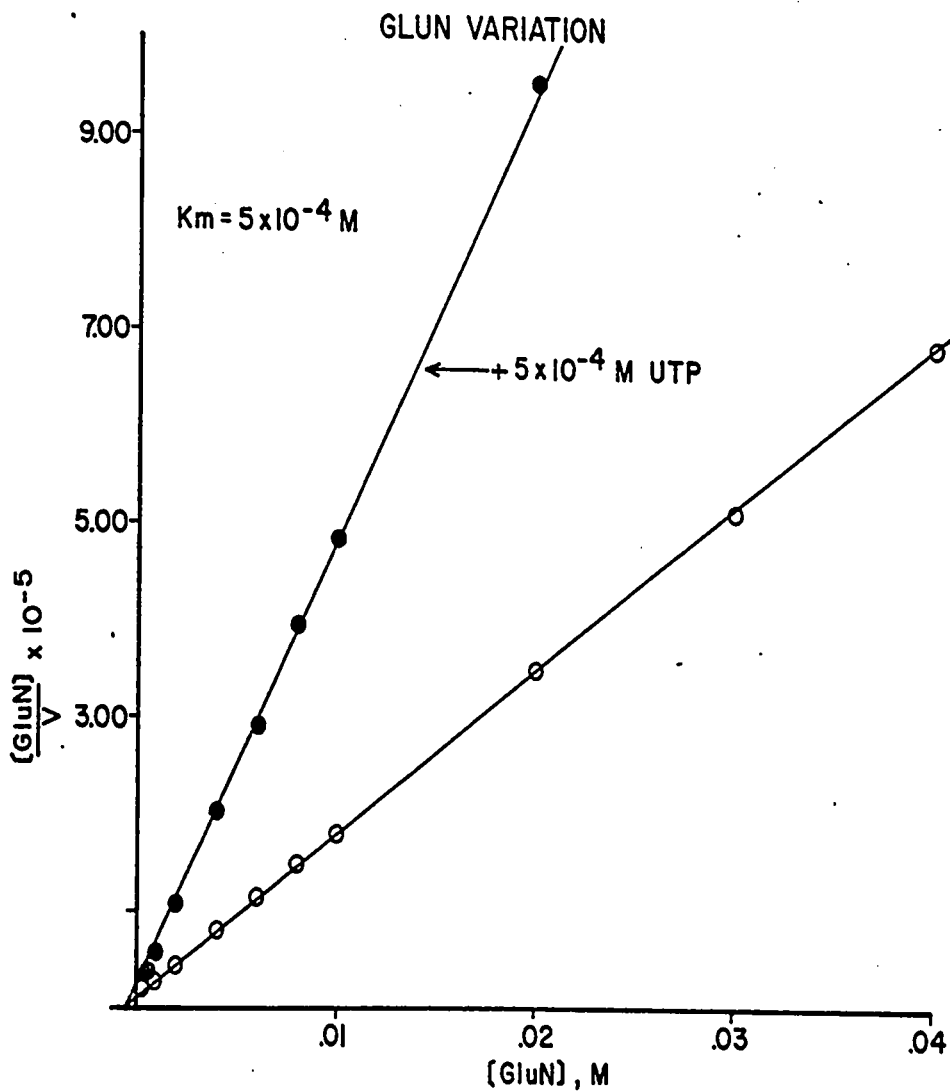


Fig. 49. Plot of (S)/V versus (S) for the estimation of the  $K_m$  for glutamine in the presence and absence of  $5 \times 10^{-4}$  UTP. Values in Fig. 48 were used for plotting this curve.

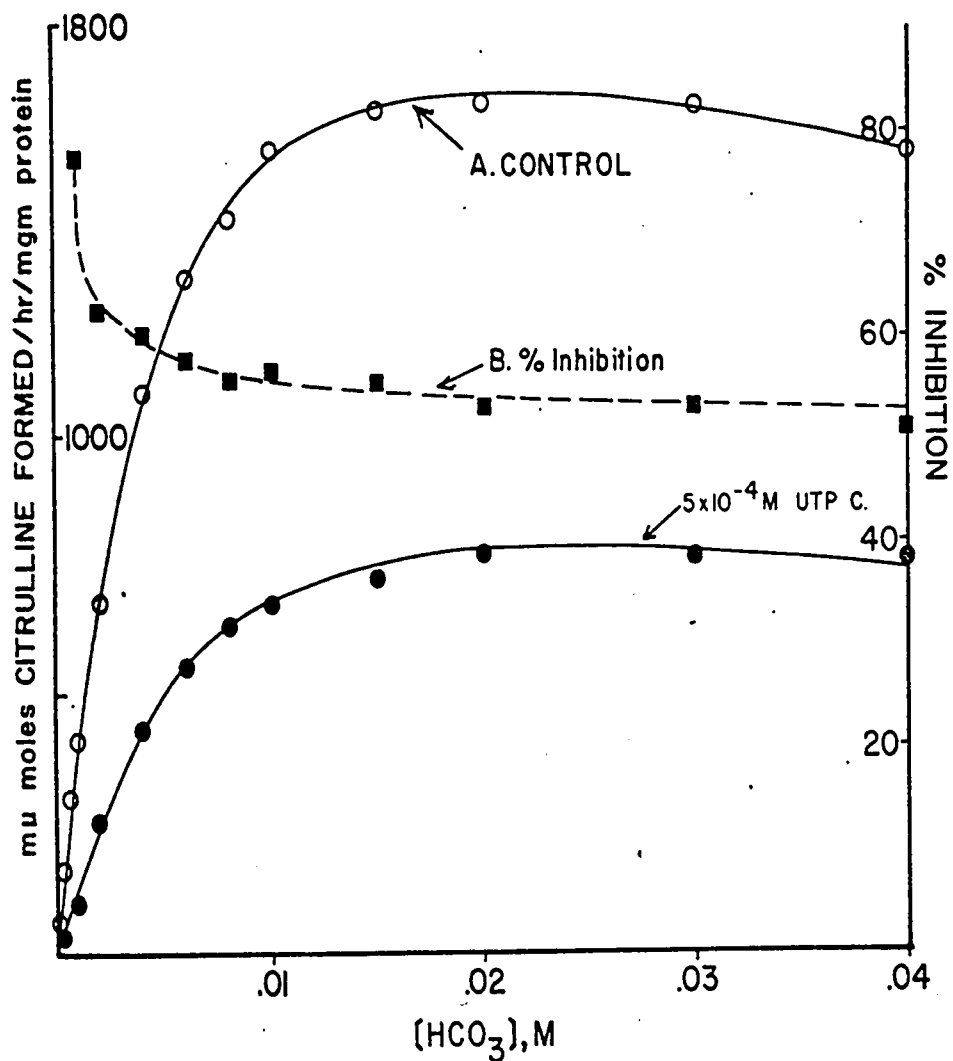


Fig. 50. Effect of various concentrations of  $\text{HCO}_3^-$  in the absence (curve A) and presence of  $5 \times 10^{-4} \text{ M UTP}$  (curve C). % inhibition is given in curve B. The reaction mixture contained:  $\text{MgSO}_4$  (20  $\mu\text{ moles}$ );  $\text{Na}_2\text{ATP}$  (20  $\mu\text{ moles}$ ); glutamine (6  $\mu\text{ moles}$ );  $\text{KCl}$  (100  $\mu\text{ moles}$ ); ornithine (6  $\mu\text{ moles}$ ); OTCase (excess); protein (0.32 mg.);  $\text{Tris-SO}_4$  buffer (100  $\mu\text{ moles}$ , pH 7.6) and varying concentrations of  $\text{NaHCO}_3$  in a final volume of 1.0 ml.

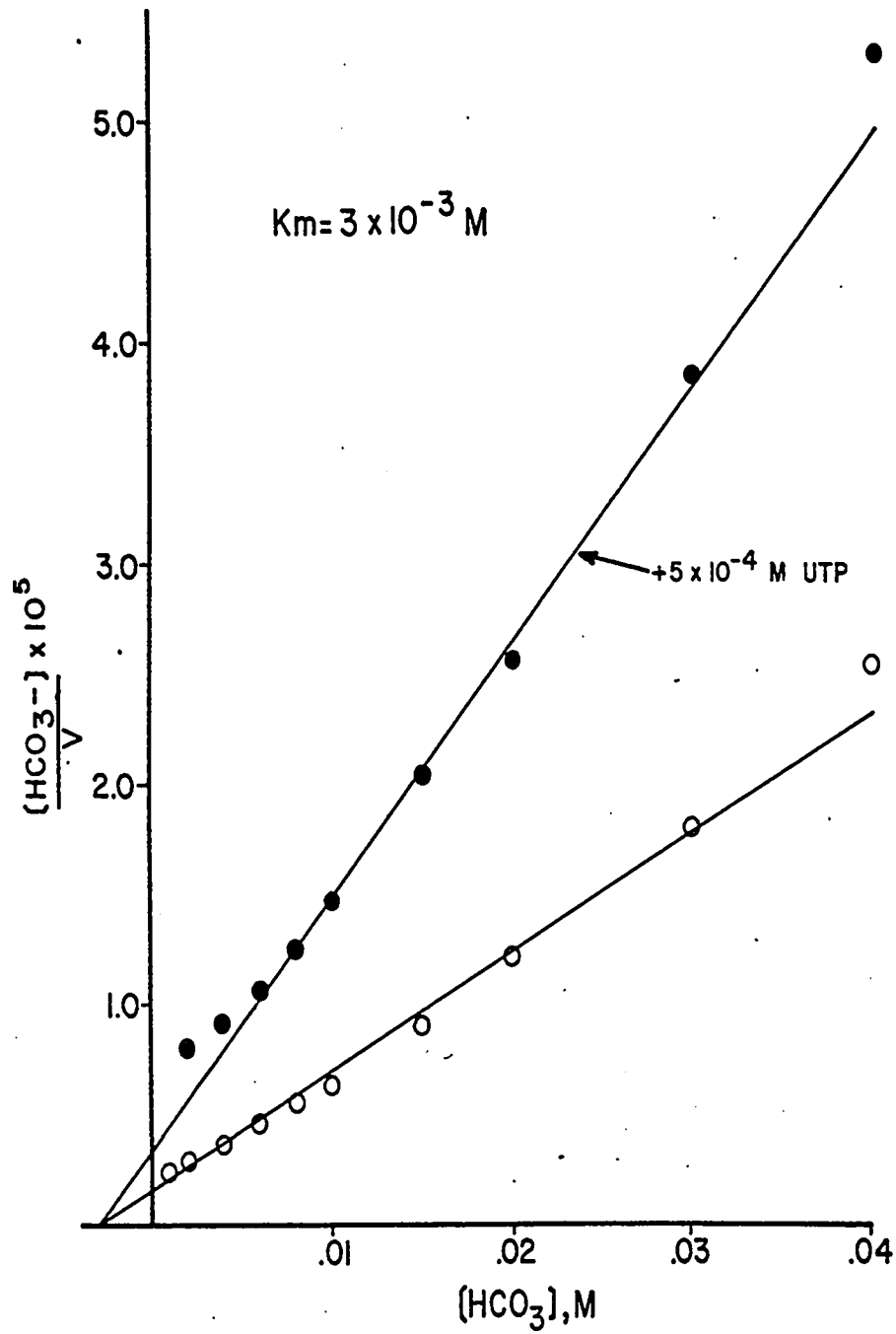


Fig. 51. Plot of (S)/V versus (S) for the estimation of the  $K_m$  for  $\text{HCO}_3^-$  in the presence and absence of  $5 \times 10^{-4} \text{ M UTP}$ . Plot was made from the values in Fig. 50.

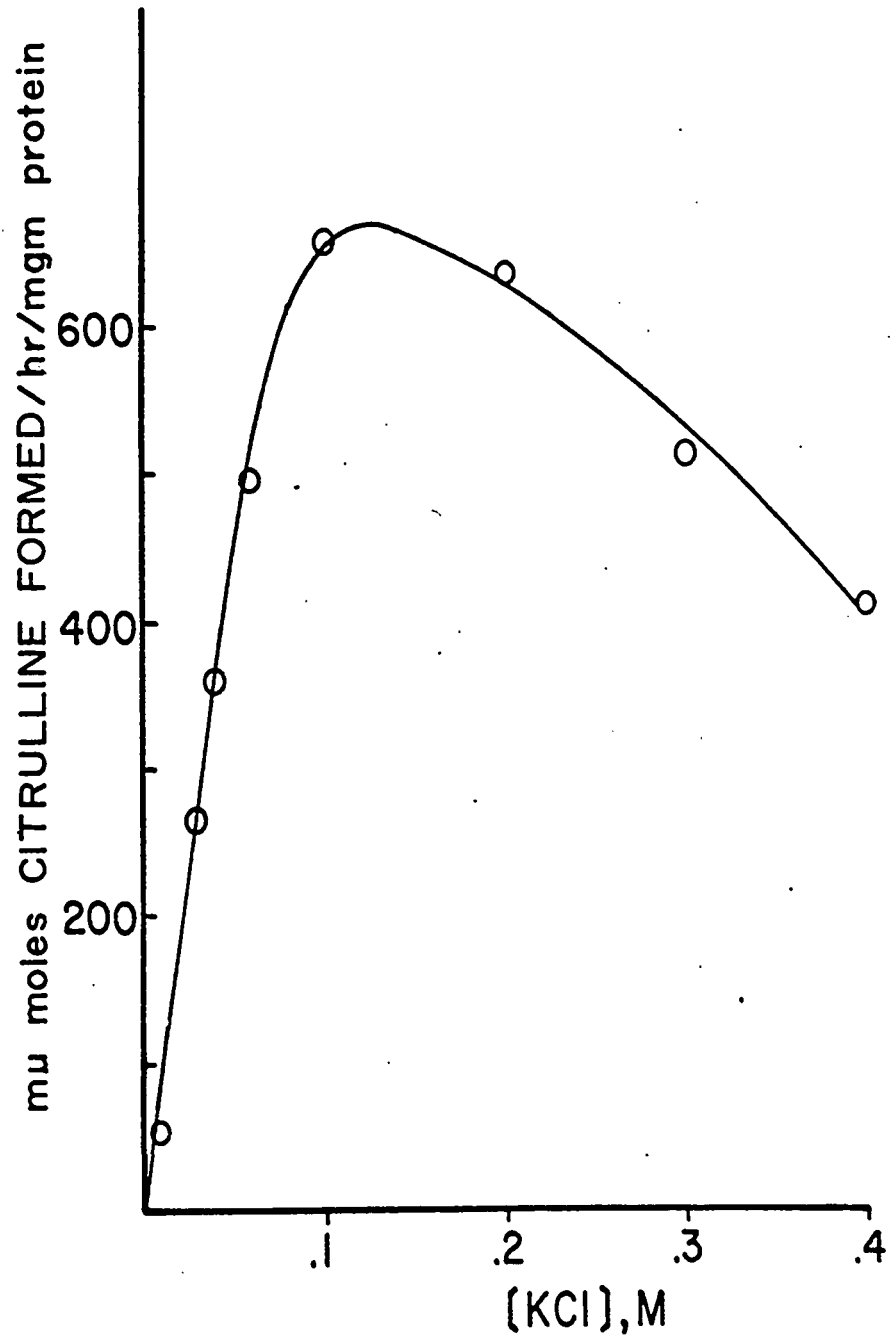


Fig. 52. Effect of various concentrations of  $K^+$  ions. The reaction mixture contained:  $MgSO_4$  (20 u moles);  $Na_2ATP$  (20 u moles); glutamine (6 u moles); ornithine (6 u moles); OTCase (excess); protein (.34 mg.);  $NaHCO_3$  (20 u moles); Tris- $SO_4$  buffer (100 u moles, pH 7.6) and varying concentrations of KCl in a final volume of 1.0 ml.

reaction.  $K^+$  was found to be an absolute requirement and could not be replaced by  $Na^+$  ions. The optimum concentration was 0.1M; higher concentrations inhibited the CPSase reaction (0.4M caused 37% inhibition).

Table 21 summarizes the  $K_m$  values as determined by the two assay systems used in this investigation. Generally, the  $K_m$  value obtained using assay system I, which contained a large excess of  $Mg^{++}$ , are 10x larger than those obtained using assay system II, which contained Mg and ATP in equal amounts. The  $K_m$  value for glutamine was 5x less in assay system I than in assay system II.

#### 7. Inhibition by UTP

Fig. 53 shows the effect of UTP concentrations on the CPSase reaction in the form of a plot of  $1/V$  against UTP, a plot for a non-competitive inhibitor (Dixon and Webb, 1964). The  $K_i$  for UTP was calculated to be  $2.4 \times 10^{-4}M$ .

Table 21

$K_m$ 's as Determined by Assay Systems I and II

Substrate	$K_m$ Values	
	Assay I	Assay II
Mg-ATP		$3.5 \times 10^{-3} M$
Glutamine	$1 \times 10^{-4}$	$5 \times 10^{-4}$
Bicarbonate	$4.2 \times 10^{-2}$	$3 \times 10^{-3}$
Mg <sup>++</sup>	$3.1 \times 10^{-2}$	
ATP	$1.3 \times 10^{-2}$	

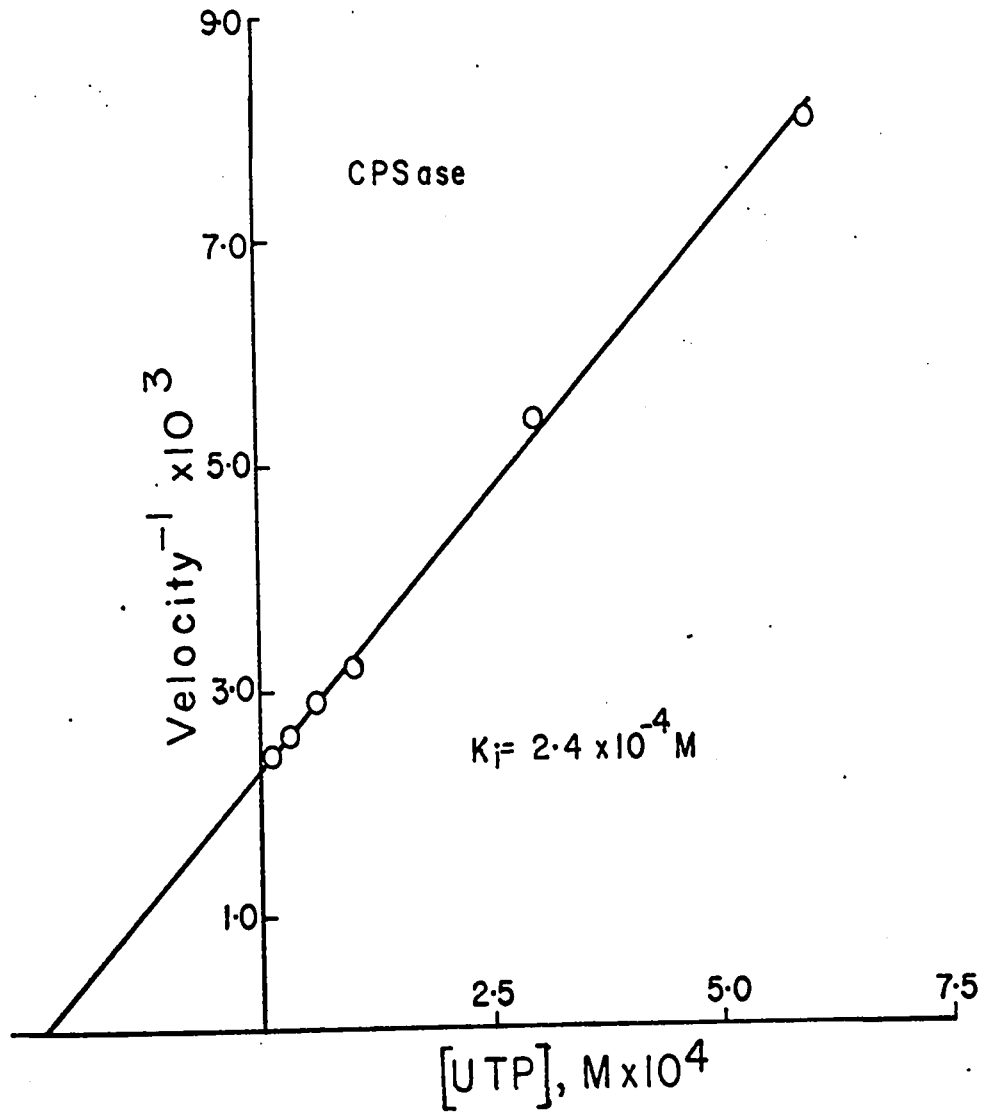


Fig. 53. Plot of  $1/V$  versus UTP concentrations for a non-competitive inhibitor to give the  $K_i$  for UTP inhibition of CPSase. The plot was made from values obtained in Fig. 54.

## VII. Regulation

### 1. Effect of Purine and Pyrimidine Nucleotides

It was pointed out previously that the regulation of pyrimidine biosynthesis must be considered with respect to cell economy, precursor availability (since CP is also utilized in arginine biosynthesis) and coupled utilization with purines in the synthesis of DNA and RNA. In E. coli, both CPSase and ATCase are affected by purines and pyrimidine nucleotides. CPSase was inhibited by UMP and activated by XMP and IMP (Anderson and Meister, 1966), whereas ATCase was inhibited by CTP and activated by ATP (Gerhart and Pardee, 1962). ATP also antagonized the inhibition by CTP of the ATCase reaction. Ornithine was found not only to prevent the inhibition by UMP but also to activate the CPSase reaction (Pierard, 1966). It thus became of interest to study the effects of purines and pyrimidine nucleotides on the yeast ATCase and CPSase activities.

(a) Aspartate Transcarbamylase

Table 22 shows the ability of pyrimidine nucleotides (column A) to inhibit the enzyme and to compete with the binding of UTP. From column C, it was seen that UTP was the most potent inhibitor, however, TTP and UDP caused substantial inhibitions. UMP and uracil were without effect. These results suggest that the two terminal phosphate groups are essential to the action of the UTP molecule. Column D shows that no compound relieved the inhibition by UTP markedly; however, CDP may have had a slight antagonizing effect. The significance of this effect of CDP on UTP inhibition remains to be justified by more experimentation. The fact that TTP was inhibitory whereas CTP was not suggests that the hydroxyl group at the C<sub>4</sub> position of UTP and TTP may be involved in binding to the enzyme. CTP possesses an amino group in this position. It is of considerable interest that the deoxyribonucleotide TTP has such marked effect on the ATCase activity.

The effects of purine nucleotides (column A) on the ATCase activity and inhibition by  $2 \times 10^{-3}M$  UTP is

Table 22

Effect of Pyrimidine Nucleosides, Mono-, Di- and Triphosphates and Uracil on ATCase activity and Feedback Inhibition by

$2 \times 10^{-3} \text{M}$  UTP

A	B	C	D
Nucleotide	Ureido-succinate synthesized (mu moles)	Relative Activity	% Inhibition by $2 \times 10^{-3} \text{M}$ UTP with added nucleotide
none	640.6	100	72
CMP	647.9	101	70
CDP	681.2	106	58
CTP	622.5	97	70
d-TMP	691.9	108	68
d-TDP	594.4	93	70
d-TTP	358.0	56	76
UTP	147.8	23	81
UDP	363.5	57	76
UMP	613.6	96	74
uracil	652.8	102	73

The reaction mixture contained L-aspartate (50 u moles), carbamyl phosphate (5 u moles), phosphate buffer (50 u moles pH 7.4), enzyme in 5 ul and nucleotide or additive (2.5 u moles) in a final volume of 0.5 ml. The ureido-succinate synthesized after incubation at 25°C for 5 min. was determined as in Methods (page 78 ).

shown in Table 23 . Unlike the case with E. coli, where ATP was found to activate the ATCase activity and to antagonize the inhibition by CTP, no purine nucleotide at a concentration of  $5 \times 10^{-3} \text{M}$  was observed to activate yeast ATCase (column C) nor to antagonize the inhibition by UTP (column D). Previously Kaplan et al. (1967) reported that high concentration of ATP ( $2-8 \times 10^{-2} \text{M}$ ) was able to reverse the inhibition by UTP. Because of the high concentration used, the physiological significance of this observation awaits studies of the intracellular concentration of ATP.

(b) Carbamyl Phosphate Synthetase

Table 24 shows the effect of various pyrimidine nucleotides on the CPSase reaction with and without  $2 \times 10^{-3} \text{M}$  UTP. As shown in column C, most of the nucleotides inhibited the activity to a slight extent in assay system II; UDP and UTP were most effective and the latter was in a class by itself. Inhibition was found to be quite consistent in magnitude from one preparation to the next, the variability noted with ATCase did not occur. In assay system I (column D) in which were present low ATP and high  $\text{Mg}^{++}$ , it was noted that only UTP was markedly inhibitory to CPSase.

Table 23

Effect of Purine Nucleosides, Mono-, Di- and Triphosphates  
on ATCase Activity and on the Feedback Inhibition by  
 $2 \times 10^{-3} \text{M}$  UTP

A Nucleotide	B Ureido- succinate synthesized (mu moles)	C Relative Activity	D % Inhibition by $2 \times 10^{-3} \text{M}$ UTP with added nucleotide
none	474.3	100	76
AMP	462.4	97.5	70
ADP	464.9	98.0	71
ATP	453.7	95.7	65
IMP	465.3	98.1	72
IDP	431.6	91.0	71
ITP	443.9	93.6	65
XMP	481.2	101.5	76
XDP	496.0	104.6	73
XTP	477.2	100.6	64
GMP	468.5	98.8	75
GDP	452.6	95.4	76
GTP	439.6	92.7	72

The reaction mixture contained L-aspartate (50 u moles), carbamyl phosphate (5 u moles), phosphate buffer (50 u moles pH 7.4) enzyme in 5 ul and nucleotide (25 u mole) in a final vol. of 0.5 ml. The ureidosuccinate synthesized after incubation at  $25^{\circ}\text{C}$  for 5 min. was determined as in Methods (page 78 ).

Table 24

Effect of Pyrimidine Nucleosides, Mono-, Di- and Triphosphates and Other Compounds on CPSase Activity and on the Feedback

Inhibition by  $2 \times 10^{-3} \text{M}$  UTP

A	B	C	D	E
Nucleotide ( $5 \times 10^{-3} \text{M}$ )	Citrulline synthesized /20 min. ( $\mu$ moles) System II	Relative Activity Assay Sys. II	Relative Activity Assay Sys. I	% Inhibition by $2 \times 10^{-3} \text{M}$ UTP with added nucleotide Assay Sys. II
none	123.2	100.0	100	79.8
CMP	118.7	96.3	86.9	90.8
CDP	92.6	75.1	92.8	85.6
CTP	76.1	61.8	99.5	90.3
UMP	90.7	73.6	98.0	84.6
UDP	69.0	56.0	100.0	87.4
UTP	11.9	9.7	18.7	96.9
uracil	82.6	67.0	-	82.5
UDPG	76.5	62.0	-	87.2
TMP			95.4	
TDP			79.4	
TTP			95.4	

When assay system II was used, equimolar concentrations of nucleotide and  $\text{MgSO}_4$  were added to a final volume of 1.0 ml. Estimation was as described in Methods.

A slight inhibition was observed with this assay system in the case of TDP. Column E shows that none of the pyrimidine nucleotides significantly affected the inhibition produced by UTP. Unlike the case with ATCase,  $7 \times 10^{-3} \text{M}$  UTP caused virtually total inhibition of the CPSase activity. The effect of pyrophosphate could not be studied since this compound precipitated with  $\text{Mg}^{++}$  ions.

Table 25 shows the effect of purine nucleotides on yeast CPSase in the presence and absence of  $2 \times 10^{-3} \text{M}$  UTP. The acid phosphates of inosine and guanine inhibited the activity slightly with the triphosphates being most effective. The inhibition by AMP was slight but a marked inhibition was observed with ADP. It was shown above that ATP, the substrate, inhibited CPSase at high concentrations. XMP and XDP caused activation of the CPSase reaction. It is obvious by comparison with the results obtained with assay system I, that much of the inhibition observed with assay system II was due to the effect of these compounds on  $\text{Mg}^{++}$  ion concentration. Under these conditions of assay, a much greater activation of CPSase by XMP was noted. Column D shows that only XMP caused any marked diminution of the

Table 25

Effect of Purine Nucleosides, Mono-, Di- and Triphosphates  
on CPSase Activity and on the Feedback Inhibition by  
 $2 \times 10^{-3} \text{M}$  UTP

A	B	C	D	E
Additive	Citrulline Synthesized /20 min. ( $\mu$ moles) System II	Relative Activity System II	Relative Activity System I	% Inhibition by $2 \times 10^{-3} \text{M}$ UTP with added nucleotide System II
none	123.2	100.0	100	79.8
AMP	103.6	84.0	74	85.6
Cyclic-AMP	72.3	58.6	-	90.8
ADP	42.3	34.3	31.1	92.1
XMP	166.5	135.1	213.1	53.0
XTP	101.3	82.2	129.3	79.3
IMP	111.0	90.0	71.1	84.0
IDP	79.4	64.4	50.1	88.7
ITP	63.6	51.6	80.7	92.1
GMP	109.0	88.5	96.9	85.8
GDP	74.2	60.2	90.8	87.4
GTP	68.1	55.2	74.8	91.2

When assay system II was used, equimolar concentrations of nucleotide and  $\text{MgSO}_4$  were added to a final volume of 1.0 ml. Estimation was as described in Methods.

apparent inhibition of CPSase by UTP, a phenomenon which seemed to merit further study.

Fig. 54 shows the effect of varying XMP and UTP concentrations on the CPSase reaction. As shown by curve A, the inhibition by UTP started levelling off at  $3-5 \times 10^{-3} \text{M}$ . Curve B illustrates the activation of CPSase activity produced by varying concentrations of XMP. In this case, a more than 3-fold activation was noted at XMP concentrations of  $2 \times 10^{-3} \text{M}$  and higher. In other experiments, these concentrations caused activations of 2-4-fold.

Fig. 55 shows the effect of XMP concentration in the presence of 3 concentrations of UTP. It will be observed that even at the highest concentration of UTP ( $2 \times 10^{-3} \text{M}$ , curve D), XMP of a given concentration was still capable of causing an activation of about the same extent as in control preparations (curve A). Fig. 56 shows the data replotted as the amount of activation produced in the presence of varying UTP concentration, and in the control, against various

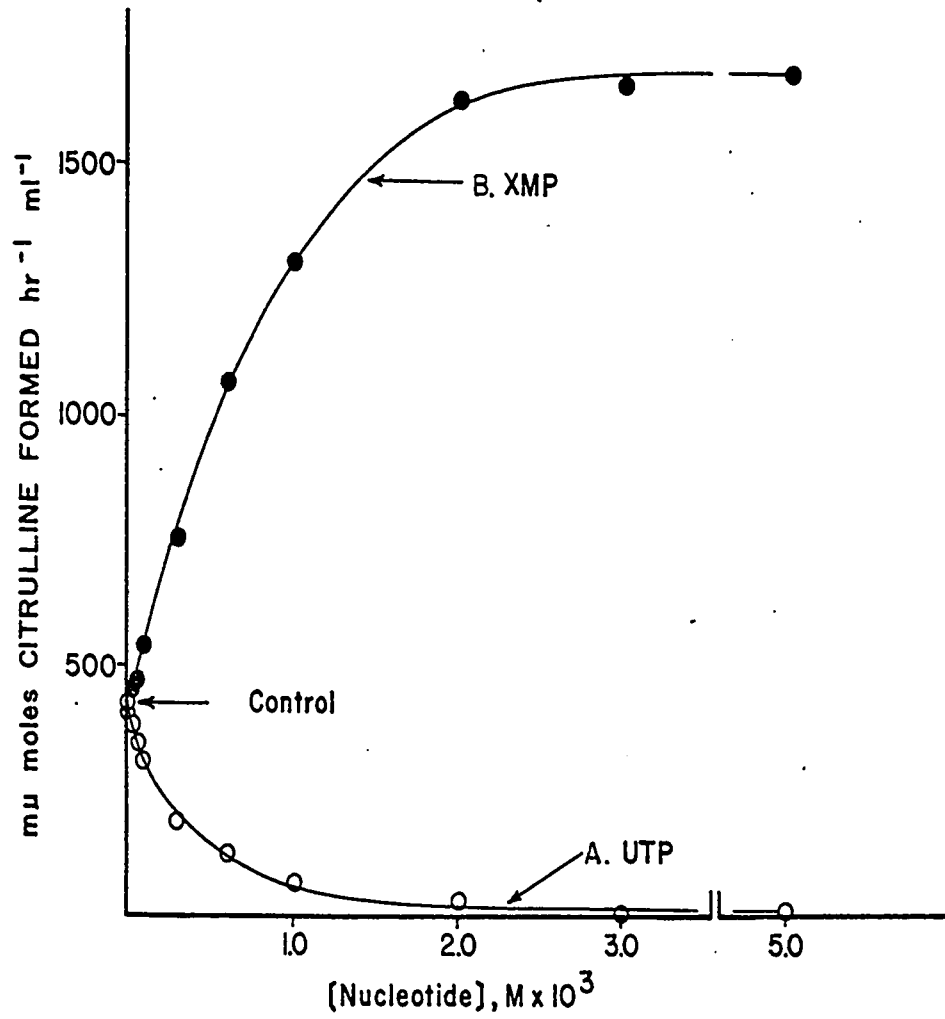


Fig. 54. Effect of various concentrations of XMP (curve B) and UTP (curve A). The reaction mixture contained  $MgSO_4$  and ATP (20 u moles); glutamine (6 u moles);  $NaHCO_3$  (20 u moles); ornithine (6 u moles); OTCase (excess); Tris- $SO_4$  buffer (100 u moles, pH 7.6); protein (1 mg.) and various concentrations of XMP or UTP in a final volume of 1.0 ml.

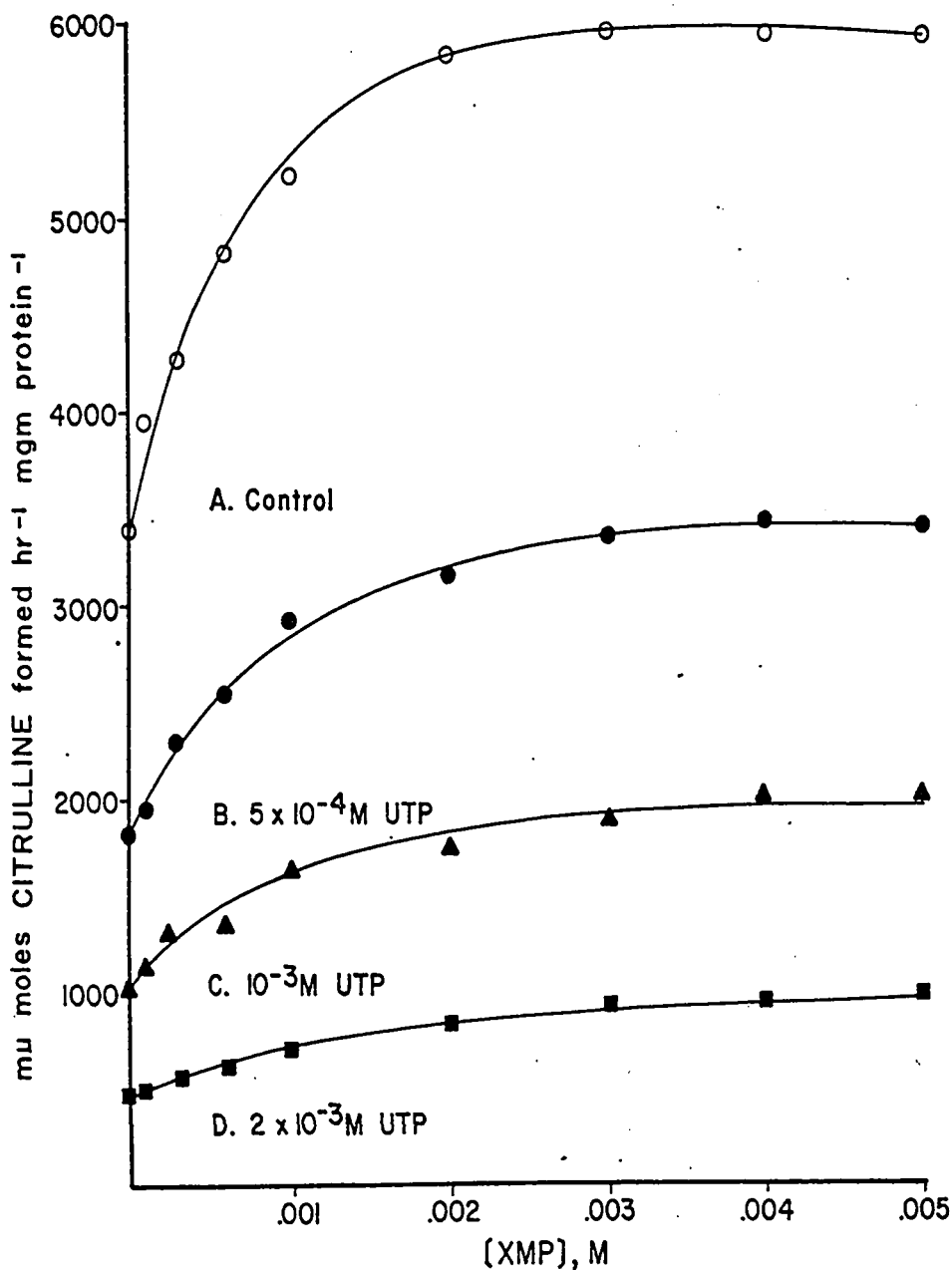


Fig. 55. Effect of various concentrations of XMP in the absence (curve A) and presence of 3 different concentrations of UTP ( $5 \times 10^{-4} \text{M}$ , curve B;  $10^{-3} \text{M}$ , curve C;  $2 \times 10^{-3} \text{M}$ , curve D). The complete reaction mixture contained:  $\text{MgSO}_4$  (20 u moles);  $\text{Na}_2\text{ATP}$  (20 u moles); glutamine (6 u moles);  $\text{NaHCO}_3$  (20 u moles); ornithine (6 u moles); OTCase (excess);  $\text{KCl}$  (100 u moles); protein (0.25 mg.);  $\text{Tris-SO}_4$  (100 u moles, pH 7.6) and varying concentrations of XMP to a final volume of 1.0 ml.

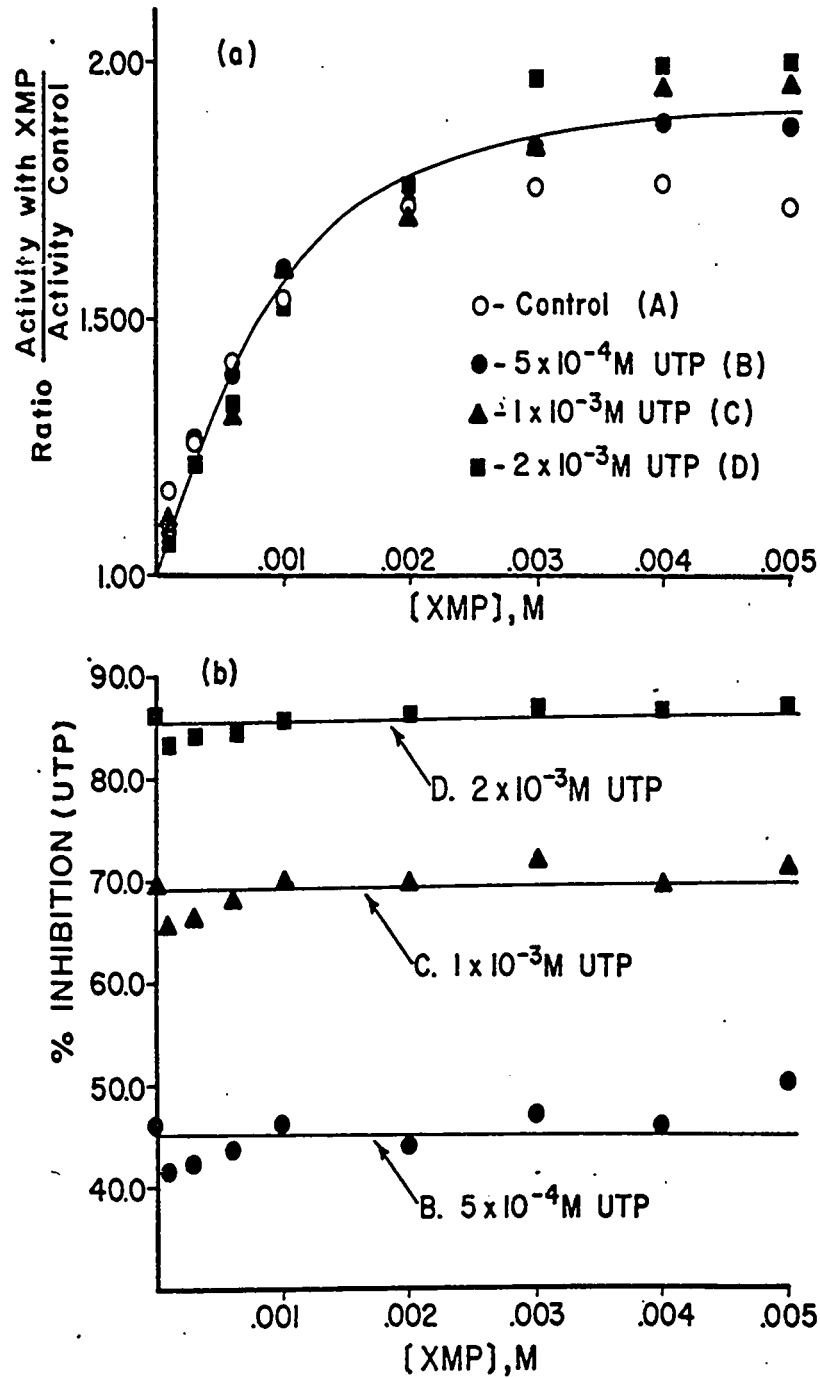


Fig. 56. (a) The relative activation by XMP in the absence (A) and presence of the various concentrations of UTP (B,C,D) versus varying concentrations of XMP. (b) The percentage inhibition caused by the different concentration of UTP at various concentrations of XMP.

concentrations of XMP. The curve was hyperbolic and fitted at least approximately all the experimental points, showing that the extent of XMP activation was independent of the presence of UTP. When the % inhibition produced by UTP was plotted against the concentration of XMP present in the assay, the curves shown in Fig. 56 were obtained. In all cases, the magnitude of the UTP effect was completely independent of the presence of XMP. These data show that the activation by XMP and inhibition by UTP occur at separate and independent sites. Indeed, preliminary experiments showed that the CPSases of mutants whose ATCases and CPSases are insensitive to inhibition by UTP (Lacroute and Slonimski, 1964) were nevertheless capable of being activated by XMP.

In view of the importance in the regulation of many enzymes by the ratio of ADP:ATP (the energy charge hypothesis, Atkinson, 1966), the effect of ADP on the CPSase reaction was further studied. Fig. 57, curve A shows that the activity falls off with increasing ADP concentrations and, as shown in curve B, an inhibition of 60% was reached at  $5 \times 10^{-3} \text{M}$  ADP. Both curves were hyperbolic. It was shown above (Table 25) that AMP

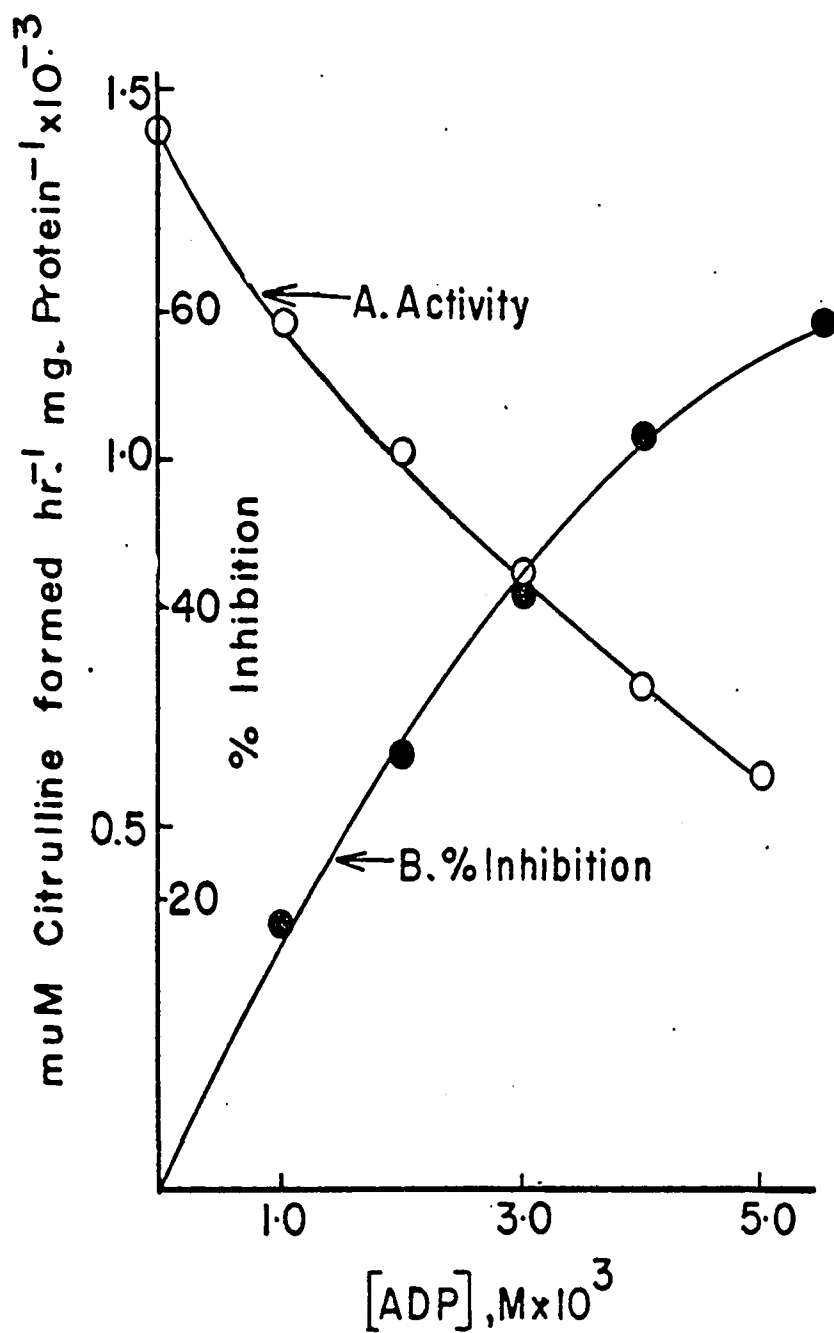


Fig. 57. Effect of various concentrations of ADP on the CPSase activity (curve A). % inhibition is given in curve B.

had no significant effect on CPSase.

The effects of ADP, XMP and UTP on a single CPSase preparation at different levels of the substrate ATP are compared in Fig. 58 ; in this experiment  $Mg^{++}$  concentration was 20mM. Activation by XMP and inhibition by UTP and ADP were apparent even at very low concentrations of ATP, where  $Mg^{++}$  was present in large excess. Thus, the activation produced by XMP occurs in the presence of excess  $Mg^{++}$ , and thus would appear to be independent of the activation (shown above, in Fig. 58 ) produced by the divalent cation. Similarly, the inhibitions produced by UTP and ADP at low ATP concentrations, occur in excess  $Mg^{++}$ , showing their effect is not due to binding of  $Mg^{++}$ ; thus, they must act directly on the enzyme protein. The ADP might well be acting as a competitive inhibitor of the substrate ATP. Attempts to demonstrate this were unsuccessful, largely because of the inhibition of the CPSase caused by higher concentrations of ATP itself.

## 2. The Physiological Role of the CPSase-ATCase Complex

Davis (1967) has suggested that the functional significance of enzyme aggregates, such as ours, might

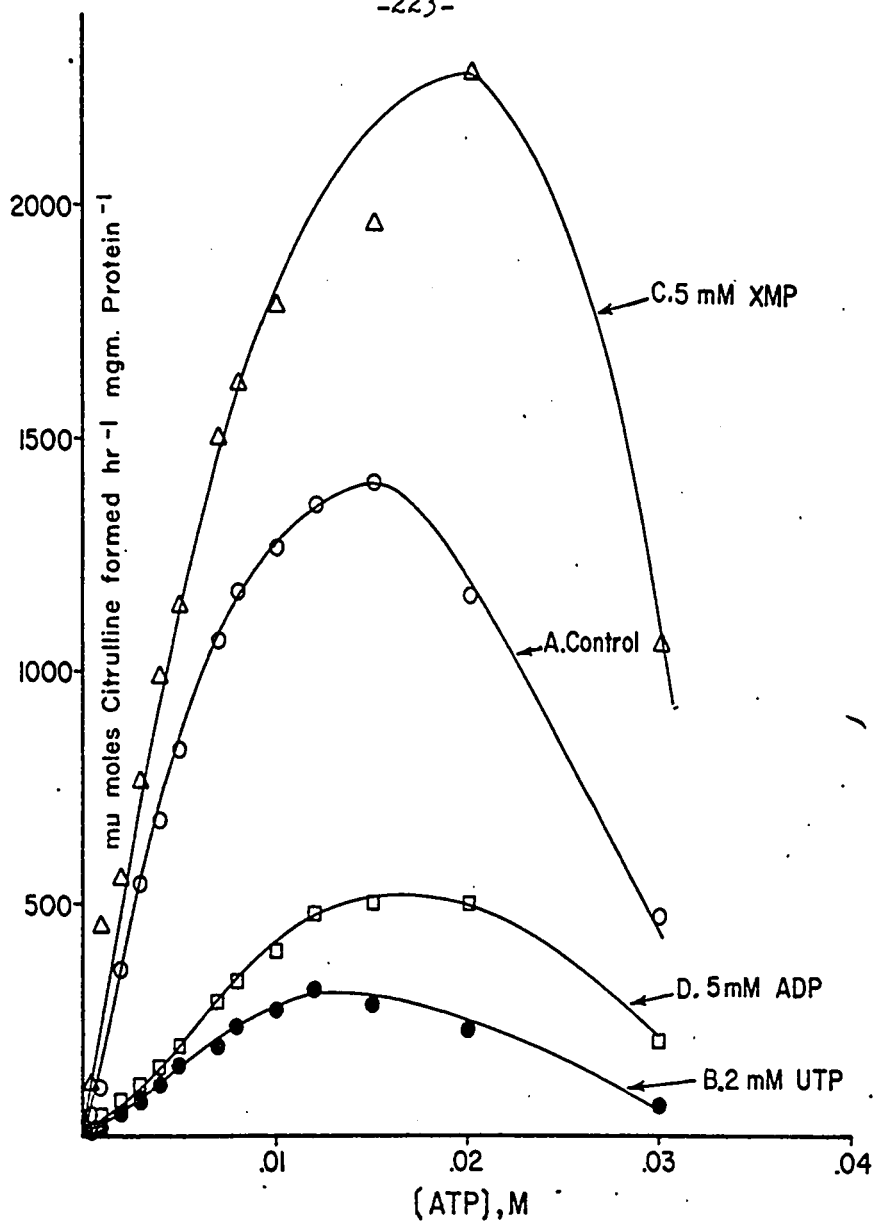


Fig. 58. Effect of various concentrations of ATP in the absence (curve A) and presence of 2mM UTP (curve B), 5mM XMP (curve C) and 5mM ADP (curve D).

be to 'channel' the product of the first enzyme preferentially into the biosynthetic pathway which produced it. One might suppose that the CP synthesized by CPSase would thus be preferentially available for US synthesis as a result of being produced by the enzyme complex which possessed the ATCase activity; hence pyrimidines would compete on a preferred basis with arginine for the CP produced by the pyrimidine-specific CPSase. This would mean operationally that the CP produced by this CPSase would be bound, all or in part, to the complex and not freely released into the medium, at least if there were sufficient aspartate in the medium to transform it into US. The following experiments were designed as a preliminary test of this hypothesis. Essentially, the test involves the use of labelled CP precursor and the tracing of the label into either US or citrulline in a reaction which includes both the CPSase-ATCase complex and a purified bacterial OTCase: in short, a molecular competition experiment.

Before such competition experiments could be done with confidence, it was necessary to show: 1, that ornithine present in the reaction mixture did not interfere with the enzymatic production of CP, especially in

view of the demonstration that this amino acid did activate the CPSase of E. coli (Anderson and Marvin, 1968); and 2, that our methods were equally sensitive and valid for the detection of the competing citrulline and US; and 3, that exogenous CP could be converted into US by ATCase while the CPSase of the complex was functioning

Table 26 , columns 1 and 2, shows that the incorporation of precursor into CP was linear with time for at least 20 min., whether the CP was subsequently transformed into citrulline or into US. It is noteworthy that in all such experiments, about 10-20% more CP on a molar basis was transformed into US by ATCase than was transformed in parallel tubes into citrulline by OTCase. This is not necessarily a demonstration that some of the CP which is available to the ATCase is not available to the OTCase, since the former activity was present in great excess. This was made necessary by the low CPSase activity of the complex. Comparison of columns 2 and 3 shows that the presence of ornithine neither activated nor inhibited the CPSase reaction.

Table 27 shows the effect of exogenously added CP on the complex while its CPSase was functionally

Table 26  
 Time Course of CP Production by CPSase Estimated as Either  
 Citrulline or Ureidosuccinate Formation; the Effect of  
 Ornithine on the CPSase Reaction

Incubation time (min.)	Column 1	Column 2	Column 3 *
	Citrulline produced (mu moles) + ornithine + OTCase - aspartate	Ureidosuccinate produced (mu moles) - ornithine - OTCase + aspartate	Ureidosuccinate produced (mu moles) + ornithine - OTCase + aspartate
5	55.5	64.2	62.6
10	114.1	141.5	130.1
15	182.7	201.6	191.1
20	239.1	274.8	256.1

\* Ornithine (6 u moles) was included in the reaction mixture. The complete reaction mixture contained MgSO<sub>4</sub> and ATP (20 u moles), NaHCO<sub>3</sub> (20 u mole), glutamine (6 u moles), KCl (100 u moles), Tris-SO<sub>4</sub> buffer (100 u moles pH 7.6) and protein (0.32 mg) to a final volume of 1.0 ml. When citrulline was to be determined, OTCase (excess) and ornithine (6 u moles) were included and aspartic acid (50 u moles) when ureidosuccinate was estimated.

Table 27  
 The Effect of Added Carbamyl Phosphate on Ureidosuccinate  
 Formation via CPSase-ATCase System

Incubation System	Carbamyl Phosphate estimated as Ureido-succinate (mu moles)		Ureido-succinate formed (mu moles)	(Expt. Control mu moles US/ 2 min. from exogenous CP)
	A	B	C	D
1. control	-	-	470.0	-
2. + 50 mu moles carbamyl phosphate	41.4	40.9	509.0	39.0
3. + 100 mu moles CP	96.2	90.4	552.6	82.6
4. + 150 mu moles CP	140.6	138.9	609.8	139.8
5. + 200 mu moles CP	181.2	177.9	657.1	187.1
6. + 250 mu moles CP	227.8	219.8	678.2	208.2

The reaction was allowed to go for 18 min., then CP was added, and the reaction stopped at 20 min. The CP estimation was also carried out for 2 min.

active. To the control, shown on line 1, no CP was added; all of the US formed must have been at the expense of endogenously synthesized CP. In lines 2-6, from 50-250  $\mu$  moles of CP were added after the reaction had proceeded for 18 minutes and then the reaction was allowed to continue for 2 min. more. Columns A and B compare the sensitivity of assay of the known amount of exogenous CP in the form of citrulline. To the tubes represented by column A, exogenous CP of the indicated concentrations were added together with the enzyme complex in excess, and excess aspartate; the reaction mixture for CPSase was not included. For column B, the same concentrations of exogenous CP were present, but excess ornithine and excess OTCase were present; again, the reaction mixture for CPSase was not present. The reaction times for both was 2 min. It will be noted that more than 80% of the CP was transformed into either US or citrulline, and that there was no significant difference between the amounts recovered as either citrulline or US.

In the experiment illustrated in column C of Table 27 the complete reaction mixture for CPSase was present, plus added exogenous CP as indicated. Comparison of

lines 1 through 6 shows that with increments in the amounts of added CP a corresponding increase in the amount of added CP is shown in column D, in which the control level of column C, line 1, was subtracted from the values of lines 2-6. Since in Table 26, it was seen that the CPSase activity under these conditions was linear with time to 20 min., these data show that the CPSase and ATCase can function simultaneously and indeed, the latter can convert practically quantitatively exogenous CP into US in the presence of excess aspartate.

Table 28 represents one of two tracer experiments, in which the effect of the presence in the reaction mixture of cold exogenous CP was examined, to see if it diminished the incorporation of  $H^{14}CO_3^-$  into US via the CPSase and ATCase reactions. The rationale of the experiments was that, if the CP produced by the CPSase was released to the medium prior to utilization by the ATCase, then cold exogenous CP should be able to replace it and the label in the final product should be diluted, since, as has been just shown, the ATCase is fully capable of utilizing external CP. The ATCase in the aliquot of enzyme complex used (2 ul) had an activity of 40.6 mu moles of US per min., or  $15 \times 40.6 = 609$  mu

Table 28

The Effect of Externally Added Carbamyl Phosphate on the Utilization of  $\text{H}^{14}\text{CO}_3^-$  in the CPSase Reaction

Incubation System	A Total acid- stable cpm/0.5 ml.	B $^{14}\text{C}$ -ureido- succinate (mu moles)
control	2560	66.3
+ 100 mu moles car- bamyl phosphate (CP)	3010	75.3
+ 500 mu moles CP	2542	63.6
+ 1000 mu moles CP	2306	57.7

The complete reaction mixture contained  $\text{MgSO}_4$  (10 u moles), ATP (7.5 u moles), KCl (50 u moles), glutamine (3 u moles),  $\text{NaH}^{14}\text{CO}_3 + \text{NaHCO}_3$  (5 u moles,  $2 \times 10^5$  cpm/u mole), aspartic acid (25 u moles), carbamyl phosphate (CP) 0, 100, 500, 1000 u moles as indicated above and enzyme (2 ul) in a final volume of 0.5 ml.

The ATCase activity of the enzyme was 40.6 mu moles per min. per 2 ul at 25°C. The incubation period was 15 min at 25°C after which time 609 mu moles of CP could be utilized.

moles during the 15 min. incubation under conditions of CP saturation.

Column A of Table 28 shows the counts recovered in the control and in the presence of 3 different concentrations of exogenous CP, the highest, 1000  $\mu$  moles per 0.5 ml., being in excess of that required to saturate the ATCase; column B shows the calculated amounts of labelled US formed under these varying conditions. It will be noted that the presence of the cold exogenous CP did not diminish much if at all the amount of label incorporated ultimately into US; only at the highest CP levels of exogenous CP was there a noticeable decrease in the counts recovered as US. Thus it would appear that the greater part of the CP endogenously formed by the CPSase is not free to exchange with cold exogenous CP, at least when excess aspartate was present.

Molecular competition experiments were performed in which the ATCase of the complex and semi-purified bacterial OTCase were allowed to compete for CP produced by the CPSase of the complex; the amounts of citrulline and US formed under these conditions were assayed subsequently. These experiments were performed by mixing ATCase and OTCase preparations of known activity in

different proportions; in one tube there was no OTCase and at the other end of the series there was a tube in which the OTCase was more than 5x as active as the ATCase present in the complex. The ratios were produced by adding a fixed volume of the CPSase-ATCase complex of known ATCase activity to all the tubes and then by adding increasing volumes of the bacterial OTCase preparations to achieve the desired ratios of activity.

One such experiment is illustrated in Fig.59. Curve A shows the total counts recovered as either citrulline or US; at differing OTCase:ATCase ratios there was considerable scatter, but the total counts recovered were fairly similar across the range of enzyme ratios utilized, as indeed they should be since both possible products were counted. Curve B illustrates the recovery of counts in US and curve C the counts as citrulline. In Fig. 60 these data are rearranged and plotted as percent total counts recovered as US versus ratio of OTCase:ATCase activities. Curve B shows the theoretical values expected were the CP free to go in either direction as dictated solely by the relative activities of the two competing enzymes. The  $K_m$  for CP of OTCase and ATCase are similar at the pH of the reaction. Curve

*What are they?*

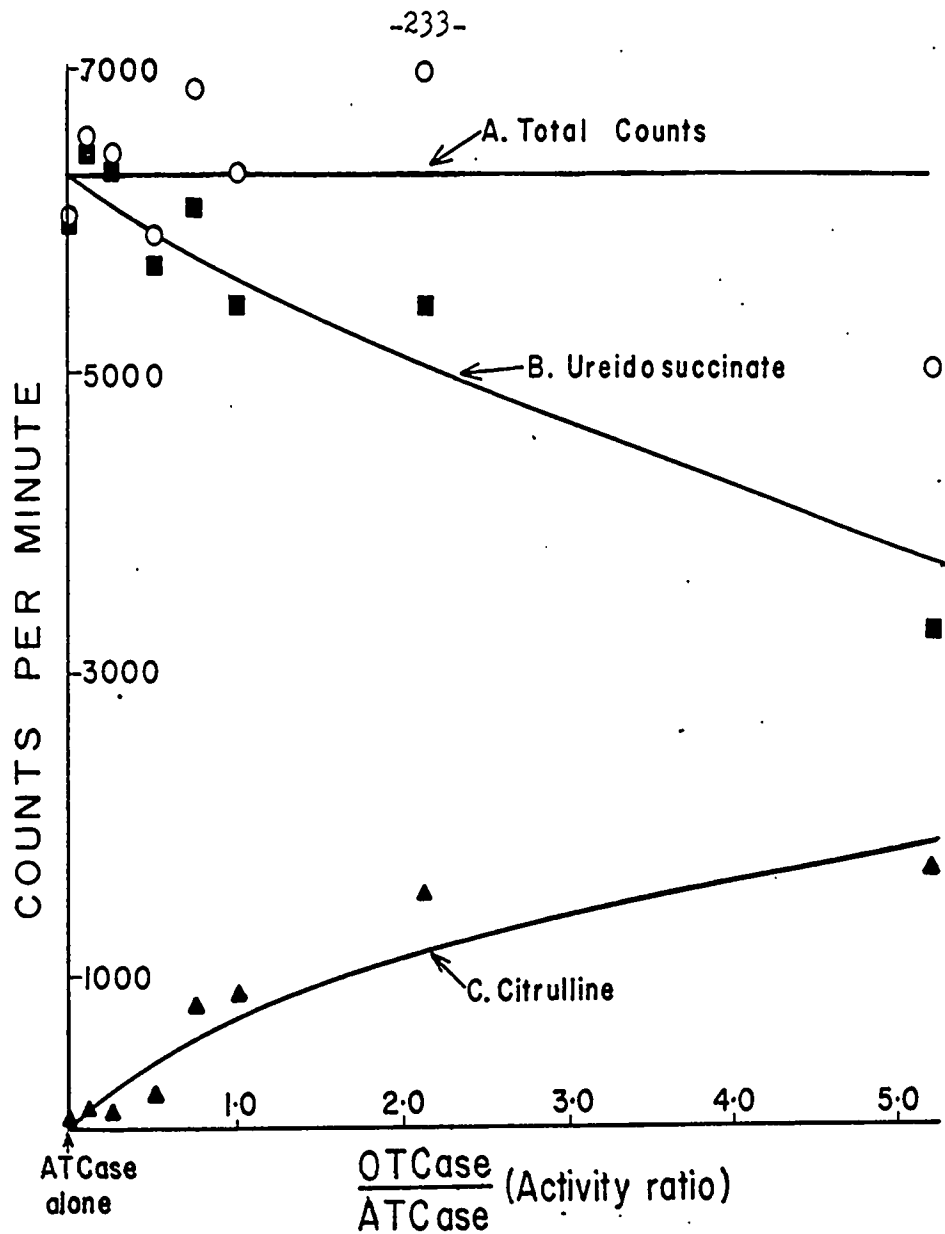


Fig. 59. Effect of variations of the ratio of activity of OTCase to ATCase on the direction of utilization of endogenously formed carbamyl phosphate.

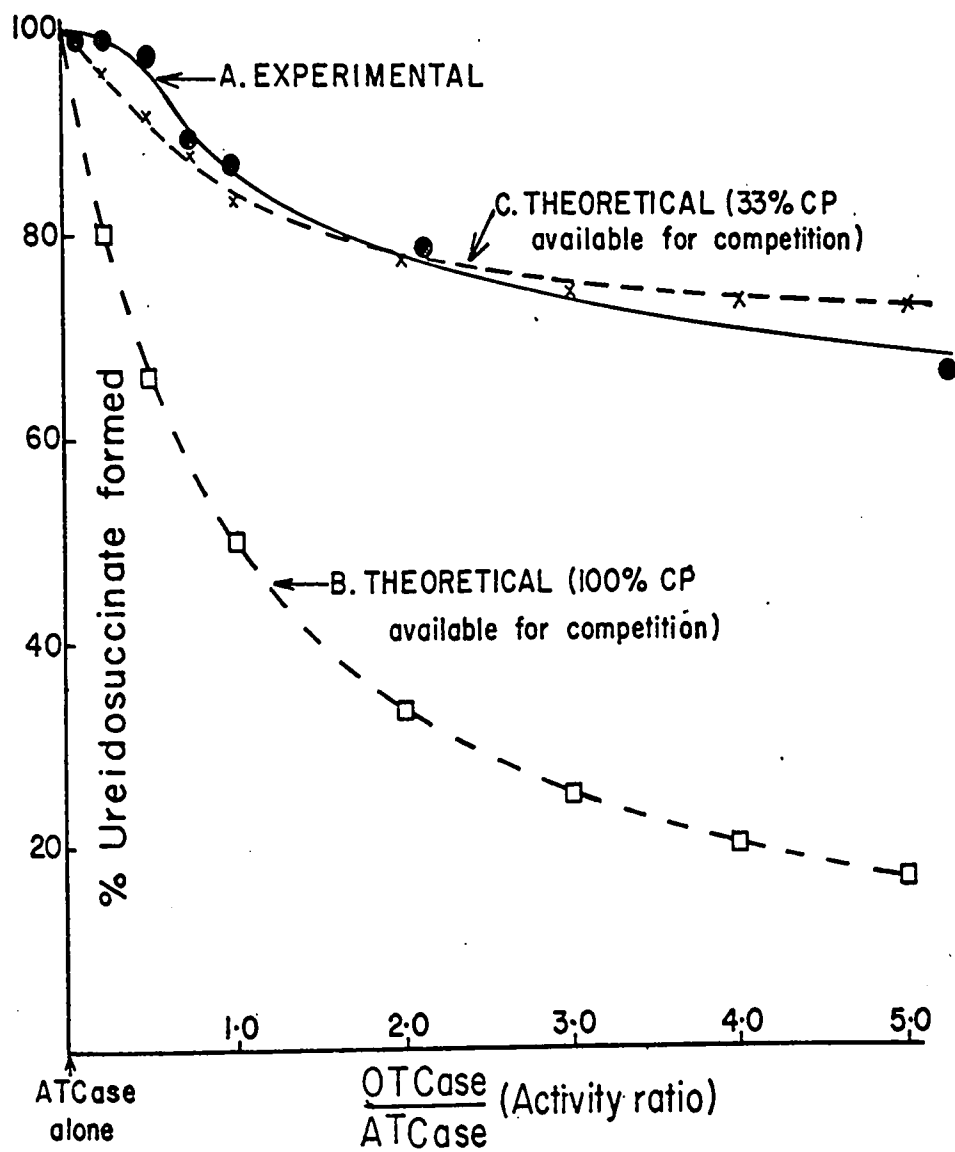


Fig. 60. Effect of variation of the ratio of activity of OTCase to ATCase on the direction of utilization of endogenously formed carbamyl phosphate (the data from the previous curve are rearranged as % ureidosuccinate formed).

A shows the experimental points actually obtained. It will be noted that a much greater proportion of the CP was channelled into US than would be expected on the basis of a 'fair' competition of the two enzymes for the substrate CP. For example, at an OTCase:ATCase activity ratio of 1:2, one would expect that without channelling 67% of the total counts would be in the form of the US, but 98% were recovered in that form.

Curve C represents what one would anticipate were two-thirds of the CP formed bound to the complex (i.e., pre-destined to US formation in the presence of excess aspartate) and one-third of the endogenously formed CP available for free competition between OTCase and CPSase. The experimental points fit this theoretical model reasonably well.

Another competition experiment is illustrated in Fig. 61, in which cold bicarbonate was utilized in the CPSase reaction and the end product, US, was determined by the standard colorimetric procedure (Methods). Otherwise the experiment was similar in concept to that of the previous figure. Citrulline could not be determined due to the interference by the high concentration of HCl required to wash the citrulline off the column.

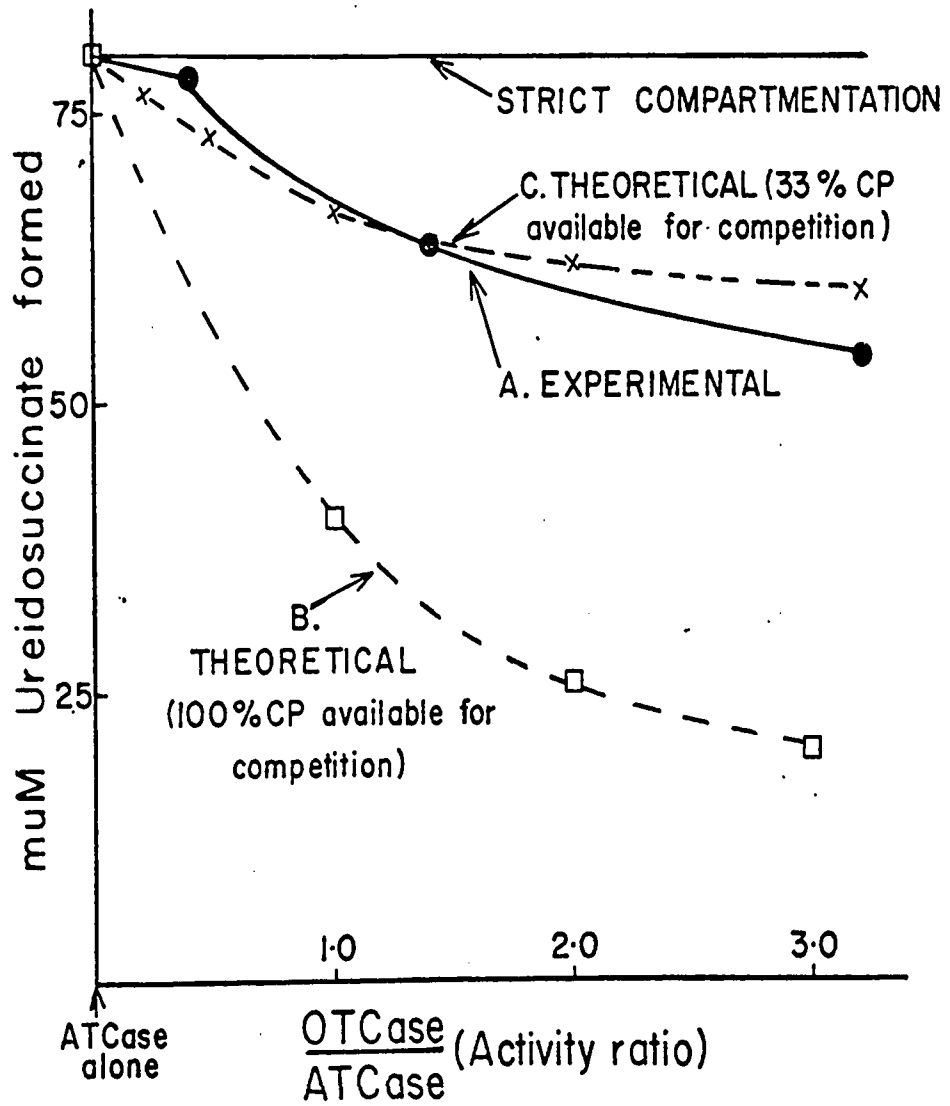


Fig. 61. Effect of variation of the ratio of activity of OTCase to ATCase on the direction of utilization of endogenously formed carbamyl phosphate (CP). Assay of ureidosuccinate was done colorimetrically.

Here, also, the experimental points of curve A were far above those expected were all the CP formed by the CPSase available to the OTCase for citrulline formation, as shown by the theoretical curve B. Curve C is the theoretical curve expected on the basis that only one-third of the endogenously produced CP is available for free competition between OTCase and CPSase; i.e., a competition exclusively controlled by the relative activities of the two enzymes. Once again, this theoretical model fits the experimental points to a first approximation. As in the previous figure, the experimental points tend to lie above the theoretical at low ratios of OTCase:ATCase and below at high ratios.

It can be tentatively concluded from these experiments that the greater part of the CP formed by the CPSase of the complex was not free to react with OTCase in the presence of excess ornithine and citrulline, but was preferentially channelled in US formation by ATCase. This is thus a model of a cellular compartmentation at the molecular level.

E. DISCUSSION

I. Enzymatic Properties

1. ATCase

Purified (20x) yeast ATCase catalyzes the irreversible condensation of CP and aspartate to yield ureidosuccinate. The optimum pH was 8.5. However, at pH 7.4, the  $K_m$  for CP was  $2 \times 10^{-3}M$  and for aspartate  $3.3 \times 10^{-2}M$ , which are in agreement with the values by Kaplan et al., (1967), using a semipurified (4x) preparation, i.e.  $4 \times 10^{-3}M$  for CP and  $2.8 \times 10^{-2}M$  for aspartate. These values are similar to those obtained in E. coli and other microorganisms (Reichard and Hanshoff, 1956; Neumann and Jones, 1964). However, the  $K_m$  for aspartate in the liver enzyme was 10x less (Bresnick and Mosse, 1966).

In the E. coli enzyme, pyrophosphate was found to be a competitive inhibitor of CP in the ATCase reaction (Kleppe, 1966), but was without effect on the mammalian enzyme (Bresnick and Mosse, 1966). However, in yeast not only is pyrophosphate a competitive inhibitor of carbamyl phosphate, but it caused the saturation curve to change from a hyperbolic to a sigmoidal form (Fig. 31).

Heat and passage on DEAE-Sephadex caused no change in  $K_m$  for CP, but did cause a loss of sigmoidicity in the presence of pyrophosphate (Fig. 34,35). These results suggest that the sigmoidicity in the presence of pyrophosphate is a function of the larger aggregated states. The mechanism involved is unknown.

Neumann and Jones (1964) studied ATCase from various bacterial sources and identified 3 kinetically distinct groups depending upon their response to pyrimidine nucleotides. Recently, Bethel and Jones (1969) found that there is a correspondence between these kinetic groups and molecular weight, as determined by Sephadex chromatography. Class A enzyme ( $K_d = 0.13$ ) yielded hyperbolic substrate curves and was inhibited by pyrimidine nucleotides. This inhibition was non-competitive with respect to aspartate, but competitive with CP. In the presence of the nucleotides, the substrate saturation curve for CP became sigmoidal. Class B enzyme ( $K_d = 0.24$ ) was represented by the E. coli enzyme, which is described in the introduction. Class C enzyme ( $K_d = 0.45$ ) yielded hyperbolic substrate curves and was not affected by nucleotides.

According to this classification, yeast ATCase

may be classified (depending upon its state of aggregation) into any of these 3 classes with respect to size; however, yeast ATCase is kinetically different in its behaviour to pyrimidine nucleotides, except in class C, where the molecular size is small and the activity insensitive to nucleotides.

## 2. CPSase

The pyrimidine-specific CPSase reaction of baker's yeast exhibited an absolute requirement for bicarbonate, potassium, magnesium, ATP and glutamine. At pH 7.6, glutamine could be replaced paritally (25%) by ammonium ions but potassium could not be replaced by sodium to any great extent. The mechanism of the potassium effect is not known.

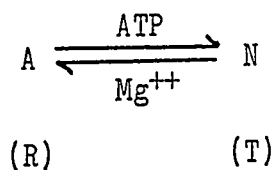
Evidence was presented to show that the true substrate of the enzyme was the Mg-ATP complex. Free  $Mg^{++}$  was found to stimulate the reaction and free ATP to inhibit it. Similar results have been observed in other  $Mg^{++}$  and ATP utilizing systems (Murray and Wong, 1967; Keech and Barritt, 1967). The  $K_m$  values for Mg-ATP, glutamine and bicarbonate were 3.5mM, 0.5mM and 3.0mM respectively. These values are similar to those obtained

in E. coli and Neurospora (Table 1). The Mg-ATP substrate saturation curve is sigmoidal. Excess  $Mg^{++}$  (10mM) induced at least 2-fold activation of the CPSase reaction (at 15mM level) by decreasing the apparent  $K_m$  for the substrate, Mg-ATP. On the other hand, ATP caused increased sigmoidicity and an increase in the apparent  $K_m$  for the substrate Mg-ATP (Fig. 47).

Murray and Wong (1967) in their study of the effect of  $Mg^{++}$  and ATP concentration on PRPP synthetase, pointed out that the "misleading kinetics of the PRPP synthetase reaction could be obtained in experiments in which the concentration of one substrate was varied in the presence of constant concentrations of the other substrate". In the present work, it was found that increasing concentration of  $Mg^{++}$  caused an increase in the apparent  $V_{max}$  and in the apparent  $K_m$  for ATP (Fig. 43). On the other hand, the reciprocal experiment (varying  $Mg^{++}$  ion concentration at 3 fixed concentrations of ATP), yielded very complex sigmoidal kinetics. The  $K_m$  values for  $Mg^{++}$  at the 3 concentrations of ATP (5, 10, 20mM) were 9, 12.5 and 21mM respectively, which were estimated as the concentration required to give one-half  $V_{max}$ . The corresponding n values (Hill coefficients) were 3, 5 and 6

respectively, suggesting increased homotropic interaction with increased ATP concentration of the substrate sites, the  $Mg^{++}$  sites, or both (Fig. 45, 46). Needless to say, the kinetic picture is somewhat confused, due to the complexity of the various reactions.

However, these results do suggest that there are at least 2 states, the activated (A) and the non-activated (N), which correspond to the R and T states of Monod et al. (1965). The presence of  $Mg^{++}$  shifts the equilibrium toward the activated state, whereas ATP shifts the equilibrium in the opposite direction.



The presence of 2 such states is also suggested by the different  $K_m$  values obtained in the two assay systems used. In assay system I, where  $Mg^{++}$  is in a large excess over ATP, the  $K_m$  values for the various substrates were 5-10x larger than that obtained in assay system II, where there was equimolar concentrations of  $Mg^{++}$  and ATP (Table 21).

The fact that linear reciprocal plots were obtained with varying ATP at fixed concentrations of  $Mg^{++}$  (Fig. 41) suggests that in the activated state, only one molecule of Mg-ATP is involved in the reaction. In addition, the rapid decrease in the reaction rate after the equimolar concentration of  $Mg^{++}$  to ATP is surpassed in favor of ATP (Fig. 45), suggests that the transition between the two states occurs via a concerted mechanism rather than a sequential mechanism; thus the model of Monod et al., (1965) is favored over that of the 'induced fit' model by Koshland and Neet (1968).

Many workers agree that the observation of sigmoidal kinetics indicate the involvement of more than 1 binding site (Sanwal et al., 1965; Cleland, 1963). It is therefore proposed that in the CPSase reaction, Mg-ATP binds at 2 or more sites, which are interdependent. This proposal together with the suggestion that only 1 molecule of ATP is involved in the reaction in the activated state suggests that  $Mg^{++}$  exerts stimulation by binding in at least 1 of the Mg-ATP sites. The possibility that there is an effector site for  $Mg^{++}$  alone is not excluded.

The CPSase reaction was optimum at pH 7.4 in the

presence of  $Mg^{++}$ ; however, the reaction was greater in the presence of  $Mn^{++}$  and exhibited a more complex pH activity curve with a peak at pH 7.0 and a shoulder at pH 8.0 (Fig. 41). Indeed, similar effects of  $Mn^{++}$  have been observed in the earthworm enzyme, where the CPSase reaction with  $Mg^{++}$  was only 30% of that observed with  $Mn^{++}$  (Bishop and Campbell, 1963). Switzer (1969) has also observed 2 pH optima in the presence of  $Mn^{++}$  and 1 with  $Mg^{++}$  in his studies on PRPP synthetase.

Studies on the stoichiometry of the reaction indicate that 2 moles of ATP and 1 mole of glutamine were utilized per mole of CP produced. These results are in agreement with the E. coli enzyme (Anderson and Meister, 1965), and also with studies with the mitochondrial enzyme from frog and mammalian liver, except that, in these cases, ammonium is the nitrogen donor (Marshall et al., 1961; Guthohrlein and Knappe, 1969).

Recently, biotin was identified as the co-factor in the mechanism of the CPSase reaction (Wellner et al., 1968), however, attempts by other workers to repeat the experiment failed to confirm the presence of biotin (Huston and Cohen, 1969). The present data on the effects of biotin and avidin indicate that yeast CPSase is not a

biotin containing enzyme. However, it is interesting that the kinetics of the CPSase reaction resemble those of biotin utilizing enzymes more than they do those of the CPSase in E. coli or liver. For example, Hatch and Stumpf (1961) found that acetyl-coenzyme A carboxylase activity was maximum when the Mg-ATP ratio was 2; similar results were obtained with pyruvate carboxylase (Keech and Barritt, 1967).

## II. Regulation

### 1. Effect of Purines and Pyrimidines

Lacroute (1968) showed that the first 2 enzymes in pyrimidine biosynthesis, CPSase and ATCase, are under control of the ur2 gene in yeast. Both are sensitive to feedback inhibition and the synthesis of both is repressed by UTP as co-repressor. To the extent that the extracted and purified complex reflects the situation in vivo, it appears that the CPSase reaction is rate-limiting, since its specific activity is only about .75% of that of the ATCase (Table 11).

Inhibition by UTP of both enzyme-substrate systems was found to be non-competitive with respect to all

the substrates. The UTP effect was on the  $V_{\max}$  and not on the  $K_m$ ; thus this is a V system (see Changeux, 1964) confirming previous results (Kaplan et al., 1967).

The case of glutamine merits a brief discussion; inhibition by UTP was found to decrease if the glutamine concentration fell below  $10^{-3}M$  (Fig. 48). This result suggests that glutamine caused a conformational change which is required for the effects of UTP to be realized. Similar results observed in the CPSase reaction of S. typhimurium were explained as being due to ordered binding of glutamine and UMP to the enzyme (Abd-El-Al and Ingraham, 1969).

The  $K_i$  for UTP in the ATCase reaction in 2 separate experiments were  $8.5 \times 10^{-4}M$ , and  $1 \times 10^{-3}M$ , whereas in the CPSase reaction, they were  $2.4 \times 10^{-4}M$  and  $2.5 \times 10^{-4}M$ . These results do not necessarily indicate that separate sites are involved in the inhibition. Since, as will be shown below, the regulatory site is more closely associated with the CPSase than with the ATCase, this dissimilarity could be due to differences in the interaction between subunits rather than to the existence of more than one UTP site. Indeed, genetic experiments by Lacroute (1966) showed that a single mutation caused loss of FI of both

activities; never was only one activity disinhibited by mutation. These data strongly support the view that only 1 UTP site is present.

Coupling of the pyrimidine biosynthetic pathway to the purine system is demonstrated by the fact that XMP stimulated the CPSase reaction and was able to overcome the inhibition by UTP to a certain degree. Ornithine and IMP were without effect, unlike the case of the E. coli system, in which these substances were found to stimulate CPSase reaction and were capable of overcoming the inhibition by UTP (Anderson and Marvin, 1968). XMP binds at a different site on the enzyme than UTP (Fig. 56) and its activation is in addition to that caused by free  $Mg^{++}$  ions (Fig. 58). These results could account for the increase in the pyrimidine pool observed by Burns (1966) when adenine was added to adenine-requiring cells grown in excess uracil. Thus, stimulation by XMP might function in maintaining a close balance between the purine and pyrimidine pools. This proposal remains to be justified in vivo; Burns (1966) was able to show by in vivo studies that the regulation of the two pools was unsymmetrical, the purine system exerting a greater influence over the pyrimidine system. Biochemical studies

on yeast PRPP amido-transferase, the first enzyme in the purine pathway, have confirmed this observation; pyrimidines and pyrimidine nucleotides were without effect on this enzyme (Satyanarayana and Kaplan, 1970).

Unlike E. coli, where ATP was found to activate the ATCase reaction and to overcome the inhibition by CTP, all purine nucleotides at 5mM were by themselves without significant effects on the yeast ATCase reaction. However, Kaplan et al. (1967) were able to prevent inhibition by UTP with high concentrations of ATP. On the other hand, the ATCase was inhibited by TTP and the inhibition by UTP was prevented somewhat by CDP. These observations are of particular interest when it is realized that CDP is the immediate precursor of the pyrimidine nucleotides of DNA and TTP the end-product of this sequence. These results may indicate the presence of further controls of the synthesis of pyrimidines.

A possible control mechanism is suggested by the fact that ADP, a product of the CPSase reaction, was itself a good inhibitor; product inhibition of this kind could serve to switch the enzyme off if excess product accumulates in the cell.

Atkinson (1966) has proposed that there is an antagonism in enzyme regulation between ATP, on the one hand, and ADP and AMP on the other. ATP tends to inhibit those reactions leading to its own production; ADP (AMP) by contrast, inhibit ATP-utilizing reactions. One might consider the ADP effect of the CPSase system as supporting Atkinson's view. Indeed, Kleckzkowski (1965) by using an ATP-generating system, found that the linearity of the CPSase reaction in pea extracts could be extended for a longer period of time.

## 2. The Occurrence of Enzyme Aggregates

A number of enzyme aggregates have been reported (Table 28). These aggregates from microorganisms usually seem to involve enzymes from a common biosynthetic pathway. Since yeast CPSase and ATCase exists in a single complex, as will be discussed below, we incline to the view that, where a substrate is common to more than one pathway, as in the case of CP, aggregation of the CPSase and ATCase may cause preferential channelling (Davis, 1967) of the common precursor into the pyrimidine pathway.

Molecular competition experiments designed to test

Table 29

## Biosynthetic Multi-functional Enzyme Complexes Identified in Various Organisms

Organism	Activities in the Aggregate	Common Precursor	End Product	References
1. <u>Neurospora</u> and yeast	pyrimidine-specific CPase aspartate transcarbamylase	carbamyl phosphate	pyrimidine	Williams and Davis (1968) in text
2. <u>E. coli</u>	threonine sensitive asparto- kinase, homo serine dehydro- genase	aspartyl phosphate	threonine	Cohen et al., (1965)
3. <u>E. coli</u>	methionine sensitive aspar- to kinase, homoserine dehyd- rogenase	aspartyl phosphate	methionine	Truffa-Bachi & Cohen (1968)
4. <u>E. coli</u> <u>A. aerogenes</u>	chorismate mutase prephenate dehydrogenase	prephenic acid	tyrosine	"
5. <u>E. coli</u> <u>A. aerogenes</u>	chorismate mutase prephenate dehydratase	prephenic acid	phenylalanine	"
6. <u>Enterobacteri-</u> <u>acae</u> <u>A. aerogenes</u> <u>S. typhimurium</u>	anthranilate synthetase anthranilate-5-phospho- ribosyl-1-pyrophosphate phosphoribosyl transferase	chorismic acid	tryptophan	"

Table 29 (cont'd)

Organism	Activities in the Aggregate	Common precursor	End Product	References
7. <u>N. crassa</u>	anthranilate synthetase, phosphoribosylanthranilate isomerase, indole glycerol phosphate synthetase		tryptophan	Gaertner and DeMoss (1969)
8. <u>N. crassa</u>	1-N-(5'-phosphoribosyl)-ATP pyrophosphohydrolase, 1-N-(5'-phosphoribosyl)-AMP cyclohydrolase, histidinal dehydrogenase	PRPP	histidine	Minson and Creaser (1969)
9. <u>N. crassa</u>	5-dihydroquinase 5-dihydroshikimic reductase shikimic acid kinase 3-enolpyruvylshikimic acid- 5-PO <sub>4</sub> synthetase 5-dihydroquinic acid synthe- tase	to separate syn- thetic from degra- dative pathways	aromatic amino acids	Giles et al. (1967)

this hypothesis showed that the CP synthesized by the CPSase is indeed preferentially utilized in the making of pyrimidines. The addition of cold CP was ineffective in diluting the amount of radioactivity incorporated as  $^{14}\text{C}$ -ureidosuccinate when  $\text{H}^{14}\text{CO}_3$  was used as a source (Table 28). Competition of E. coli OTCase and the ATCase activity of the complex ( $K_m$  values are similar) for  $^{14}\text{C}$ -carbamyl phosphate synthesized by the CPSase portion of the complex showed that only one-third was available for free competition and that two-thirds was preferentially channelled into ureidosuccinate production (Fig. 60, 61).

It is therefore tentatively concluded that the physiological importance of the CPSase-ATCase complex lies in the preferential utilization of the CP, synthesized by the first activity, into pyrimidine biogenesis.

In Neurospora, as in yeast, a single gene also controls both ATCase and CPSase activities (Davis and Woodward, 1962), and in preliminary experiments, both were also co-purified, suggesting the presence of a single complex (Williams and Davis, 1968).

In rat liver preparations, Oliver et al. (1969) identified 2 major components of ATCase, corresponding to molecular weights of 900,000 and 600,000. In view of

these high molecular weights, it is conceivable that the CPSase activity might be in some way associated with these ATCase-active components. On the other hand, Natale and Tremblay (1969) showed that the CP produced in the mitochondria is not compartmentally isolated. Thus, the mitochondrial CPSase may serve as a source of CP for the synthesis of pyrimidines and in the formation of citrulline.

### III. Purification

#### 1. Molecular Weights and States of Aggregation

##### (a) Ultracentrifugation Studies

A method for the purification of yeast ATCase from strains MD169(c) and MD170-7X resulted in co-purification of the CPSase activity based on the activities obtained at step 3 (Table 11). Elution of the enzyme preparation from Sepharose 6B in the presence of UTP resulted in co-elution of both activities in a large symmetrical peak. After centrifugation on sucrose gradients, such preparations showed a single peak for both activities corresponding to a sedimentation coefficient of 26.3S and a molecular weight of approx. 800,000 (Martin and Ames, 1961),

provided that UTP,  $Mg^{++}$  and glutamine were included in the gradient. With either Sepharose or sucrose gradients, both activities remained highly sensitive to UTP. It is therefore concluded that both activities are associated with a single protein molecule in which form they are co-extracted, co-purified, co-eluted and co-sedimented.

That the enzyme complex is composed of many individual subunits is supported by the following findings: omission of UTP from the sucrose density gradients (but including  $Mg^{++}$  and glutamine) resulted in dissociation of the aggregate into 'half molecules' of molecular weight 380,000 (15.6S), possessing both activities, but whose ATCase possessed reduced sensitivity to FI (20-35% inhibition by  $2 \times 10^{-3}M$  UTP); the ATCase activity of the high molecular weight aggregate could be inhibited to 75-85% by a similar concentration of UTP. On the other hand, the sensitivity to UTP of the CPSase of the 15.6S material remained as high as that of the 26.3S material.

Omission of  $Mg^{++}$  and glutamine, as well as UTP from the gradients, caused the CPSase peak (molecular weight 250,000 approx.) to trail behind the ATCase peak (molecular weight 380,000 approx.). It is apparent that this effect

was due to the disaggregation of the complex and not to contamination by the CPSase of the arginine pathway, since similar results were obtained with a mutant lacking the arginine-specific CPSase. These results were also confirmed in gel filtration studies from Sepharose 6B, where in the absence of UTP, the molecular weights of CPSase and ATCase were estimated as approx. 240,000 and 360,000 respectively. Although it seems theoretically possible to separate completely the CPSase-active fraction from the ATCase activity, such fractionation was never achieved. Furthermore, since the CPSase activity was found invariably to remain highly inhibitable by UTP regardless of the purification procedure, the regulatory site seems to be more closely associated with the CPSase activity.

Treatment of the purified preparations either on DEAE-Sephadex at room temperature, or by heat caused dissociation into units possessing a molecular weight of approx. 140,000. Such preparations had no detectable CPSase activity and their very active ATCase was completely insensitive to FI by UTP. These results demonstrate the actual presence of subunits in the ATCase-CPSase complex, confirming the previous conclusion based on intragenic

complementation studies (Lacroute, 1966), and on the combined effects of temperature and dilution (Kaplan and Messmer, 1969). It is also clear that UTP is necessary for the maintenance of the complex as a large molecular aggregate. Kaplan et al. (1969) had shown that UTP either in the cell or in the extraction medium was essential to stabilize the feedback site. It follows from these studies that the capacity of the ATCase activity to be inhibited by UTP is indicative of the state of aggregation present. In other words, a preparation whose ATCase activity is highly inhibitable by UTP (75-85% by  $2 \times 10^{-3}M$  UTP) is largely composed of the enzyme as a large aggregate, whereas a less sensitive preparation (20-50%) may consist of a mixture of the different states of aggregation. An ATCase preparation of high specific activity which is totally insensitive to UTP is in an even more dissociated state (molecular weight 130,000-140,000).

It should be noted that with each step of purification the ATCase of the discarded fractions became progressively less sensitive to inhibition by UTP, as opposed to the increased sensitivity of the retained fractions. These findings explain the curious fact that during purification

there was always a greater purification of the CPSase than of the ATCase. The crude extract contained a mixture of the enzyme in various degrees of aggregation; the purification procedure was selective for the larger aggregates (Lue and Kaplan, 1969).

Similar sedimentation behaviour has been observed with homoserine dehydrogenase in the presence and absence of threonine, its end-product inhibitor (Datta et al., 1964).

It was shown previously (Kaplan et al., 1967) and confirmed in these studies, that 5 min. at 50°C caused a sharp decrease in the sensitivity of the ATCase to UTP; this decrease without a corresponding decrease in specific ATCase activity suggests that disaggregation has occurred. Indeed, sucrose density gradient centrifugation confirmed that this treatment caused a dissociation of the complex into smaller, non-inhibited units of approx 140,000 (approx. one-fourth the molecular weight of original, which had originally been estimated to be about 600,000 \* Lue and Kaplan, 1969). Together with this disaggregation process, there was a more or less parallel decline in

\* Footnote: This molecular weight was incorrectly calculated. From the data of Lue and Kaplan, 1969, the correct MW corresponding is 900,000.

CPSase activity. Furthermore it was observed that at any duration of heating, the residual CPSase activity remained fully sensitive to FI by UTP. These results were therefore taken to support the postulated structural model of Kaplan and Messmer (1969); according to which the *ur2* gene, which controls the CPSase and ATCase, produces but one polypeptide chain (a subunit with ATCase activity alone). The CPSase and the regulatory site were supposed to be acquired upon aggregation (Lue and Kaplan, 1970). In other words, both the CPSase activity and the sensitivity of the activities to UTP were presumed to be functions of the aggregated state.

However, in view of the finding that there was a trailing of the CPSase peak of 250,000 daltons behind the ATCase peak in elution from Sepharose columns, and in sucrose gradient studies lacking UTP,  $Mg^{++}$  and glutamine, the structural model of Kaplan and Messmer (1969) seems improbable. Thus, the thermal effects are probably due to a high degree of lability of the CPSase activity; this explanation was proposed as an alternate possibility (Lue and Kaplan, 1970).

A number of preparations were studied in the analytical ultracentrifuge (model E). The best gave

schlieren patterns similar to that of Fig. 16. The major peak is the heaviest (minimum molecular weight 600,000) and the minor peak about half. A lighter shoulder was always present. It is thus not possible to make any claims as to the purity of these preparations. The lighter might well correspond to the partially dissociated 'half molecule' which has been demonstrated by density gradient studies, or it might be a contaminant.

(b) Gel Filtration

Molecular weight estimation by gel filtration is known to be less precise than estimates by centrifugation (Fischer, 1969). Studies on Sepharose 6B and Sephadex G-200 indicated that the molecular weight was approx. 800,000.

Determann (1968) has indicated that Sephadex G-200 is suitable for molecular weight determination of globular proteins from 10,000 to 300,000 (Andrews, 1965). The ATCase complex was eluted almost at the void volume outside the range of linearity; thus, the estimation of molecular weight here is of questionable significance. However, these results serve to indicate that the molecular weight is close to the exclusion limit of 800,000 (Determann, 1968) since, the ATCase complex did

not behave like E. coli cells, nor like blue dextran, which are totally excluded from the gel. On the other hand it migrated somewhat ahead of thyroglobulin (670,000).

The elution data from Sepharose 6B showed catalase to behave abnormally, confirming the results of other workers (Fischer, 1969) and yielded a molecular weight of  $1 \times 10^6$  daltons for the ATCase-CPSase complex. This estimate of molecular weight suffers from a number of disadvantages: although the enzyme was eluted within the limits of Sepharose 6B ( $1.0 \times 10^4$  -  $1.5 \times 10^6$ ), there was a lack of marker proteins above 400,000 daltons which would have served to increase the accuracy. Also elution of the complex was in the range where small differences in  $K_{av}$  would result in a large difference in molecular weight estimates. Passage of the enzyme preparation on Sepharose 6B in the absence of UTP resulted in separate activity for ATCase and CPSase, the former corresponding to a molecular weight of  $3.6 \times 10^5$  and the latter  $2.4 \times 10^5$ , confirming the results from sucrose density gradient.

#### IV. Genetic Data

Lacroute (1968) suggested that there were 3 possibilities involving 1 operon which are in agreement with his genetic results. 1. The operon consists of 3 cistrons, one for CPSase, one for ATCase and the other for the regulatory subunit. 2. The operon consists of 2 cistrons, one making a polypeptide with CPSase and ATCase activities and the other for the UTP subunit. 3. The operon consists of one cistron coding for one chain bearing both activities and the UTP site. It is apparent from these studies that possibilities 2 and 3 can be rejected since subunits possessing ATCase activity only were isolated from the complex.

In this laboratory, Denis-Duphil (pers. comm.) has studied the fine structure of the *ur2* gene by means of meiotic recombination. She found that all the ATCase mutants mapped in a cluster in one extremity of the gene, whereas the CPSase and the doubles were scattered mainly to one side of this ATCase region; furthermore, none was found to overlap this ATCase region. The fact that the doubles (ATCase<sup>-</sup>, CPSase<sup>-</sup>) map in the CPSase part of the locus and not in the ATCase region indicated a

Leaf 261 omitting in page numbering.

direction of reading going from CPSase → ATCase. The double mutants have been found by Lacroute (pers. comm.) to be nonsense mutations, since he found that they were all suppressible by suppressor genes.

Denis-Duphil (pers. comm) also observed complementation between the ATCase and the CPSase mutants and also between one ATCase single mutant (ur<sub>2-20</sub>) and all the other ur<sub>2</sub> (ATCase<sup>-</sup>) mutants except one. Complementation of 2 ATCase<sup>-</sup> mutants indicates that the ATCase enzyme has a polymeric structure. Since such studies with different CPSase single mutants were without success, indications are that the CPSase does not possess a polymeric structure. The fact that Lacroute (1968) was able to isolate a mutant (ura-2-27) which lacks both sensitivity to UTP and ATCase activity, but which possesses CPSase, suggests that the region corresponding to the UTP site is located after the CPSase region. Since synthesis occurs from CPSase to ATCase, the question arises as to whether the UTP region is before the ATCase. Denis-Duphil (pers. comm.) found that some of the CPSase and the double mutants mapped very close to the ATCase region. If the UTP region were before the ATCase region, these doubles should be like ura-2-27 (above), but they are not.

Thus the probable existence of the UTP region before the ATCase locus is excluded. However, it is curious to find the UTP site so intimately associated with the CPSase activity in these biochemical studies, both in sucrose density gradient and in the effects of heat.

Thus, the *ur2* gene may be viewed as an operon corresponding to 3 polypeptide chains - 1 for CPSase, 1 for ATCase and 1 for the UTP site. The indicated sequence is the probable direction of synthesis. The possibility that the operon corresponds to 2 polypeptide chains - 1 for CPSase and the UTP site and 1 for the ATCase, is not altogether excluded.

#### V. Structural Model

The molecular weight data have led to the following speculative but plausible model. The data indicate the existence of at least 2 states where ATCase and CPSase co-sedimented (Fig. 62). State I, with a molecular weight of approx. 800,000 consists of 2 state II, with a molecular weight of approx. 400,000. State II is in turn composed of a CPSase active subunit (C) with a regulatory subunit (R), giving a molecular weight of

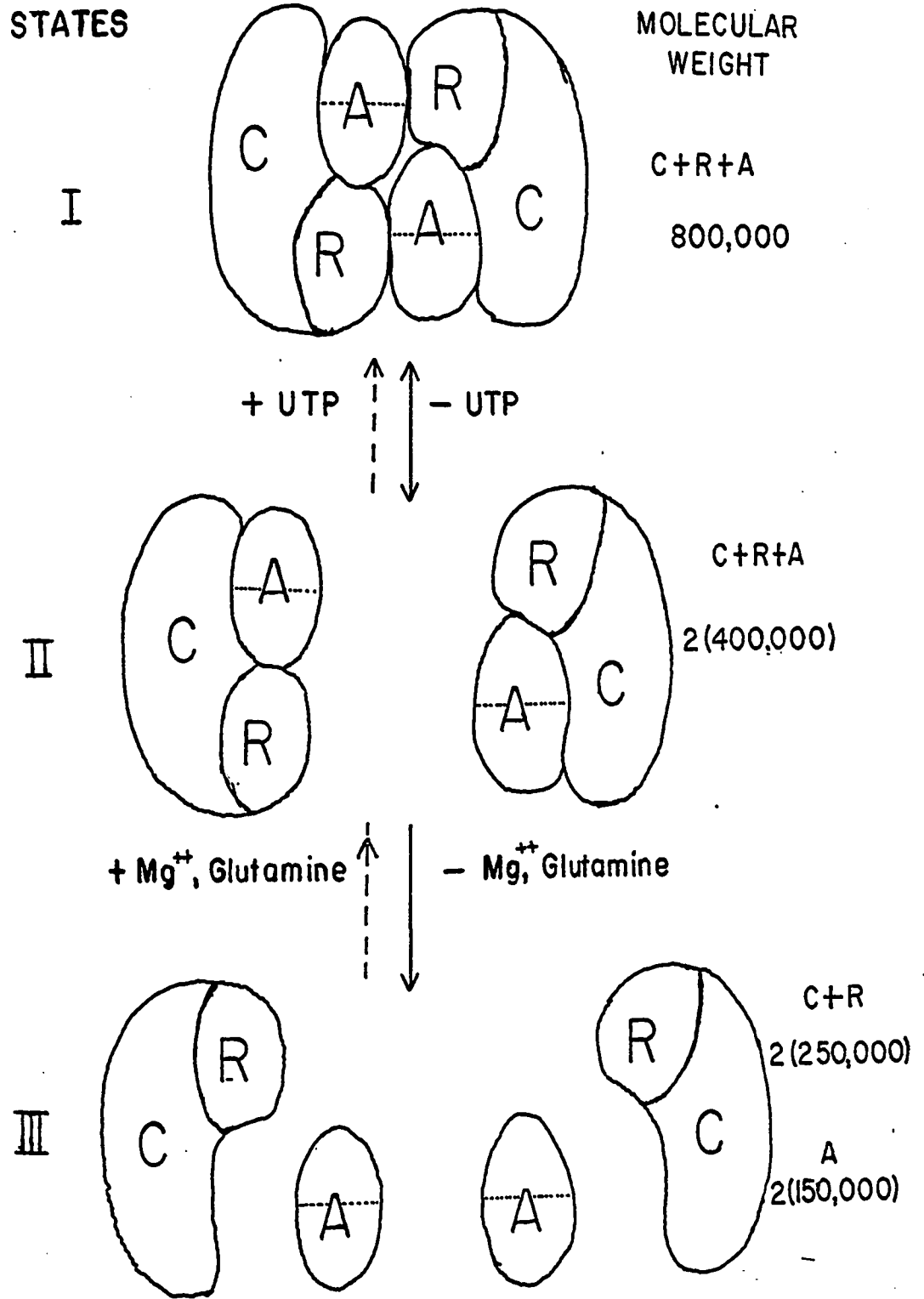


FIG.62 STRUCTURAL MODEL OF THE ATCase-CPSase COMPLEX

250,000 and an ATCase-active (A) subunit of molecular weight 150,000, i.e. state III.

The presence of a regulatory subunit as such and the reversibility between these states remains to be investigated. This structural model is of course highly speculative and it does not exclude the possibility that other small molecules apart from UTP, glutamine and  $Mg^{++}$  are necessary for aggregation. However, to date it complies with most of the data.

A state of high ATCase activity and low CPSase was identified in sucrose gradient studies using MD170-7X. It is conceivable that such a state might occur if one of the CPSase subunits (C) dissociated from the state I complex. A molecular weight of 550,000 would result, which agrees reasonably well with the experimental of 520,000.

REFERENCES

1. Abd-El-Al, A. and Ingraham, J.L. (1969).  
Control of carbamyl phosphate synthesis in  
Salmonella typhimurium.  
J. Biol. Chem., 244, 4033-4038.
2. Ames, B.N. and Martin, R.G. (1964).  
Biochemical aspects of genetics: the operon.  
Ann. Rev. Biochem., 33, 235-258.
3. Anderson, P.M. and Marvin, S.V. (1968).  
Effect of ornithine, IMP, and UMP on carbamyl  
phosphate synthetase from Escherichia coli.  
Biochem. Biophys. Res. Commun., 32, 928-934.
4. Anderson, P.M. and Meister, A. (1965).  
Evidence for an activated form of carbon dioxide  
in the reaction catalyzed by Escherichia coli  
carbamyl phosphate synthetase.  
Biochem., 4, 2803-2809.
5. Anderson, P.M. and Meister, A. (1966).  
Control of Escherichia coli carbamyl phosphate  
synthetase by purine and pyrimidine nucleotides.  
Biochem., 5, 3164-3169.
6. Andrews, P. (1965).  
The gel-filtration behaviour of proteins related  
to their molecular weights over a wide range.  
Biochem. J., 96, 595-606.
7. Arnstein, H.R.V. (1965).  
Mechanism of protein biosynthesis.  
Brit. Med. Bull., 21, 217-222.
8. Arvidson, H., Eliasson, N.A., Hammarsten, E.,  
Reichard, P., von Ubisch, H., and Bergstrom, S. (1949).  
Orotic acid as a precursor of pyrimidines in the  
rat.  
J. Biol. Chem., 179, 169-173.

9. Atkinson, D.E. (1965).  
Biological feedback control at the molecular level.  
*Science*, 150, 851-857.
10. Atkinson, D.E. (1966).  
Regulation of enzyme activity.  
*Ann. Rev. Biochem.*, 35, 85-124.
11. Barber, E.J. (1966)  
Calculation of density and viscosity of sucrose  
solutions as a function of concentration and  
temperature.  
*Natl. Canc. Inst. Monogram*, 21, 219.
12. Barnes, F.W. Jr. and Schoenheimer, R. (1943)  
On the biological synthesis of purines and pyrimidines.  
*J. Biol. Chem.*, 151, 123-139.
13. Beadle, G.W. and Tatum, E.L. (1941).  
Genetic control of biochemical reactions in *Neurospora*.  
*Proc. Nat. Acad. Sci., Wash.*, 27, 499-506.
14. Beckwith, J.R., Pardee, A.B., Austrian, R., and  
Jacob, F. (1962).  
Coordination of the synthesis of the enzymes in the  
pyrimidine pathway of *E. coli*.  
*J. Molecular Biol.*, 5, 618.
15. Belser, W.L. (1961).  
Uracil biosynthesis in *Serratia marino*rubra.  
*Biochem. Biophys. Res. Commun.*, 4, 56-60.
16. Berg, P. and Joklik, W.K. (1954).  
Enzymatic phosphorylation of nucleoside diphosphates.  
*J. Biol. Chem.*, 210, 657-672.
17. Bernhard, S. (1968).  
The Structure and Function of Enzymes.  
W.A. Benjamin, Inc., New York, N.Y. pp. 324.
18. Bethel, M.R. and Jones, M.E. (1969).  
Molecular size and feedback-regulation characteristics  
of bacterial aspartate transcarbamylase.  
*Arch. Biochem. Biophys.*, 134, 352-365.

19. Bethell, M.R., von Fellenberg, R., Jones, M.E. and Levine, L. (1968).  
Immunological studies of aspartate transcarbamylase I. Characterization of the native enzyme, catalytic and regulatory subunits, immune systems.  
*Biochem.*, 7, 4315-4322.
20. Bethell, M.R., Smith, K.E., White, J.S. and Jones, M.E. (1968)  
Carbamyl phosphate: an allosteric substrate for aspartate transcarbamylase of *Escherichia coli*.  
*Proc. Natl. Acad. Sci.*, 60, 1442-1449.
21. Biscaro, G. and Belloni, E. (1905).  
Über die orotsäure.  
*Chem. Zentr.* 2, 64.
22. Bishop, B.S. (1966).  
Digital computation of sedimentation coefficient in zonal centrifuges.  
*Nat. Canc. Inst. Monogram.*, 21, 175.
23. Bishop, S.H. and Campbell, J.W. (1963).  
Carbamyl phosphate synthesis in the earthworm *Lumbricus terrestris*.  
*Science*, 142, 1583-1585.
24. Brenner, S. (1965).  
Theories of gene regulation.  
*Brit. Med. Bull.*, 21, 244-248.
25. Bresnick, E. and Mosse, H. (1966).  
Aspartate carbamoyl transferase from rat liver.  
*Biochem. J.* 101, 63-69.
26. Brzozowski, T.H. and Kalman, S.M. (1966).  
Carbamyl phosphate and acetyl phosphate synthesis in *Escherichia coli*: analysis of associated enzyme activities by an antibody to acetokinase.  
*J. Bacteriol.* 91, 2286-2290.
27. Burns, V.W. (1966).  
Regulation of pyrimidine biosynthesis and its strong coupling to the purine system.  
*Biophys. J.* 6, 787-800.

28. Chakraborty, K.P. and Hurlbert, R.B. (1961).  
Role of glutamine in the biosynthesis of cytidine nucleotides in Escherichia coli.  
Biochim. Biophys. Acta 47, 607-609.
29. Changeux, J.P. (1964).  
Allosteric interactions interpreted in terms of quaternary structure.  
Brookhaven Symposia in Biology 17, 232.
30. Changeux, J.P., Gerhart, J.C. and Schachman, H.K. (1968).  
Allosteric interaction in aspartate transcarbamylase I. Binding of specific ligands to the native enzyme and its isolated subunits.  
Biochem. 7, 531-538.
31. Changeux, J.P. and Rubin, M.M. (1968).  
Allosteric interactions in aspartate transcarbamylase III. Interpretation of Experimental Data in terms of the model of Monod, Wyman and Changeux.  
Biochem. 7, 553-561.
32. Chantrenne, H. (1955).  
Effets d'un inhibiteur de la catalase sur la formation induite de cet enzyme chez la levure.  
Biochim. Biophys. Acta 16, 410-417.
33. Chattaway, F.W. (1944).  
Growth stimulation of L. casei E. by pyrimidines.  
Nature 153, 250-251.
34. Cleland, W.W. (1963).  
The kinetics of enzyme-catalyzed reactions with two or more substrates or products.  
II. Inhibition: nomenclature and theory.  
Biochim. Biophys. Acta 67, 173- .
35. Cline, A.L. and Bock, R.M. (1966).  
Translational control of gene expression.  
Cold Spr. Harb. Symp. Quant. Biol. 31, 321-333.
36. Cohen, G.N. (1968).  
The Regulation of Cell Metabolism.  
Holt, Rinehart and Winston, Inc., Paris, France  
pp. 240.

37. Cohen, G.N., Patte, J.C. and Truffa-Bachi, P. (1965). Parallel modification caused by mutations in two enzymes concerned with the biosynthesis of threonine in Escherichia coli. Biochem. Biophys. Res. Comm. 19, 546-550.
38. Cohn, M., Cohen, G.N. and Monod, J. (1953). L'effet inhibiteur spécifique de la methionine dans la formation de la methionine-synthase chez Escherichia coli. Compt. rend. 236, 746-748.
39. Collins, K.D. and Stark, G.R. (1969). Aspartate transcarbamylase. Studies of the catalytic subunit by ultraviolet difference spectroscopy. J. Biol. Chem. 244, 1869-1877.
40. Cooper, C., Wu, R. and Wilson, D.W. (1955). Studies of some precursors of pyrimidines. J.B.C. 216, 37-49.
41. Datta, P. (1969). Regulation of branched biosynthetic pathways in bacteria. Science 165, 556-562.
42. Datta, P., Gest, H. and Segal, H.L. (1964). Effects of feedback modifiers on the state of aggregation of homoserine dehydrogenase of Rhodospirillum rubrum. Proc. Natl. Acad. Sci. 51, 125-130.
43. Davis, R.H. (1963). Neurospora mutants lacking in arginine-specific carbamyl phosphokinase. Science 142, 1652-1654.
44. Davis, R.H. (1964). Disc electrophoresis. Ann. N.Y. Acad. Sci. 121, 409-413.
45. Davis, R.H. (1967). in "Organizational Biosynthesis". (H. Vogel, J.O. Lampen and V. Bryson eds.) Academic Press, New York pp. 303.

46. Davis, R.H. and Woodward, V.W. (1962).  
The relationship between gene suppression and aspartate transcarbamylase activity in pyr-3 mutants of *Neurospora*.  
*Genetics* 47, 1075-1083.
47. Determann, H. (1968)  
in "Gel Chromatography".  
Springer-Verlag New York Inc.
48. Dixon, M. and Webb, E.C. (1964).  
in "Enzymes", 2nd edition.  
Longman's, Green and Co. Ltd., London.
49. Dratz, E.A. and Calvin, M. (1966).  
Substrate- and inhibitor-induced changes in the optical rotatory dispersion of aspartate transcarbamylase.  
*Nature* 211, 497-501.
50. Edmonds, M., Delluva, A.M. and Wilson, D.W. (1952).  
The metabolism of purines and pyrimidines by growing yeast.  
*J. Biol. Chem.* 197, 251-259.
51. Epstein, W. and Beckwith, J.R. (1968).  
Regulation of gene expression.  
*Ann. Rev. Biochem.* 37, 411-436.
52. Epstein, C.J., Goldberger, R.F. and Anfinsen, C.B. (1963).  
The genetic control of tertiary protein structure: Studies with model systems.  
*Cold Spr. Harb. Symp. Quant. Biol.* 28, 439-449.
53. Felix, K. (1960)  
Protamines.  
*Advances in Protein Chemistry* 15, 1-56.
54. Fischer, L. (1969).  
An introduction to gel chromatography.  
*Lab. Tech. in Biochem. and Mol. Biol.*, Vol. 1  
Work, T.S. and Work, E., Eds.  
North-Holland, pp. 288-291.

55. Gaertner, F.H. and DeMoss, J.A. (1969).  
Purification and characterization of a multi-enzyme complex in the tryptophan pathway of Neurospora crassa.  
J. Biol. Chem. 244, 2716-2725.
56. Galzy, P. and Slonimski, P.P. (1957).  
Variations physiologiques de la levure au cours de la croissance sur l'acide lactique comme seule source de carbone.  
Compt. Rend. Acad. Sci. 245, 2423-2426.
57. Geiduschek, E.P. and Haselkorn, R. (1969).  
Messenger RNA.  
Ann. Rev. Biochem. 38, 647-676.
58. Gerhart, J.C. (1964).  
Subunits for control and catalysis in aspartate transcarbamylase.  
Brookhaven Symposia in Biology 17, 222-231.
59. Gerhart, J.C. and Holoubek, H. (1967).  
The purification of aspartate transcarbamylase of Escherichia coli and separation of its protein subunits.  
J. Biol. Chem. 242, 2886-2892.
60. Gerhart, J.C. and Pardee, A.B. (1962).  
The enzymology of control by feedback inhibition.  
J. Biol. Chem. 237, 891-896.
61. Gerhart, J.C. and Pardee, A.B. (1963).  
The effect of feedback inhibitor, CTP, on subunit interactions in aspartate transcarbamylase.  
Cold Spr. Harb. Symp. Quant. Biol. 28, 491-496.
62. Gerhart, J.C. and Pardee, A.B. (1964).  
Aspartate transcarbamylase, an enzyme designed for feedback inhibition.  
Fed. Proc. 23, 727-735.
63. Gerhart, J.C. and Schachman, H.K. (1965).  
Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase.  
Biochem. 4, 1054-1062.

64. Gerhart, J.C. and Schachman, H.K. (1968).  
Allosteric interactions in aspartate transcarbamylase. II. Evidence for different conformational states of the protein in the presence and absence of specific ligands.  
Biochem. 7, 538-552.
65. Gilbert, W. and Muller-Hill, B. (1966).  
Isolation of the lac repressor.  
Proc. Natl. Acad. Sci. 56, 1891-1898.
66. Giles, N.H., Case, M.E., Partridge, C.W.H. and Ahmed, S.I. (1967).  
A gene cluster in Neurospora crassa coding for an aggregate of five aromatic synthetic enzymes.  
Proc. Natl. Acad. Sci. 58, 1453, 1460.
67. Gorini, L. and Kalman, S.M. (1963).  
Control by uracil of carbamyl phosphate synthesis in Escherichia coli.  
Biochim. Biophys. Acta 69, 355-360.
68. Gorini, L. and Maas, W.K. (1957).  
The potential for the formation of a biosynthetic enzyme in Escherichia coli.  
Biochim. Biophys. Acta 25, 208-209.
69. Grisolia, S. and Cohen, P.P. (1952).  
The catalytic role of carbamyl glutamate in citrulline biosynthesis.  
J. Biol. Chem. 198, 561-571.
70. Grisolia, S. and Cohen, P.P. (1953).  
Catalytic role of glutamate derivatives in citrulline biosynthesis.  
J. Biol. Chem. 204, 753.
71. Grisolia, S. and Harmon, P. (1962).  
Acetyl phosphate utilization with animal and bacterial enzymes.  
Biochem. Biophys. Res. Comm. 7, 357-360.
72. Guthohrlein, G. and Knappe, J. (1968).  
Structure and function of carbamylphosphate synthetase.

72. (cont'd) 1. Transitions between two catalytically inactive forms and the active form.  
Eur. J. Biochem. 7, 119-127.
73. Guthohrlein, G. and Knappe, J. (1969).  
Structure and function of carbamylphosphate synthetase on the mechanism of bicarbonate activation.  
Eur. J. Biochem. 8, 207-214.
74. Hartman, S.C. and Buchanan, J.M. (1958).  
Biosynthesis of the purines.  
XXI. 5-phosphoribosylpyrophosphate amidotransferase.  
J. Biol. Chem. 233, 451-455.
75. Hatch, M.D. and Stumpf, P.K. (1961).  
Fat metabolism in higher plants.  
XVI. Acetyl coenzyme A carboxylase and acyl coenzyme A-malonyl coenzyme A transcarboxylase from wheat germ.  
J. Biol. Chem. 236, 2879-2885.
76. Hayward, W.S. and Belser, W.L. (1964).  
Regulation of pyrimidine biosynthesis in Serratia marescens.  
*Shelby?* Proc. Nat. Acad. Sci. 53, 1483-1489.
77. Heinrikson, R.L. and Goldwasser, E. (1964).  
Studies on the biosynthesis of 5-ribosyluracil-5'-monophosphate in Tetrahymena pyriformis.  
J. Biol. Chem. 239, 1177-1187.
78. Herve, G.L. and Stark, G.R. (1967).  
Aspartate transcarbamylase. Amino-terminal analyses and peptide maps of the subunits.  
Biochem. 6, 3743-3747.
79. Hill, A.V. (1910).  
The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves.  
J. Physiol. 40, 4P.
80. Horowitz, N.H. and Metzberg, R.L. (1965).  
Biochemical aspects of genetics  
Ann. Rev. Biochem. 34, 527-564.

81. Hurlbert, R.B. and Potter, V.R. (1952).  
A survey of the metabolism of orotic acid in the rat.  
J. Biol. Chem. 195, 257-270.
82. Huston, B.R. and Cohen, P.P. (1969).  
On the reported presence of biotin in carbamyl  
phosphate synthetase.  
Biochem. 8, 2658-2661.
83. Jacob, F. and Monod, J. (1961)  
Genetic regulatory mechanisms in the synthesis  
of proteins.  
J. Mol. Biol. 3, 318-356.
84. Jacob, F., Perrin, D., Sanchez, C. and Monod, J.  
(1960).  
L'operon: groupe de genes a expression coordonnee  
par u operateur.  
C.R. Acad. Sci. (Paris) 250, 1727-1729.
85. Jones, M.E. (1963).  
Carbamyl phosphate.  
Science 140, 1373-1379.
86. Jones, M.E. (1965).  
Amino acid metabolism.  
Ann. Rev. Biochem. 34, 381.
87. Jones, M.E. and Hager, S.E. (1966).  
Source of carbamyl phosphate for pyrimidine  
biosynthesis in mouse ehrlich ascites cells and  
rat liver.  
Science 154, 422.
88. Jones, M.E. and Lipmann, F. (1960).  
Chemical and enzymatic synthesis of carbamyl phosphate.  
Proc. Nat. Acad. Sci. U.S. 46, 1194.
89. Jones, M.E., Spector, L., and Lipmann, F. (1955).  
Carbamyl phosphate, the carbamyl donor in enzymatic  
citrulline synthesis.  
J. Am. Chem. Soc. 77, 819.

90. Kalman, S.M., Duffield, P.H. and Brzozowski, T. (1965).  
Identity in Escherichia coli of carbamyl phosphokinase and an activity which catalyses amino group transfer from glutamine to ornithine in citrulline synthesis.  
Biochem. Biophys. Res. Comm. 18, 530.
91. Kalman, S.M., Duffield, P.H. and Brzozowski, T. (1966).  
Purification and properties of a bacterial carbamyl phosphate synthetase.  
J. Biol. Chem. 241, 1871-1877.
92. Kammen, H.O. and Hurlbert, R.B. (1958).  
Amination of uridine nucleotides to cytidine nucleotides by soluble mammalian enzymes: role of glutamine and guanosine nucleotides.  
Biochim. et Biophys. Acta 30, 195.
93. Kanazir, D., Barner, H.D., Flaks, J.B. and Cohen, S.S. (1959).  
Some physiological and genetic properties of a strain of Escherichia coli requiring thymine, arginine and uracil.  
Biochim. et Biophys. Acta 34, 341.
94. Kaplan, J.G., Duphil, M., and Lacroute, F. (1967).  
A study of the aspartate transcarbamylase activity of yeast.  
Arch. Biochem. Biophys. 119, 541-551.
95. Kaplan, J.G., Lacroute, F. and Messmer, I. (1969).  
On the loss of feedback inhibition of yeast aspartate transcarbamylase during derepression of pyrimidine biosynthesis.  
Arch. Biochem. Biophys. 129, 539-544.
96. Kaplan, J.G. and Messmer, I. (1969).  
The combined effects of temperature and dilution on the activity and feedback inhibition of yeast aspartate transcarbamylase.  
Can. J. Biochem. 47, 477-479 (1969).

97. Kaziro, Y., Leone, E. and Ochoa, S. (1960).  
Biotin and propionyl carboxylase.  
Proc. Nat. Acad. Sci. U.S.A. 46, 1319-
98. Keech, B. and Barritt, G.J. (1967).  
Allosteric activation of sheep kidney pyruvate  
carboxylase by the magnesium ion ( $Mg^{++}$ ) and  
the magnesium adenosine triphosphate ion ( $Mg-ATP^{--}$ )  
J. Biol. Chem. 242, 1983-1987.
99. Kleczkowski, K. (1965).  
  
Acta Biochem. Pol. 12, 243-
100. Kleppe, K. (1965).  
Inorganic pyrophosphate and cellular control  
mechanisms: inhibition of aspartate transcarbamylase.  
Biochem. Biophys. Res. Comm. 19, 468-472.
101. Kleppe, K. (1966).  
Aspartate transcarbamylase from Escherichia coli.  
I. Inhibition by inorganic anions.  
Biochim. Biophys. Acta 122, 450-461.
102. Kleppe, K. and Spaeren, U. (1966).  
Aspartate transcarbamylase from Escherichia coli.  
II. Interaction of metal ions with substrates,  
inhibitors and activators.  
Biochim. Biophys. Acta 123, 199-202.
103. Kleppe, K. and Spaeren, U. (1967).  
Feedback inhibition and ionizing radiation.  
Mechanism of inactivation of allosteric properties  
of aspartate transcarbamylase by x-rays.  
Biochem. 6, 3497-3502.
104. Kleppe, K. Sanner, T., and Pihl, A. (1966).  
Preferential inactivation of the allosteric  
function of aspartate transcarbamylase.  
Biochim. Biophys. Acta 118, 210-211.
105. Koritz, S.B. and Cohen, P. (1954).  
Colorimetric determination of carbamylamino acids  
and related compounds.  
J. Biol. Chem. 209, 145-150.

106. Kornberg, A., Liebermann, I. and Simms, E.S. (1955).  
Enzymatic synthesis and properties of 5-phospho-  
ribosyl pyrophosphate.  
J. Biol. Chem. 215, 389.
107. Koshland, D.E. Jr. (1958).  
Application of a theory of enzyme specificity to  
protein synthesis.  
Proc. Nat. Acad. Sci. U.S.A. 44, 98-104.
108. Koshland, D.E. Jr. (1968).  
The catalytic and regulatory properties of enzymes.  
Ann. Rev. Biochem. 37, 359-410.
109. Lacroute, F. (1964A).  
Un cas de double retrocontrole: la chaine de  
biosynthese de l'uracile chez la levure.  
C.R. Acad. Sc. Paris 259, 1357-1359.
110. Lacroute, F. (1964B).  
Regulation des enzymes de biosynthese de l'uracile  
chez la levure.  
C.R. Acad. Sci. Paris 258, 2884-2886.
111. Lacroute, F. (1966).  
Thesis - Regulation de la chaine de biosynthese  
de l'uracile chez Saccharomyces cerevisiae.  
Thesis, University of Paris.
112. Lacroute, F. (1968).  
Regulation of pyrimidine biosynthesis in  
Saccharomyces cerevisiae.  
J. Bacterial. 95, 824-832.
113. Lacroute, F. and Slonimski, P.P. (1964).  
Etude physiologique des mutants resistant au  
5-fluorouracite chez la levure.  
C.R. Acad. Sci. Paris 258, 2172-2174.
114. Lacroute, F., Pierard, A., Grenson, M. and Wiame, J.M.  
(1965).  
The biosynthesis of carbamyl phosphate in  
Saccharomyces cerevisiae.  
J. Gen. Microbiol. 40, 127-142.

115. Lévenberg, B. (1962).  
Role of L-glutamine as donor of carbamyl nitrogen  
for the enzymatic synthesis of citrulline in  
Agaricus bisporus.  
J. Biol. Chem. 237, 2590-2598.
116. Liebermann, I. (1955).  
Enzymatic amination of uridine triphosphate to  
cytidine triphosphate.  
J. Am. Chem. Soc. 77, 2661.
117. Liebermann, I. (1956).  
Enzymatic amination of uridine triphosphate  
to cytidine triphosphate.  
J. Biol. Chem. 222, 765.
118. Liebermann, I. and Kornberg, A. (1954).  
Enzymatic synthesis and breakdown of a pyrimidine,  
orotic acid.  
J.B.C. 207, 911.
119. Liebermann, I., Kornberg, A. and Simins, E.S.  
(1955).  
Enzymatic synthesis of nucleoside diphosphates  
and triphosphates.  
J. Biol Chem. 215, 429-440
120. Light, R.J. and Hager, L.P. (1968).  
Molecular parameters of 6-methylsalicylic acid  
synthetase from gel filtration and sucrose density  
gradient centrifugation.  
Arch. Biochem. Biophys. 125, 326-333
121. Lineweaver, H. and Burk, D. (1934).  
The determination of enzyme dissociation constant.  
J. Am. Chem. Soc. 56, 658-666.
122. Long, C.W. and Pardee, A.B. (1967).  
Cytidine triphosphate synthetase of Escherichia coli B  
I. Purification and kinetics.  
J. Biol. Chem. 242, 4715-4721.
123. Loring, H.S. and Pierce, J.G. (1944).  
Pyrimidine nucleosides and nucleotides as growth  
factors for mutant strains of Neurospora.  
J. Biol. Chem. 153, 61-69.

124. Lou, M.F. and Herrman, R.L. (1967).  
A pyrimidine-specific carbamate kinase in Neurospora.  
Biochim. Biophys. Acta 139, 199-201.
125. Lowenstein, J.M. and Cohen, P.P. (1954).  
The formation of carbamyl aspartic acid by rat  
liver preparations.  
J. Am. Chem. Soc. 76, 5571-5572.
126. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and  
Randall, R.J. (1951).  
J. Biol. Chem. 193, 265-275.
127. Lue, P.F. and Kaplan, J.G. (1969).  
The aspartate transcarbamylase and carbamoyl  
phosphate synthetase of yeast: A multifunctional  
enzyme complex.  
Biochem. Biophys. Res. Comm. 34, 426-433.
128. Lue, P.F. and Kaplan, J.G. (1970)  
Heat-induced dissociation of a multi-functional  
enzyme complex catalyzing the first steps in  
pyrimidine biosynthesis in baker's yeast.  
Can. J. Biochem. in press.
129. Maas, W.K. and McFall, E. (1964).  
Genetic aspects of metabolic control.  
Am. Rev. Microbiol. 18, 95-110.
130. Magasanik, B. (1962).  
Biosynthesis of purine and pyrimidine nucleotides.  
in "The Bacteria", edited by I.C. Gunsalus and  
R.Y. Stanier, Vol. III Biosynthesis  
Acad. Press pp. 295.
131. Marshall, M. and Cohen, P. (1966).  
A kinetic study of the mechanism of crystalline  
carbamate kinase.  
J. Biol. Chem. 241, 4197-4208.
132. Marshall, M., Metzenberg, R.L. and Cohen, P.  
(1958).  
Purification of carbamyl phosphate synthetase  
from frog liver.  
J. Biol. Chem. 233, 102.

133. Marshall, M., Metzenberg, R.L. and Cohen, P. (1961).  
Physical and kinetic properties of carbamyl phosphate  
synthetase from frog liver.  
J. Biol. Chem. 236, 2229-2237.
134. Martin, R.G. and Ames, B.N. (1961).  
A method for determining the sedimentation  
behaviour of enzymes: application to protein  
mixtures.  
J. Biol. Chem. 236 1372-1379.
135. McClintock, D.K. and Markus, G. (1968).  
Conformational changes in aspartate transcarbamylase  
I. Proteolysis of the intact enzyme.  
J. Biol. Chem. 243, 2855-2862.
136. McClintock, D.K. and Markus, G. (1969).  
Conformation changes in aspartate transcarbamylase  
II. concerted or sequential mechanism.  
J. Biol. Chem. 244 36-42.
137. Metzenberg, R.L., Marshall, M. and Cohen, P.  
(1958).  
Carbamyl phosphate synthetase: Studies on the  
mechanism of action.  
J. Biol. Chem. 233, 1560-1564.
138. Minson, A.C. and Creaser, G.H. (1969).  
Purification of a trifunctional enzyme catalyzing  
three steps of the histidine pathway, from Neuro-  
spora crassa.  
Biochem. J. 114, 49-56.
139. Mitchell, H.K. and Houlahan, M.B. (1947).  
Investigations on the biosynthesis of pyrimidine  
nucleosides in Neurospora.  
Fed. Proc. 6, 506.
140. Mitchell, H.K., Houlahan, M.B. and Nye, J.F. (1948).  
The accumulation of orotic acid by a pyrimidine-less  
mutant of Neurospora.  
J. Biol. Chem. 172, 525.
141. Moldave, K.  
Nucleic acids and protein biosynthesis.  
Ann. Rvw. Biochem. 34, 419-448,

142. Monod, J. and Cohen-Bazire, G. (1953).  
L'effet d'inhibition specifique dans la biosyn-  
these de la tryptophane-demase chez Aerobacter  
aerogenes.  
Compt. rend. 236, 530-532.
143. Monod, J., Changeux, J. and Jacob, F. (1963).  
Allosteric proteins and cellular control systems.  
J. Mol. Biol. 6, 306-329.
144. Monod, J., Wyman, J. and Changeux, J.P. (1965).  
On the nature of allosteric transitions: a  
plausible model.  
J. Mol. Biol. 12, 88-118.
145. Morgan, V.E. and Chichester, D.F. (1935).  
Properties of the blood of the domestic fowl.  
J. Biol. Chem. 110, 285-298.
146. Mortimer, R.K. and Hawthorne, D.C. (1966).  
Genetic mapping in Saccharomyces.  
Genetics 53, 165-173.
147. Moyed, H.S. and Magasanik, B. (1957).  
Enzymes essential for the biosynthesis of nucleic  
acid guanine; xanthosine 5-phosphate aminase of  
Aerobacter aerogenes.  
J. Biol. Chem. 226, 351.
148. Muirhead, H., and Perutz, M.F. (1963).  
Structure of reduced human hemoglobin.  
Cold Spr. Harb. Symp. Quant. Biol. 28, 451-459.
149. Murray, A.W. and Wong, P.C.L. (1967).  
5-phosphoribosyl pyrophosphate synthetase from  
ehrlich ascites-tumour cells: effect of magnesium  
and ATP concentration on the enzyme activity.  
Biochem. Biophys. Res. Comm. 29, 582-587.
150. Natale, P.J. and Tremblay, G.C. (1969).  
On the availability of intramitochondrial carba-  
moyl phosphate for the extramitochondrial bio-  
synthesis of pyrimidines.  
Biochem. Biophys. Res. Comm. 37, 512-517.

151. Neuman, J. and Jones, M.E. (1964).  
End-product inhibition of aspartate transcarbamylase  
in various species.  
Arch. Biochem. Biophys. 104, 438-447.
152. Novick, R.P. and Maas, W.K. (1961).  
Control of endogenously synthesized arginine of  
the formation of ornithine transcarbamylase in  
Escherichia coli.  
J. Bacteriol. 81, 236-240.
153. Oliver, I.T., Koskimies, D., Hurwitz, R. and  
Kretchmer, N. (1969).  
Macromolecular forms of aspartate transcarbamylase  
in rat liver.  
Biochem. Biophys. Res. Comm. 37, 505-511.
154. O'Neal, D. and Naylor, A.W. (1969).  
Partial purification and properties of carbamyl  
phosphate synthetase of alaska pea (Pisum sativum  
L. cultivar Alaska): purification and general  
properties.  
Biochem. J. 113, 271-279.
155. O'Sullivan, W.J. and Perrin, D.D. (1964).  
The stability constants of metal-adenine nucleotide  
complexes.  
Biochem. (Wash.) 3, 18-26.
156. Peng, L. and Jones, M.E. (1969).  
Effect of avidin on vertebrate carbamyl phosphate  
synthetases.  
Biochem. Biophys. Res. Comm. 34, 335-339.
157. Peterson, E.A. and Sober, H.A. (1962).  
Column chromatography of proteins: substituted  
celluloses.  
In "Methods in Enzymology", S.P. Colowick and N.O.  
Kaplan eds, Vol. 5, Academic Press, New York, p 3-27.
158. Pierard, A. (1966).  
Control of the activity of Escherichia coli  
carbamoyl phosphate synthetase by antagonistic  
allosteric effectors.  
Science 154, 1572-1573.

159. Pierard, A., Glansdorff, N., Mergeay, M., and Wiame, J.M. (1965).  
Control of the biosynthesis of carbamyl phosphate in *Escherichia coli*.  
*J. Mol. Biol.* 14, 23-36.
160. Pierard, A. and Wiame, J.M. (1964).  
Regulation and mutation affecting a glutamine dependent formation of carbamyl phosphate in *Escherichia coli*.  
*Biochem. Biophys. Res. Comm.* 15, 76-81.
161. Porter, R.W., Modebe, M.O. and Stark, G.R. (1969).  
Aspartate transcarbamylase  
Kinetic studies of the catalytic subunit.  
*J. Biol. Chem.* 244, 1846-1859.
162. Ptashne, M. (1967a).  
Isolation of the phage repressor.  
*Proc. Natl. Acad. Sci.* 57, 306-313.
163. Ptashne, M. (1967b).  
Specific binding of the phage repressor to DNA.  
*Nature*, 214, 232-234.
164. Raijman, L. and Grisolia, S. (1964).  
Enzymatic synthesis of acetyl phosphate and formyl phosphate by carbamyl phosphate synthetase.  
*J. Biol. Chem.* 239, 1272.
165. Ramos, F., Stalon, V., Pierard, A. and Wiame, J.M. (1967).  
The specialization of the two ornithine carbamoyl transferase of *Pseudomonas*.  
*Biochim. Biophys. Acta* 139, 98-106.
166. Reichard, P. (1952).  
The function of orotic acid in the biogenesis of pyrimidines in slices from regenerating liver.  
*J. Biol. Chem.* 197, 391-398.
167. Reichard, P. (1954).  
Enzymatic synthesis of ureidosuccinic acid.  
*Acta Chem. Scand.* 8, 1102.

168. Reichard, P. (1955).  
Biosynthesis of purines and pyrimidines.  
in "The Nucleic Acids", E. Chargaff and J.N.  
Davidson, eds., Academic Press, Inc., New York,  
N.Y., Vol. 1, pp. 277.
169. Reichard, P. (1959).  
The enzymatic synthesis of pyrimidines.  
Adv. Enzym. 21, 263.
170. Reichard, P. and Bergstrom, S. (1951).  
Synthesis of polynucleotides in slices from  
regenerating liver.  
Acta Chem. Scand. 5, 190-191.
171. Reichard, P. and Hanshoff, G. (1955).  
Synthesis of ureidosuccinic acid with soluble  
enzymes from liver mitochondria and Escherichia  
coli.  
Acta Chem. Scand. 9, 519-530.
172. Reichard, P. and Hanshoff, G. (1956).  
Aspartate carbamyl transferase from Escherichia  
coli.  
Acta Chem. Scand. 10, 548-566.
173. Reichard, P. and Lagerkvist, U. (1953).  
The biogenesis of orotic acid in liver slices.  
Acta Chem. Scand. 7, 1207.
174. Reissig, J.L., Issaly, A.S. and de Issaly, I.M.  
(1967).  
Arginine-pyrimidine pathways in microorganisms.  
Nat. Canc. Inst. Monogr. 27, 259-271.
175. Roberts, R.B., Abelson, P.H., Cowie, D.B.,  
Bolton, E.T. and Britten, R.J. (1955).  
Studies of biosynthesis in Escherichia coli.  
Carnegie Inst. of Washington, publ. no. 607.
176. Roepke, R.R., Libby, R.L. and Small, M.H. (1944).  
Mutation or variation of Escherichia coli with  
respect to growth requirements.  
J. Bacteriol. 48, 401.

177. Rogers, H.J. (1944).  
Importance of pyrimidine derivation in the growth of group C (Lancefield) Streptococci upon a simplified medium.  
Nature 153, 251.
178. Rogers, P. and Novelli, G.D. (1962).  
Purification of ornithine transcarbamylase from derepressed cells of Escherichia coli W.  
Arch. Biochem. Biophys. 96, 398-407.
179. Rutman, R.J., Cantarow, A. and Paschkis, K.E. (1954).  
Studies in 2-acetylaminofluorene carcinogenesis. III. The utilization of uracil-2-<sup>14</sup>C by preneoplastic rat liver and rat hepatoma.  
Cancer Research 14, 119.
180. Sadler, J.R. and Novick, A. (1965).  
The properties of repressor and the kinetics of its action.  
J. Mol. Biol. 12, 305-327.
181. Sanwal, B.D., Stachow, C.S. and Cook, R.A. (1965).  
A kinetic model for the mechanism of allosteric activation of nicotinamide-adenine dinucleotide-specific isocitric dehydrogenase.  
Biochemistry (Wash.) 4, 410-421.
182. Satyanarayana, T., Umbarger, H.E. and Lindegren, G.  
Biosynthesis of branched-chain amino acids in yeast: Correlation of biochemical blocks and genetic lesions in leucine auxotrophs.  
J. Bacteriol. 96, 2012-2017.
183. Satyanarayana, T. and Kaplan, J.G. (1970)  
Regulation of the purine pathway in yeast: Phosphoribosyl pyrophosphate amidotransferase.  
Fed. Proceed. in press.
184. Schmidt, P.G., Stark, G.R. and Baldeschwieler, J.D. (1969).  
Aspartate transcarbamylase: A nuclear magnetic resonance study of the binding of inhibitors and substrates to the catalytic subunit.  
J. Biol. Chem. 244, 1860-1868.

185. Schweet, R. and Heintz, R. (1966).  
Protein synthesis.  
Ann. Rev. Biochem. 35, 723-758.
186. Sheperdson, M. and Pardee, A.B. (1960).  
Production and crystallization of aspartate  
transcarbamylase.  
J. Biol. Chem. 235, 3233-3237.
187. Shumaker, V.N. (1967).  
Zone centrifugation.  
Adv. in Biol. and Med. Physics 11, 245-339.
188. Siegel, L.M. and Monty, K.J. (1966).  
Determination of molecular weights and frictional  
ratios of proteins in impure systems by use of  
gel filtration and density gradient centrifugation .  
Application to crude preparations of sulfite and  
hydroxylamine reductases.  
Biochim. Biophys. Acta 112, 346-362.
189. Slade, H.D. and Slamp, W.C. (1952).  
The formation of arginine dihydrolase and by  
Streptococci and some properties of the enzyme  
system.  
J. Bacteriol. 64, 455-466.
190. Stadtman, E.R. (1963).  
Enzyme multiplicity and function in the regulation  
of divergent metabolic pathways.  
Bacteriol. Rev. 27, 170-181.
191. Stadtman, E.R. (1966).  
Allosteric regulation of enzyme activity.  
Adv. Enzymol. 28, 41-154.
192. Stent, G. (1964).  
The operon: on its third anniversary.  
Science 144, 816-820.
193. Strecker, H.J. (1955).  
L-glutamine dehydrogenase from liver .  
in "Methods in Enzymology", Colowick and Kaplan eds.,  
Vol. II, pp. 220-225.

194. Sundararajan, T.A. and Sarma, P.S. (1959).  
Substrate specificity of phosphoprotein phosphatase from spleen.  
Biochem. J. 71, 537-544.
195. Sweeny, J.R. and Fisher, J.R. (1968).  
An alternative to allosterism and cooperativity in the interpretation of enzyme kinetic data.  
Biochem. 7, 561-565.
196. Switzer, R.L. (1969).  
Regulation and mechanism of phosphoribosylpyrophosphate synthetase. I. Purification and properties of the enzyme from Salmonella typhimurium.  
J. Biol. Chem. 244, 2854-2863.
197. Tatibana, M. and Ito, K. (1967).  
Carbamyl phosphate synthetase of the hemopoietic mouse spleen and the control of pyrimidine biosynthesis.  
Biochem. Biophys. Res. Comm. 26, 221-227.
198. Taylor, A.L., Beckwith, J.R., Pardee, A.B., Austrian, R. and Jacob, F. (1964).  
The chromosomal location of the structural gene for orotydic acid pyrophosphorylase in Escherichia coli.  
J. Mol. Biol. 8, 771.
199. Thorne, K.J.I. and Jones, M.E. (1963).  
Carbamyl and acetyl phosphokinase activities of Streptococcus faecalis and Escherichia coli.  
J. Biol. Chem. 238, 2992.
200. Truffa-Bachi, P. and Cohen, G.N. (1968).  
Some aspects of amino acid biosynthesis in microorganisms.  
Ann. Rev. Biochem. 37, 79-108.
201. Umbarger, H.E. (1956).  
Evidence for a negative-feedback mechanism in the biosynthesis of isoleucine.  
Science, 123, 848.

202. Umbarger, H.E. (1964).  
Intracellular regulatory mechanisms.  
Science 145, 674-679.
203. Vallee, B.L. and Hoch, F.L. (1955).  
Zinc, a component of yeast alcohol dehydrogenase.  
Proc. Natl. Acad. Sci. 41, 327.
204. Vogel, H.J. (1957).  
in "The Chemical Basis of Heredity", W.D. McElroy  
and B. Glass, eds., The John Hopkins Press,  
Baltimore, pp. 276.
205. Vogel, H.J. and Vogel, R.H. (1967).  
Regulation of protein synthesis.  
Ann. Rev. Biochem. 36, 519-538.
206. Weber, K. (1968).  
New structural model of E. coli aspartate trans-  
carbamylase and the amino-acid sequence of the  
regulatory polypeptide chain.  
Nature 218, 1116-1119.
207. Weed, L.L. and Cohen, S.S. (1951).  
The utilization of host pyrimidines in the synthesis  
of bacterial viruses.  
J. Biol. Chem. 192, 693-700.
208. Weed, L.L., Edmonds, M. and Wilson, D.W. (1950).  
Conversion of radioactive orotic acid into  
pyrimidine nucleotides of nucleic acid by slices  
of rat liver.  
Proc. Soc. Exptl. Biol. Med. 75, 192-193.
209. Weed, L.L. and Wilson, D.W. (1951).  
The incorporation of  $C^{14}$ -orotic acid into nucleic  
acid pyrimidines in vitro.  
J. Biol. Chem. 189, 435-442.
210. Wellner, V.P., Santos, J.I. and Meister, A. (1968).  
Carbamyl phosphate synthetase. A biotin enzyme.  
Biochem. 7, 2848-2851.

211. Weitzman, P.D.J. and Wilson, I.B. (1966).  
Studies on aspartate transcarbamylase and its  
allosteric interaction.  
J. Biol. Chem. 241, 5481-5488.
212. Whitaker, J.R. (1963).  
Determination of molecular weights of proteins  
by gel filtration on Sephadex.  
Anal. Chem. 35, 1950-1953.
213. Wiley, D.C. and Lipscomb, W.N. (1968).  
Crystallographic determination of aspartate  
transcarbamylase.  
Nature 218, 1119-1121.
214. Williams, L.G. and Davis, R.H. (1968).  
Genetic and physical relationship between two  
early steps of pyrimidine synthesis.  
Genetics 60, 238.
215. Wong, G.C. and Burns, V.W. (1969).  
The inhibition of aspartate transcarbamylase  
activity by ATP in a mutant of baker's yeast S1237.  
Biochim. Biophys. Acta 192, 118-123.
216. Wyman, J. Jr. (1948).  
Heme proteins.  
Adv. Protein Chem. 4, 407-531.
217. Wyman, J. Jr. (1963).  
Allosteric effects in hemoglobin.  
Cold Spr. Harb. Symp. Quant. Biol. 28, 483-489.
218. Yasphe, J. and Gorini, L. (1965).  
Phosphorylation of carbamate in vivo and in vitro.  
J. Biol. Chem. 240, 1681-1686.
219. Yates, R.A. and Pardee, A.B. (1956a).  
Pyrimidine biosynthesis in Escherichia coli.  
J. Biol. Chem. 221, 743-756.
220. Yates, R.A. and Pardee, A.B. (1956b).  
Control of pyrimidine biosynthesis in Escherichia  
coli by a feedback mechanism.  
J. Biol. Chem. 221, 757-770.