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IN NEUROSPORA CRASSA.

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THE GENETIC CONTROL OF ASPARTATE TRANSCARBAMYLASE
BY THE PYR-3 LOCUS IN NEUROSPORA CRASSA

by

John Mayes Hill, Jr.

A THESIS
SUBMITTED TO THE FACULTY
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Thesis Director's signature:

A handwritten signature in dark ink, appearing to read "Val A. Woodward", written over a horizontal line.

Houston, Texas
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INTRODUCTION

Mutant strains of *Neurospora* requiring pyrimidines as growth factors were first described by Loring and Pierce (1944). These strains were produced by X-ray irradiation and had been obtained from the laboratories of G.W. Beadle and E.L. Tatum, and of F.P. Hungate. Other mutant strains of *Neurospora* requiring pyrimidines as growth factors were isolated by Beadle and Tatum using X-ray irradiation, ultra-violet treatment, and neutron bombardment (Beadle and Tatum, 1945).

Variations of nutritional requirement among the pyrimidine requiring strains of *Neurospora* were described by Mitchell and Houlahan (1947). They suggested that the responses of pyrimidine requiring strains to an unlinked suppressor mutation, s, could be used as an indication of allelism (Houlahan and Mitchell, 1947). Three mutant strains, isolation numbers 37301, 37815, and 67605, were found to respond to s, and were designated pyr-3a, pyr-3b and pyr-3c, respectively. The presence of s in the same genome with one of these mutations will render that mutation independent of pyrimidine requirement.

The pyr-3 genetic locus was shown to be in the fourth linkage group by Houlahan, Beadle, and Calhoun (1949). By using classical breeding tests the position of the pyr-3 locus was determined to be about 15 map units to the right of

the centromere in linkage group IV (Mitchell and Mitchell, 1954; Barratt, Newmeyer, Perkins, and Garnjobst, 1954).

A new pyr-3 mutant strain was described by Mitchell and Mitchell in 1956 which did not respond to s (Mitchell and Mitchell, 1956). This strain, pyr-3d, was compared with pyr-3a and pyr-3b using classical breeding tests and heterocaryon formation. No prototrophs were recovered in breeding tests with pyr-3a and pyr-3b as parental types while occasional prototrophs appeared in breeding tests with either pyr-3a or pyr-3b and pyr-3d. Heterocaryons between pyr-3a and pyr-3b are only partially relieved of their pyrimidine requirement while heterocaryons between either pyr-3a or pyr-3b and pyr-3d are independent of a pyrimidine requirement.

Extensive genetic analysis of the pyr-3 locus was carried out by Suyama, Munkres, and Woodward (1959), using thirty-two ultraviolet induced, independently isolated mutant strains. The locus consists of thirteen single site mutations arranged in a one-dimensional map as determined by classical breeding tests, and of at least two functionally distinct types as determined by heterocaryon complementation. The division into distinct functional types was supported by the responses of these mutant strains to s; one complementing type was suppressed by s while the other complementing type and the non-complementing type were not.

Munkres, Suyama, and Woodward (1959) found that the

pyr-3 mutant strains do not accumulate any of the intermediates of the pyrimidine pathway and suggested that they are unable to carry out the initial step of pyrimidine synthesis, the carbamylation of aspartate to form carbamyl aspartate. Davis (1960) confirmed this when he showed that strain pyr-3d lacks the enzyme aspartate transcarbamylase (ATC); however, pyr-3a and pyr-3b were found to have the ability to synthesize in vitro carbamyl aspartate from aspartate and carbamyl phosphate (CAP).

Davis and Woodward (1962) extended the study of ATC activity to the thirty-two pyr-3 mutant strains isolated and genetically analyzed by Suyama et al. (1959) and found that with no exceptions one complementing type has ATC activity while the other complementing type and the non-complementing type do not have activity. It is the ATC positive complementing type that is suppressed by s.

Woodward (1962) observed that growth resulting from heterocaryon formation is vigorous only when one homocaryon has in vitro ATC activity and the other homocaryon does not. No growth or rudimentary growth always occurred where the two homocaryons were of the same type with respect to ATC activity, and sometimes occurred when the homocaryons were of opposite types. When vigorous heterocaryon growth is compared with no growth or rudimentary growth the pyr-3 locus is divided into two distinct functional regions: one region consists of sites of complementing ATC negative

mutations which are not suppressible by s, and the other region consists of sites of complementing ATC positive mutations which are suppressible by s. The non-complementing ATC negative mutational sites are interspersed through both regions.

The pyr-3 locus which controls the initial step in pyrimidine synthesis apparently possesses two functions as demonstrated by the types of mutant strain which result from mutations at this locus: the ATC positive mutant strains are defective in one function, the ATC negative complementing mutant strains are defective in the other function, and the non-complementing mutant strains are defective in both functions (Davis, 1962; Davis and Woodward, 1962). This theory is supported by the fact that one of the non-complementing mutant strains which lacks ATC activity was converted by ultraviolet irradiation into a mutant strain, designated b₂C₇, of the complementing type possessing ATC activity in vitro (Woodward and Davis, 1963). The altered mutant also responded as the original ATC positive mutants with respect to suppression by s. The same non-complementing mutant was altered in the opposite way with ultraviolet irradiation producing a mutant strain which complements with ATC positive types and which lacks ATC activity in vitro (Dutta and Woodward, 1964).

Since the s mutation suppressed the ATC positive pyr-3 mutant strains, it was apparent that the nature of its

genetic lesion may shed light on the functions of the pyr-3 locus. Davis (1962) found that s is a mutation which controls the structure of ornithine transcarbamylase (OTC), the enzyme in the arginine biosynthetic pathway which couples carbamyl phosphate with ornithine to yield citrulline.

It had been known for several years that two unlinked loci seemed to control the reaction converting ornithine to citrulline. Srb and Horowitz (1944) isolated two mutant strains of Neurospora crassa which respond to exogenous citrulline but not to ornithine. However, Fincham (1960) later showed that both mutant strains, arg-2 and arg-3 (isolation numbers 33442 and 30300, respectively), possess OTC activity in vitro. Recently, Davis (1963) demonstrated that the arg-3 locus controls the production of the enzyme carbamyl phosphokinase (CPK) which synthesizes CAP in the arginine biosynthetic pathway. The function of the arg-2 locus is still unknown, but an abundance of genetic data suggest that it controls an enzyme which catalyzes a reaction prior to the action of CPK in the biosynthesis of CAP.

These findings point to the s mutation as the structural locus for OTC. To finally demonstrate this, mutant strains lacking in vitro OTC activity were isolated (Woodward, 1963; Davis-Thwaites, 1963), and it was shown that these mutants are allelic with s (Woodward and Schwarz, 1964). The locus is called arg-12 and it is located near the centromere on linkage group II.

The arg-12 mutations lacking in vitro OTC activity do not suppress the ATC positive pyr-3 mutations. The reason for this is explained as follows: complete blocks at the arg-12 locus introduce a requirement for exogenous arginine, and it has been shown that exogenous arginine prevents the suppression of the ATC positive pyr-3 mutations. The mutations on the other hand is only a partial block at the arg-12 locus and can produce sufficient endogenous arginine to sustain growth.

It was found that the complementing ATC negative mutants also act as suppressor loci affecting the arg-3 mutations (McDougall and Woodward, 1965). The action of the reciprocal suppression of the two pathways at the transcarbamylase reaction was thought to proceed through the common substrate, CAP. This reciprocal suppression implies the second function at the pyr-3 locus is the synthesis of CAP. arg-3 mutant strains apparently can obtain CAP from the pyrimidine pathway when the pyrimidine pathway is blocked at the step utilizing CAP, i.e., the step at which the complementing ATC negative mutations of the pyr-3 locus are postulated to be blocked. The ATC positive mutants of the pyr-3 locus apparently can obtain CAP from the arginine pathway when the arginine pathway is partially blocked at the analogous step, i.e., at the arg-12 locus. A possible site of action for exogenous arginine in blocking suppression of the ATC positive pyr-3 mutations is the repression or inhibition of CPK there by

cutting off the supply of CAP which could have effected the suppression.

Definitive evidence on the answer to the question about the function of the pyr-3 locus other than the trans-carbamylase function could be provided if the enzyme in the pyrimidine pathway with the ability to synthesize carbamyl phosphate would be assayed; however, an extensive search in several laboratories has failed to provide an assay for this enzyme in spite of the fact that this activity has been inferred with radioactive tracer techniques in the intact cell (Davis, 1963).

The present study attempts to answer the question of whether the pyr-3 locus controls the synthesis of one enzyme which performs both functions or whether it controls the synthesis of two enzymes. Lacking an assay for the carbamyl phosphate synthesizing enzyme in the pyrimidine pathway, the answer to this question becomes difficult. An alternative to an assay for the second function has been to examine the enzymes having ATC activity from mutant strains known to be defective in the second function. An alteration of the properties of the ATC molecule due to mutations at the second functional region would indicate that one protein is produced by the pyr-3 locus possessing both functions.

MATERIALS AND METHODS

Mutant Strains

St. Lawrence strain 74A was used as the wild-type strain of *Neurospora* in these studies. Mutant strains pyr-3a, pyr-3b, and pyr-3c were obtained from the Fungal Genetics Stock Center at Dartmouth College, Hanover, New Hampshire, and the pyr-3 mutants prefixed KS- were from our laboratory. Strain b₂C₇ was also from our laboratory.

Chemical Reactions and Enzyme Assays

Carbamyl aspartate is formed by the coupling of CAP and aspartate in the presence of ATC (Jones, Spector, and Lipmann, 1956) with the stoichiometry as shown below:

ATC activity was determined with the colorimetric assay for carbamyl-amino acids of Koritz and Cohen (1954) (Appendix 1) and with the titration of the hydrogen ion released in a Sargent pH Stat (Appendix 2).

Citrulline is formed in a similar reaction between CAP and ornithine in the presence of OTC (Jones, Spector, and Lipmann, 1955) with the following stoichiometry:

OTC activity was also determined by assay for the carbamyl amino acid produced in the reaction using the method of Koritz and Cohen (Appendix 1) and by assay with the Sargent pH Stat (Appendix 2).

CAP was synthesized from potassium cyanate and potassium phosphate according to the method of Spector, Jones, and Lipmann (1957) (Appendix 3) and assayed according to the labile phosphate determination of Fiske and SubbaRow (1925) (Appendix 4) or with the Sargent pH Stat (Appendix 2).

Enzyme Purification

Neurospora was grown on citrate medium, harvested, dried, and prepared for extraction (Appendix 5). ATC and OTC were partially purified by the standard protein fractionation methods (Appendix 6) to obtain enzyme preparations which were relatively free from other enzymes utilizing aspartate, ornithine and CAP. Protein concentrations were determined by a modification of the Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951) (Appendix 7). The degree of purity of the enzyme preparations was estimated by starch gel electrophoresis (Appendix 8) and polyacrylamide disc electrophoresis (Appendix 9).

Enzyme Characterization

The first property of the transcarbamylase enzymes studied was the pH optimum of each enzyme. The second property was the kinetic characteristics which were determined

at a pH near these optima and at 30°C unless otherwise indicated.

The kinetic analysis involved the determination of initial velocity (v_o) as a function of substrate concentration. When the plot of v_o as a function of substrate concentration proceeded from a first-order to a zero-order reaction on increasing substrate concentration a Lineweaver-Burk reciprocal plot (Lineweaver and Burk, 1934) was used to determine the maximum velocity (V_m) (Appendix 10). When the plot of v_o as a function of substrate concentration was not a first-order equation in limiting concentrations of substrate, V_m was taken directly from the plot at saturating substrate concentrations. The orders of the transcarbamylase reactions were determined using plots of $\log v_o/(V_m - v_o)$ as a function of $\log$ substrate concentration according to the method of Atkinson, Hathaway and Smith (1965) (Appendix 11). The slope of such a plot gives the order of the reaction directly.

RESULTS

The pH optimum was determined for ATC from strain 74A and from each ATC positive mutant strain in the Sargent pH Stat at a concentration of 0.015 molar aspartate and 0.00025 molar CAP. Reaction blanks were determined which consisted of the hydrolysis of CAP in the absence of aspartate but in the presence of ATC. The pH optimum was determined for OTC from strain 74A in the Sargent pH Stat at a concentration of 0.005 molar ornithine and 0.0005 molar CAP. A reaction blank was determined at each point which consisted of the hydrolysis of CAP in the absence of ornithine but in the presence of OTC. These data are tabulated (Table 1) and illustrated (Figure 1). Further analysis of the trans-carbamylase enzymes was carried out at pH 8.5 and 30°C.

ATC has an affinity for CAP which allows the enzyme to be saturated with CAP at a concentration of 25 μ M at optimum concentrations of aspartate (Figure 2); however, high concentrations of aspartate interfere with the saturation of the enzyme with CAP (Figure 3). The high affinity of ATC for CAP makes it difficult to determine accurately the initial velocity as a function of CAP concentration. As a result this determination would not be a sensitive indication of alterations in the structure of the enzyme.

ATC is saturated in a 0.01 molar solution of aspartate. This was considered a high enough concentration that the

order of reaction at various aspartate concentrations was used as the kinetic parameter to indicate alterations in the structure of the enzyme.

The complete series of ATC reactions at various concentrations of aspartate for each strain are represented (Figure 4) as they appear on the Sargent pH Stat. Each curve demonstrates the high affinity of ATC for CAP by being linear except at extremely low concentrations of CAP at the end of the reaction. At high concentrations of aspartate the affinity is reduced and can be observed in the reduction of slope which becomes more pronounced toward the end of the reaction. At the beginning of each reaction there is a secondary, more rapid reaction, which may represent an attachment reaction between CAP and the enzyme. This becomes more noticeable as the concentration of aspartate increases.

The initial velocity is taken from the slope of the reaction after the secondary reaction has ended and before carbamyl phosphate has become limiting. At very low concentrations of aspartate the representations of the reaction (Figure 4) become more curved since the aspartate concentration is decreased during the reaction and aspartate is not at a saturating concentration. The values of v_0 as a function of substrate concentration are tabulated (Table 2) and illustrated (Figure 5).

To determine the order of the reaction as a function

of aspartate concentration, the values of v_o as a function of substrate concentration (S) are converted into $v_o/(V_m-v_o)$ (Table 2) and plotted as a function of S on log-log coordinates (Figure 6). It was determined that the various orders found for ATC from the various pyr-3 mutant strains were not due to the degree of purification or enzyme concentration. This was done by comparing ATC from strain 74A in two very different degrees of purity (ammonium sulfate fractionation and complete purification procedure) and two concentrations of the extract with the greater specific activity. The data (Table 3) indicate that the points on the log $v_o/(V_m-v_o)$ versus log S graph are superimposable.

The variety of slopes at a given concentration of aspartate indicating different orders of the transcarbamylase reaction carried out by pyr-3 mutant strains has been taken as evidence that the pyr-3 locus produces one enzyme which performs two functions. Mutations in one functional region cause a block in the biosynthetic pathway which includes this locus and causes alterations in the kinetic properties of the other function.

Strain pyr-3b is a temperature sensitive mutant which requires pyrimidine above 34°C and is independent of pyrimidine requirement below 34°C. The kinetic curve of ATC from strain pyr-3b is identical to that of strain 74A at 30°C and at 37°C and is the only pyr-3 mutant strain which shows a similarity to wild-type.

OTC does not have the great affinity for CAP which makes kinetic analysis of ATC difficult. A concentration of 400 μ M CAP is limiting at optimum concentrations of ornithine (Figure 7). High concentrations of ornithine did not inhibit enzymatic action while high concentrations of CAP had an inhibitory effect (Figures 8 and 9).

To characterize OTC, v_o as a function of each substrate was determined holding the other substrate constant. The complete series of OTC reactions at various concentrations of each substrate is represented as they appear on the Sargent pH Stat (Figures 10 and 11). The data of v_o as a function of CAP concentration at three concentrations of ornithine are tabulated (Table 4) and illustrated (Figure 8). The maximum velocity, V_m , was taken directly from these data and used to calculate $v_o/(V_m-v_o)$ (Table 4). The order of the reaction as determined by $\log v_o/(V_m-v_o)$ plotted as a function of $\log$ CAP concentration (Figure 12) changes with the concentration of ornithine; however, the same value of $v_o/(V_m-v_o)$ may be found at a higher molarity of CAP, i.e., the curves are identical but displaced with respect to CAP concentration.

The data of v_o as a function of ornithine concentration at three concentrations of CAP are tabulated (Table 5) and illustrated (Figure 9). The initial velocity as a function of ornithine concentration proceeds from a first-order to a zero-order reaction on increasing ornithine

concentration and V_m is determined by extrapolation on a Lineweaver-Burk reciprocal plot (Figure 13). The maximum velocity (Table 5) is used to calculate $v_o/(V_m-v_o)$ as a function of ornithine concentration (Table 5). An illustration of the first order OTC reaction detected when the CAP concentration is held constant even in the presence of inhibitory amounts of CAP emphasizes the differences between OTC and ATC (Figure 14).

DISCUSSION

By several criteria the pyr-3 locus appears complex. It is subdivisible into many sites by genetic recombination; it is at least duplex in function according to complementation by heterocaryosis; the enzyme, aspartate transcarbamylase, under its structural control is present in extracts of many pyr-3 mutants and absent in the majority; the ATC positive mutants are suppressed by arg-12^s and two of the ATC negative mutants suppress arg-2 and arg-3; and non-complementing mutants that lack ATC, by partial reversion, can be converted into one or the other of the two complementing types. These data have led to the hypothesis that the protein structured by the pyr-3 locus is bifunctional. One of the functions is the transferase function which catalyzed the coupling of carbamyl phosphate and aspartic acid to yield carbamyl aspartate. The other function involves the synthesis of carbamyl phosphate, presumably from NH_3 , CO_2 and ATP (this reaction has not been demonstrated in *Neurospora*).

The prime motive of the present work was to distinguish between the alternative hypotheses inferred above, i.e., does the pyr-3 locus structure one protein with two functions or two proteins each with one function. Two methods have been proposed in our laboratory for testing these hypotheses: first, the primary structure of wild type aspartate transcarbamylase will be compared with the primary structure of

the protein with aspartate transcarbamylase activity found in the ATC positive mutants. If the lesion effecting the pyrimidine-requiring phenotype is on the same protein with the ATC site the fingerprints may differ; if the lesion is on a different protein the fingerprints will never differ. This experiment cannot be executed until a method for purification of ATC is refined. The second method uses the same logic but demands kinetic analyses of the mutant enzymes rather than fingerprints. With this method we have demonstrated that ATC proteins from pyr-3 mutants possess widely divergent kinetic properties, and from these data we have concluded that the ATC proteins differ kinetically because of a mutation inactivating the carbamyl phosphate synthesizing site of the protein and secondarily changing the conformational properties of the transferase site.

This conclusion is supported by all of the genetic and biological data. The complementation pattern would argue for three types of pyr-3 mutant: those inactivated at both sites, those inactivated at the transferase site, and those inactivated at the carbamyl phosphate synthesizing site. The latter two types complement in heterocaryons. The reciprocal suppression pattern is explained by metabolic cross feeding of carbamyl phosphate between the arginine and pyrimidine pathways. arg-12^s accumulates carbamyl phosphate which can be used only by pyr-3 mutants defective at the carbamyl phosphate synthesizing site. The analogous

pyr-3 mutants would in like manner provide carbamyl phosphate for arg-3, the mutant known to possess defective carbamyl phosphokinase.

It is of interest that the analogous steps in the arginine pathway are under different genetic and enzymatic control. The arg-3 locus structures carbamyl phosphokinase, the enzyme catalyzing the synthesis of carbamyl phosphate, and the arg-12 locus structures ornithine transcarbamylase, the enzyme that couples carbamyl phosphate with ornithine to yield citrulline. Neither locus is complex as measured by complementation. In this pathway the two functions are under separate genetic control. Of the possible locus-enzyme relationships which have been described (Loper, Grabner, Stahl, Hartman, and Hartman, 1964) two are applicable to a comparison of the transcarbamylase step in the pyrimidine biosynthetic pathway with the transcarbamylase step in the arginine biosynthetic pathway. These are as follows:

One Locus - One Complementation Unit - One Enzyme

Mutations within a locus fail to complement one another but complement all other mutations located outside this locus. The enzyme is composed of a single polypeptide chain. arg-3 and arg-12 are most likely of this type.

One Locus - Two or More Complementation Units - One Enzyme

Some mutations in this locus complement while others fail to complement. Most of the complementation data can be expressed on a linear complementation map. Non-complementing mutations are scattered throughout the genetic region. The enzyme is a polymer of identical subunits. pyr-3 is a locus of this type.

Another difference between these analogous pathways is the response of each transferase to its substrates. OTC gives a first-order reaction at constant CAP concentration and varying low ornithine concentrations while ATC is an allosteric enzyme and gives a multiple and varying order reaction at constant CAP concentration and varying, low aspartate concentrations.

The differences between the genetic control and kinetic behavior of two enzymes which perform similar functions poses this question: What is the cause of the diversity? If the unity found in complex biological systems arises from the common evolutionary origin of all life then the diversity found in the basic chemical machinery could be the result of an adaptation leading to more efficient control of cellular function. Control of function is reflected in the loci controlling the steps in the chemical machinery.

An investigation of these analogous steps in more primitive organisms might indicate whether or not they had a common origin and if they did, what diverging paths they took in reaching their present dissimilar state.

SUMMARY

The pyr-3 locus in Neurospora crassa has previously been shown to be a complex locus by several genetic and biological criteria. Two metabolic functions may be ascribed to this locus: the synthesis of carbamyl phosphate and the coupling of carbamyl phosphate with aspartic acid to form carbamyl aspartate. A defect in one function will alter the in vitro kinetic characteristics of the other indicating that the locus produces one enzyme with two active sites. The structure of this enzyme permits the association of the two functions when mutant enzymes, enzymes defective in opposite functions, are combined in the same cytoplasm. This enzyme is an allosteric enzyme which gives a multiple- and varying-order reaction at low substrate concentrations.

REFERENCES

- Archibald, R.M., 1944, Determination of citrulline and allantoin and demonstration of citrulline in blood plasma: J. Biol. Chem. 156: 121-142.
- Atkinson, D.E., J.A. Hathaway, and C. Smith, 1965, Kinetics of regulatory enzymes: Kinetic order of the yeast diphosphopyridine nucleotide isocitrate dehydrogenase reaction and a model for the reaction. Personal Communication.
- Barratt, R.W., D. Newmeyer, D.D. Perkins, and L. Garnjobst, 1954, Map Construction in Neurospora crassa: Advances in Genetics 6: 1-93.
- Beadle, G.W. and E.L. Tatum, 1945, Neurospora II. Methods of producing and detecting mutations concerned with nutritional requirements: Am. Jour. Botany 32: 678-686.
- Davis, R.H., 1960, An enzymatic difference among pyr-3 mutants of Neurospora crassa: Proc. Natl. Acad. Sci., U.S., 46: 677-682.
- _____, 1962, Consequences of a suppressor gene effective with pyrimidine and proline mutants of Neurospora: Genetics 47: 351-360.
- _____, 1963, Neurospora mutant lacking an arginine specific carbamyl phosphokinase: Science 142, 1652-1654.
- Davis, R.H. and V.W. Woodward, 1962, The relationship between gene suppression and aspartate transcarbamylase activity in pyr-3 mutants of Neurospora: Genetics 47: 1075-1083.
- Davis, R.H. and W.M. Thwaites, 1963, Structural gene for ornithine transcarbamylase in Neurospora: Genetics 48: 1551-1558.
- Dutta, S.K. and V.W. Woodward, 1964, The alteration of a complementation pattern among pyr-3 mutants of Neurospora: Neurospora Newsletter 6: 13.
- Finchem, J.R.S., 1960, Genetically controlled differences in enzyme activity: Advances in Enzymol. 22: 1-43.
- Fiske, C.H. and Y. SubbaRow, 1925, Determination of phosphorus: J. Biol. Chem. 66: 375-400.

- Houlahan, M.B., G.W. Beadle, and H.G. Calhoun, 1949, Linkage studies with biochemical mutants of Neurospora crassa: Proc. Natl. Acad. Sci., U.S., 34: 493-507.
- Houlahan, M.B. and H.K. Mitchell, 1947, A suppressor in Neurospora and its use as evidence for allelism: Proc. Natl. Acad. Sci., U.S., 33: 223-229.
- Jones, M.E., L. Spector, and F. Lipmann, 1955, Carbamyl phosphate, the carbamyl donor in enzymatic citrulline synthesis: J. Am. Chem. Soc. 77: 819-820.
- _____, _____, and _____, 1956, Carbamyl phosphate: Proc. 3rd Intern. Congr. Biochem., Brussels, 1955, pp. 278-281.
- Koritz, S.B. and P.P. Cohen, 1954, Colorimetric determination of carbamylamino acids and related compounds: J. Biol. Chem. 209: 145-150.
- Lineweaver, H. and D. Burk, 1934, The determination of enzyme dissociation constants: J. Am. Chem. Soc. 56: 658-666.
- Loper, J.C., M. Grabnar, R.C. Stahl, Z. Hartman, and P.E. Hartman, 1964, Genes and proteins involved in histidine biosynthesis in Salmonella: Brookhaven Symposia in Biology 17: 15-52.
- Loring, H.S. and J.G. Pierce, 1944, Pyrimidine nucleosides and nucleotides as growth factors for mutant strains of Neurospora: J. Biol. Chem. 153: 61-69.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent: J. Biol. Chem. 193: 265-275.
- McDougall, K.J. and V.W. Woodward, 1964, Properties of pyr-3 suppressor mutations of Neurospora (abstr.): Genetics 50: 295-296.
- Michaelis, L. and M.L. Menten, 1913, Die Kinetik der Invertinwirkung: Biochem. Z. 48: 333-369.
- Mitchell, H.K., M.B. Houlahan, 1947, Investigations of the biosynthesis of pyrimidine nucleosides in Neurospora: Federation Proceedings 6: 506-509.
- Mitchell, M.B. and H.K. Mitchell, 1954, A partial map of linkage group D in Neurospora crassa: Proc. Natl. Acad. Sci., U.S., 40: 436-440.

- Mitchell, M.B. and H.K. Mitchell, 1956, Tests for non-allelism at the pyrimidine-3 locus of Neurospora: Genetics 41: 319-326.
- Munkres, K.D., Y. Suyama, and V.W. Woodward, 1957, unpublished data.
- Srb, A.M. and N.H. Horowitz, 1944, The ornithine cycle in Neurospora and its genetic control: J. Biol. Chem. 154: 129-139.
- Suyama, Y., K.D. Munkres, and V.W. Woodward, 1959, Genetic analysis of the pyr-3 locus of Neurospora crassa: The bearing of recombination and gene conversion upon intraallelic linearity: Genetics 30: 293-311.
- Woodward, V.W., 1962, Complementation and recombination among pyr-3 heteroalleles of Neurospora crassa: Proc. Natl. Acad. Sci., U.S., 48: 348-356.
- _____, 1963, Ornithine transcarbamylaseless mutants of Neurospora (abstr.): Proc. 11th Intern. Cong. Genetics 1: 51.
- Woodward, V.W. and R.H. Davis, 1963, Co-ordinate changes in complementation, suppression and enzyme phenotypes of a pyr-3 mutant of Neurospora crassa: Heredity 18: 21-25.
- Woodward, V.W. and P. Schwarz, 1964, Neurospora mutants lacking ornithine transcarbamylase: Genetics 49: 845-853.

TABLE 1

pH Optima: Reaction Rate Given as $(v_o/V_{\text{optimum}}) \times 100$

pH	ATC 74A		ATC KS-10		ATC KS-16	
	Reaction	Blank	Reaction	Blank	Reaction	Blank
7.4	-	-	-	1	6	2
7.6	10	1	-	1	18	4
7.8	26	1	12	1	30	5
8.0	40	1	33	1	48	6
8.2	66	1	61	1	73	7
8.4	88	1	85	1	92	8
8.5	93	1	93	1	96	9
8.6	99	3	99	3	100	11
8.7	100	4	100	4	99	14
8.8	94	10	97	9	93	17
9.0	71	17	72	15	73	19
9.2	20	20	19	19	30	21

pH	ATC KS-123		OTC 74A		pyr-3b	
	Reaction	Blank	Reaction	Blank	Reaction	Blank
7.4	7	6	-	-	-	-
7.6	14	7	11	4	16	10
7.8	26	10	36	5	36	11
8.0	42	12	61	5	53	13
8.2	58	15	90	6	71	14
8.4	75	17	98	7	85	16
8.5	83	17	100	7	92	17
8.6	93	18	97	8	97	17
8.7	97	18	89	8	100	18
8.8	100	19	66	9	96	19
9.0	78	20	37	11	73	20
9.2	40	21	11	11	-	-

TABLE 2

Initial Velocity (v_o) as a Function of Aspartate Concentration for ATC

74A Blank: 7.5 V_{max} : 47.5			KS-10 Blank: 0.0 V_{max} : 125.0		
Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$	Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$
0.0355	80.9	-	0.0325	79.6	-
0.0218	95.0	-	0.0196	97.6	-
0.0164	97.8	-	0.0178	99.2	-
0.0136	100.0	-	0.0157	100.0	-
0.0086	97.3	40.6	0.0111	96.0	24.0
0.00480	82.7	5.58	0.00824	89.6	8.62
			0.00552	76.0	3.17
0.00300	51.8	1.50	0.00460	66.4	1.98
0.00212	34.5	0.735	0.00244	42.6	0.739
0.000889	13.6	0.345	0.001000	20.0	0.250
0.000435	6.36	0.214	0.000513	12.0	0.135

KS-16 Blank: 3.0 V_{max} : 84.5			KS-123 Blank: 5.5 V_{max} : 42.0		
Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$	Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$
0.0357	87.6	-	0.0344	85.0	-
0.0204	100	-	0.0276	93.0	-
			0.0256	96.4	-
0.0151	100	-	0.0192	98.4	-
0.0138	99.4	168.0	0.0184	100	-
0.0122	99.4	168.0	0.0101	100	-
0.0086	86.4	6.35	0.00667	88.0	5.77
0.0060	69.8	2.36	0.00472	77.4	3.42
0.00480	56.8	1.32	0.00350	67.8	2.11
0.00400	50.9	1.05	0.00250	53.6	1.15
0.00300	40.2	0.673	0.00150	32.1	0.474
0.00212	31.6	0.462	0.00104	17.8	0.217
0.000876	13.6	0.158	0.00053	3.60	0.037
0.000443	6.50	0.0667			

Table 2 (Continued)

<u>pyr-3a</u> Blank: 9.5 V_{max} : 64.5			<u>pyr-3b</u> Blank: 7.0 V_{max} : 61.5		
Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$	Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$
0.03072	79.1	-	0.0248	92.7	-
0.0241	89.1	-	0.0173	99.2	-
0.0176	100	-	0.0121	100	-
0.0143	100	-	0.00890	97.8	40.0
0.00893	89.1	8.21	0.00600	89.4	8.46
0.00800	86.8	6.59	0.00422	79.7	3.92
0.00598	76.7	3.30	0.00222	47.2	0.892
0.00419	60.5	1.53	0.00092	27.6	0.382
0.00300	48.1	0.925	0.00047	1.6	0.016
0.00222	36.4	0.573			
0.00092	13.2	0.152			
0.00050	6.2	0.066			

<u>pyr-3b</u> Blank: 16.0 V_{max} : 103.0 37°C			<u>pyr-3c</u> Blank: 9.5 V_{max} : 141.5		
Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$	Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$
0.0250	100	-	0.0342	83.0	-
0.173	100	-	0.0261	85.9	-
0.122	99.0	102.0	0.0177	91.5	-
0.0910	87.4	7.24	0.0124	100	-
0.00604	72.8	2.68	0.00911	95.8	22.58
0.00424	49.0	0.962	0.00605	72.4	2.63
0.00222	23.3	0.304	0.00429	51.6	1.07
0.00092	9.7	0.108	0.00223	30.0	0.429
0.00047	5.8	0.062	0.00092	10.7	0.120
			0.00046	3.0	0.031

Table 2 (Continued)

b ₂ C ₇ Blank: 15.5 V _{max} :24.5			74A 37°C Blank:24.0 V _{max} :198.0		
Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$	Molarity Aspartate	v_o/V_m X100	$v_o/(V_m-v_o)$
0.0333	100	-	0.0304	100	-
0.0250	100	-	0.0240	100	-
0.0163	100	-	0.0190	100	-
0.0127	100	-	0.0089	89.9	8.90
0.00901	83.7	5.12	0.0060	72.7	2.67
0.00600	71.4	2.50	0.0042	54.5	1.20
0.00417	51.0	1.04	0.0022	31.3	0.456
0.00351	38.8	0.630	0.00091	12.6	0.144
0.00200	22.4	0.289	0.00045	0.7	0.008
0.00091	11.4	0.129			
0.00047	4.1	0.043			

TABLE 3

 $v_o/(V_m - v_o)$ for 74A ATC of Two Preparations and Two Concentrations

Ammonium Sulfate Fractionation		Entire Purification Procedure		
		0.094 Units ATC	0.180 Units ATC	
S Molarity Aspartate	$v_o/(V_m - v_o)$	S Molarity Aspartate	$v_o/(V_m - v_o)$	S Molarity Aspartate $v_o/(V_m - v_o)$
0.004	4.00	0.004	3.95	0.004 4.15
0.003	1.50	0.003	1.54	0.003 1.54
0.002	0.670	0.002	0.678	0.002 0.683
0.001	0.244	0.001	0.253	0.001 0.268

TABLE 4

Initial Velocity (v_o) as a Function of CAP Concentration for OTC
from 74A: v_o given in Stat Units* per 20 Minutes

0.00105 M Ornithine $V_{\max}$: 510.0			0.000535 M Ornithine $V_{\max}$: 124.0			0.0000545 M Ornithine $V_{\max}$: 34.0		
Molarity CAP	v_o	$v_o/(V_m - v_o)$	Molarity CAP	v_o	$v_o/(V_m - v_o)$	Molarity CAP	v_o	$v_o/(V_m - v_o)$
0.000415	470.5	-	0.000423	102.6	-	0.000388	22.0	-
0.000340	510.0	-	0.000346	124.0	-	0.000307	22.5	-
0.000260	492.0	27.33	0.000265	124.0	-	0.000224	31.5	-
0.000200	454.0	8.107	0.000181	118.6	21.90	0.000138	33.5	-
0.000177	410.0	4.100	0.000092	106.4	6.04	0.000047	29.5	6.56
0.000160	400.0	3.636	0.000070	92.0	2.88	0.000024	21.0	1.62
0.000140	365.0	2.517	0.000060	81.0	1.88	0.000016	16.0	0.889
0.000120	328.0	1.802	0.000047	61.6	0.987	0.000010	11.5	0.511
0.000090	268.2	1.110	0.000030	53.0	0.746			
0.000046	140.0	0.378	0.000020	37.0	0.425			
0.000020	68.0	0.154	0.000010	18.0	0.170			
0.000010	35.0	0.074						

* One Stat unit is equivalent to 0.01895 μ -mole hydrogen ions released by the reaction.

TABLE 5

Initial Velocity (v_o) as a Function of Ornithine Concentration for OTC from 74A

v_o given in Stat units* per 20 minutes

0.00045 <u>M</u> CAP		Blank: 19.0 V_{max} : 370.0		
<u>M</u> orn.	$(1/\text{orn}) \times 10^{-3} \underline{M}^{-1}$	v_o	$1/v_o$	$v_o/(V_m - v_o)$
0.002510	0.399	227.0	0.0044	1.32
0.001550	0.645	193.0	0.0052	1.09
0.001050	0.953	179.0	0.0056	0.94
0.000532	1.88	109.0	0.0092	0.418
0.000427	2.34	91.5	0.0109	0.329
0.000320	3.12	69.0	0.0145	0.230
0.000215	4.65	51.0	0.0196	0.160
0.000108	9.30	27.0	0.0370	0.079
0.000054	18.50	18.5	0.0540	0.051

0.00024 <u>M</u> CAP		Blank: 9.0 V_{max} : 120.5		
<u>M</u> orn.	$(1/\text{orn}) \times 10^{-3} \underline{M}^{-1}$	v_o	$1/v_o$	$v_o/(V_m - v_o)$
0.001110	0.9	95.5	0.0105	3.82
0.000837	1.19	89.0	0.0112	2.82
0.000562	1.78	79.0	0.0127	1.90
0.000450	2.22	74.0	0.0135	1.59
0.000340	2.94	66.0	0.0152	1.21
0.000227	4.41	51.5	0.0194	0.746
0.000114	8.78	34.0	0.0294	0.393

0.00012 <u>M</u> CAP		Blank: 9.6 V_{max} : 114.3		
<u>M</u> orn.	$(1/\text{orn}) \times 10^{-3} \underline{M}^{-1}$	v_o	$1/v_o$	$v_o/(V_m - v_o)$
0.001117	0.895	68.0	0.0147	1.47
0.000908	1.101	75.4	0.0133	1.94
0.000686	1.458	68.9	0.0145	1.52
0.000461	2.167	39.4	0.0168	1.08
0.000348	2.877	30.4	0.0198	0.789
0.000233	4.296	40.4	0.0248	0.547
0.000117	8.547	24.4	0.0410	0.271

* One Stat unit is equivalent to 0.01895 μ -mole hydrogen ions released by the reaction.

FIGURE 1. pH OPTIMA of ATC and OTC (horizontal coordinate pH units)

FIGURE 2. AFFINITY of ATC for CAP

FIGURE 3. INITIAL VELOCITY versus CAP MOLARITY for ATC

FIGURE 4. ATC at VARIOUS ASPARTATE CONCENTRATIONS
 2.0×10^{-4} M CAP
 Reaction begun by addition of CAP*

74 A

g

FIGURE 4 b. KS-10

FIGURE 4c. KS-16

FIGURE 4d. KS-123

FIGURE 4 e. Pyr-3 a

FIGURE 4f. Pyr-3b 30°C

FIGURE 4g. Pyr-3b 37°C

FIGURE 4 h. Pyr-3c

FIGURE 41. $b_2 C_7$

FIGURE 4 j. 74 A 37°C

FIGURE 5a. INITIAL VELOCITY versus ASPARTATE for ATC
(horizontal coordinate molarity asp.)

FIGURE 5b.

FIGURE 5c.

FIGURE 6a. ORDERS of ATC REACTIONS

FIGURE 6b.

FIGURE 6c.

FIGURE 7. AFFINITY of OTC for CAP

FIGURE 8. INITIAL VELOCITY versus CAP MOLARITY for OTC

FIGURE 9. INITIAL VELOCITY versus ORNITHINE MOLARITY for OTC

FIGURE 10. OTC at VARIOUS CAP CONCENTRATIONS
 5.0×10^{-4} M Ornithine
 Reaction begun by addition of CAP*

FIGURE II. OTC at VARIOUS ORNITHINE CONCENTRATIONS
 $2.0 \times 10^{-4} M$ CAP
 Reaction begun by addition of CAP *

FIGURE 12. ORDERS of OTC REACTIONS

FIGURE 13. DETERMINATION of V_m for OTC

FIGURE 14 ORDER of OTC REACTIONS

APPENDIX 1

Colorimetric assays for ATC and OTC

Reference: Archibald, R.M. (1944), J. Biol. Chem. 156: 121-142.

Koritz, S.B. and P.P. Cohen (1954), J. Biol. Chem. 209: 145-150.

Reagents: 0.05 M Tris-HCl buffer pH 8.5
50% concentrated sulfuric acid
Diacetylmonoxime 11.25 gm. per 500 ml. H₂O
Sodium diphenylamine-p-sulfonate 0.57 gm. per 500 ml. 0.1 N HCl
Potassium persulfate 1.25 gm. per 500 ml. H₂O

L-Aspartic acid standard solution
aspartic acid is dried over phosphorus pentoxide in a vacuum desicator. 0.6655 gm. is dissolved in 50 ml. 0.10 M NaOH and diluted to 100 ml. This provides neutralized 0.05 M aspartate.

L-Ornithine standard solution
ornithine is dried over phosphorus pentoxide in a vacuum desicator. 0.0844 gm. is dissolved in 2.5 ml. 0.1 N NaOH and diluted to 10 ml. to provide neutralized 0.05 M ornithine.

Carbamyl phosphate
0.0204 gm. is dissolved in 25 ml. cold, 0.05 M Tris-HCl buffer pH 8.5 (MW carbamyl phosphate 162.89).

Carbamyl aspartate standard solution
Carbamyl aspartate is dried over phosphorus pentoxide in a vacuum desicator. 0.0352 gm. is dissolved in 200 ml. H₂O to give a concentration of 1 μ mole per ml.

Citrulline standard solution
citrulline is dried over phosphorus pentoxide in a vacuum desicator. 0.0175 gm. is dissolved in 100 ml. to give 1 μ mole per ml.

Apparatus: Water baths 60°C and 30°C and Spectronic-20 spectrophotometer or Klett-Summerson photoelectric colorimeter.

Appendix 1 (Continued)

Procedure: The activity of the transcarbamylase enzymes may be determined by the reaction of the carbamyl-amino acids produced with diacetylmonoxime in the presence of an aromatic amine and comparing the color produced with a standard graph of the product. The reaction volume of the transcarbamylase assay is 0.5 ml. This same volume is taken from dilutions of a standard solution of the product, i.e., carbamyl aspartate or citrulline, and treated in the same way as the reaction mixture. To the reaction mixture at the end of the reaction, and to the dilutions of the standard solution of the product, 2.5 ml. of a mixture of 50% concentrated sulfuric acid, aqueous diacetylmonoxime, and sodium diphenylamino-p-sulfonate in a ratio of 3:1:1 is added. This mixture is allowed to react for 30 minutes at 60°C and then is cooled to 30°C. To the mixture 0.5 ml. of aqueous potassium persulfate is added and allowed to develop for 20 minutes. The solutions are cooled and shielded from direct sunlight. The color intensity is determined at 550 mμ on a Spectronic-20 or determined on a Klett-Summerson colorimeter and compared to a standard graph.

The ATC reaction mixture consists of 0.05 M Tris-HCl buffer pH 8.5, 0.25 μmoles of carbamyl phosphate, 10 μmoles of aspartate, and an enzyme preparation in a

Appendix 1 (Continued)

volume of 0.5 ml. These concentrations of substrates will allow a reaction rate of 0.018 μ moles of carbamyl aspartate per minute (one unit of enzyme produces one μ mole of product per minute) for 10 minutes without either substrate becoming limiting.

The OTC reaction mixture contains 0.05 M Tris-HCl buffer pH 8.5, 0.25 μ moles of carbamyl phosphate, 0.50 μ moles of ornithine, and an enzyme preparation in a volume of 0.5 ml. Neither substrate is saturating at these concentrations.

Examples: ATC

A series of tubes containing 0.2 ml. 0.05 M aspartate, 0.15 ml. 0.05 M Tris-HCl buffer pH 8.5, and 0.10 ml. enzyme preparation containing 0.018 units of ATC was prepared. At zero time 0.05 ml. of 0.005 M CAP was added to each tube to start the reactions. The reactions were stopped at various times by the addition of 2.5 ml. of the acid, diacetyl, aromatic amine mixture. The same amount of this mixture was also added to a series of tubes containing 0.5 ml. of the various dilutions of the carbamyl aspartate standard solution. The resulting mixtures were allowed to react at 60°C for 30 minutes, cooled, and allowed to equilibrate at 30°C before the addition of 0.5 ml. of the aqueous potassium persulfate solution. After 20 minutes the color intensity

Appendix 1 (Continued)

was measured on the Klett-Summerson photoelectric colorimeter. The results are tabulated below with the color intensity given in Klett units. It may be observed that the initial rate was 0.018 μ moles of carbamyl aspartate per minute. One unit of enzyme is that concentration which releases one μ mole of product under given conditions.

ATC Reaction		Standard Solution	
Minutes from zero time	Klett Units	μ moles carbamyl aspartate/ml.	Klett Units
0	57	0.00	57
		0.01	66
2	95	0.02	75
4	115	0.04	90
6	150	0.06	106
8	211	0.08	124
10	248	0.10	137
12	266	0.20	224
14	269	0.30	296
16	270	0.40	380
18	270		

OTC

A series of tubes containing 0.01 ml. 0.05 M ornithine, 0.34 ml. 0.05 M Tris-HCl buffer pH 8.5, and 0.10 ml. enzyme preparation containing approximately 0.02 units of OTC, i.e., that concentration which will release 0.02 μ moles of citrulline per minute under these conditions. The reaction is started with the

Appendix 1 (Continued)

addition of 0.05 ml. of 0.005 M CAP. The reactions were stopped at various times as in the ATC assay and a series of dilutions of the standard citrulline solution prepared. The results are tabulated below with the color intensity given in Klett units. It may be observed that the initial rate was 0.0262 μ moles citrulline per minute.

OTC Reaction		Standard Solution	
Minutes from zero time	Klett Units	μ moles citrul- line/ml.	Klett Units
0	45	0.00	45
2	189	0.02	57
4	299	0.04	69
6	380	0.06	81
8	443	0.08	93
12	510	0.10	107
16	530	0.20	155
20	540	0.40	310
24	540	0.60	395
		0.80	540
		1.00	670

APPENDIX 2

Assays for ATC, OTC, and CAP made with the Sargent pH Stat

Reagents: 0.005 M Sulfamic acid primary standard solution.
Sulfamic acid is dried at 100°C for 24 hours before weighing.

0.01 M NaOH standardized against sulfamic acid.

0.05 M Aspartate standard solution.
Aspartic acid is dried over phosphorus pentoxide in a vacuum desiccator. 0.665 gm. is dissolved in 50 ml. 0.10 M NaOH and diluted to 100 ml.

0.05 M Ornithine standard solution.
Ornithine is dried over phosphorus pentoxide in a vacuum desiccator. 0.0844 gm. is dissolved in 2.5 ml. 0.1 M NaOH and diluted to 10 ml.

0.005 M Carbamyl phosphate.
0.0204 gm. is dissolved in 25 ml. cold HOH and is kept in an ice bath. Good for 12 hours.

H₂O boiled and capped to exclude CO₂ from atmosphere.

Protein solution dialyzed by passing through G-75 Sephadex which has been equilibrated in deionized H₂O adjusted to pH 8.5 with NaOH.

Apparatus: Sargent pH Stat equipped with the following:

2.5 ml. barrel and plunger,
10 ml. container for reaction covered with stopper with holes for electrodes, thermocouple, thermometer, NaOH delivery tube, nitrogen delivery tube, and reaction delivery syringe.

Nitrogen cylinder with gauge and pre-purified nitrogen. Nitrogen is passed through 0.5 M NaOH (400 ml.) to remove CO₂ before being introduced into reaction container.

Procedure: The Sargent pH Stat is calibrated against standard pH 8.0 buffer at 30°C. The buffer is thoroughly washed

Appendix 2 (Continued)

out of the reaction vessel and nitrogen is started flowing through the vessel. The barrel is filled with 0.01 M NaOH. Water, enzyme, and aspartate (or ornithine) are introduced into the reaction vessel and the mixture titrated to pH 8.5. All volumes must be accurately recorded to calculate the concentrations of the substrates in the reaction mixture. The reaction is begun with the addition of CAP. Sensitivity of the assay can be increased by setting the pH Stat to record five times the volume of NaOH delivered. At the end of the assay an aliquot of standard 0.005 M sulfamic acid is titrated under the conditions of the assay to standardize the NaOH. From this titration the number of umoles of hydrogen ions released in the assay can be calculated. CAP can be assayed by letting the reaction go to completion, i.e., allow all CAP to be converted to a carbamyl amino acid, and determine the total number of umoles of hydrogen ions released.

Examples: ATC

Into the reaction vessel were introduced 2.00 ml. 0.05 M aspartate, 0.180 unit of enzyme, H₂O and 0.01 M NaOH to a final volume of 7.15 ml. and a final pH of 8.5. To start the reaction 0.50 ml. of 0.00425 M CAP was added to the reaction mixture. At the end of the reaction the

Appendix 2 (Continued)

volume was 7.85 ml. It was then determined that the NaOH was 0.00947 M when titrated against standard sulfamic acid under the conditions of the assay. As determined on the pH Stat the reaction required 0.1945 ml. 0.00947 M NaOH per 10 minutes initially to remain at pH 8.5, i.e., the initial velocity of the total reaction was 0.1843 μ mole hydrogen ions produced per minute. However, a blank consisting of the reaction mixture lacking the aspartate showed a CAP hydrolysis of 0.0057 μ mole hydrogen ions produced per minute giving an initial velocity of 0.1786 μ mole per minute. An identical reaction was run in the pH Stat and 0.05 ml. aliquots were taken out and developed by the colorimetric assay. The initial velocity was 0.180 μ moles carbamyl aspartate per minute. This close agreement between assays indicates that the pH Stat is a valid indication of the activity.

OTC

Into the reaction vessel were introduced 0.05 ml. 0.05 M ornithine, 0.160 unit of enzyme, H₂O and 0.01 M NaOH to a final volume of 6.35 ml. and a final pH of 8.5. To start the reaction 0.50 ml. of 0.00425 M CAP was added to the reaction mixture. The initial velocity determined on the pH Stat was 0.161 μ mole per minute

Appendix 2 (Continued)

less 0.012 μ moles per minute for the blank giving
0.159 μ mole citrulline per minute. The same reaction
with aliquots taken out at timed intervals and assayed
with the colorimetric method gave an initial velocity of
0.158 μ mole citrulline per minute.

APPENDIX 3

The Synthesis of Carbamyl Phosphate

Reference: Spector, L., M.E. Jones, and F. Lipmann (1957),
Methods in Enzymology III. Sidney P. Colowick and
Nathan O. Kaplan, editors: Academic Press Inc., Pub.,
New York, 1957, p. 653.

Reagents: Lithium hydroxide
 KH_2PO_4
 KCNO
 60% perchloric acid
 absolute ethanol

Apparatus: Fisher Freeze Dryer
 Vacuum desicator

Procedure: Carbamyl phosphate is formed through the equilibrium reaction of KH_2PO_4 with KCNO . Eight grams of KCNO and 13.6 grams of KH_2PO_4 are combined in a reaction volume of 100 ml. of H_2O at 30°C . After 30 minutes the solution is cooled to 4°C . The remainder of the steps are carried out at 4°C . A solution of lithium perchlorate is made by adding 7.2 grams of lithium hydroxide to 21.7 ml. of 60% perchloric acid, diluting to 100 ml. and cooling to 4°C . This solution is added to the crude potassium carbamyl phosphate to remove the potassium and inorganic phosphate as the perchlorate and as the lithium salt, respectively. The mixture is stirred for 2 hours at 4°C and filtered in a Büchner funnel. Seventy ml. of cold, absolute ethanol is added, dropwise, to the stirred filtrate. The precipitate is removed by filtration and discarded. To the filtrate

Appendix 3 (Continued)

55 ml. of cold absolute ethanol is added and the precipitate is collected and dried in the Fisher Freeze Dryer.

The dilithium salt of carbamyl phosphate is stable if stored in a desicator over phosphorus pentoxide. In solution the stability of carbamyl phosphate varies with pH and temperature. In acid solutions inorganic phosphate is released from carbamyl phosphate by hydrolysis. In neutral or basic solutions the predominant reaction is a splitting of carbamyl phosphate to inorganic phosphate and cyanate.

APPENDIX 4

Colorimetric Assay for Carbamyl Phosphate

Reference: Fiske, C.H. and Y. SubbaRow (1925), J. Biol. Chem. 66: 375.

Reagents: Standard phosphate solution
 KH_2PO_4 0.0136 gm. per 10 cc H_2O

10 N Sulfuric acid
450 ml. concentrated sulfuric acid
1300 ml. H_2O

15% Sodium bisulfite
30 gram sodium bisulfite
200 ml. H_2O

20% Sodium sulfite
20 gram anhydrous sodium sulfite
100 ml. H_2O

Molybdate solution
25 gm. ammonium molybdate
200 ml. H_2O
500 ml. 10 N sulfuric acid

dissolve the ammonium molybdate in acid and dilute to final total volume of 1000 ml.

Aminonaphtholsulfonic acid solution
0.5 grams 1,2,4-aminonaphtholsulfonic acid

195 ml. 15% sodium bisulfite
5 ml. 20% sodium sulfite

store at 4°C -- good for 4 weeks

Procedure: Carbamyl phosphate is for practical purposes

stable on short exposure to the conditions of the Fiske-SubbaRow colorimetric phosphate assay and may be measured by differential phosphate analysis. A solution of carbamyl phosphate containing approximately 10 μ moles

Appendix 4 (Continued)

per ml. is kept at 0°C. One ml. is delivered to each of two 100 ml. volumetric flasks containing 50 ml. of H₂O. To one flask 10 ml. of the molybdate solution are added and mixed thoroughly followed in 30 seconds by the addition of 4 ml. of the aminonaphtholsulfonic acid solution. The mixture is diluted to 100 ml. with water and read on a Klett-Summerson colorimeter after 5 minutes. The other flask is heated in a boiling water bath for 5 minutes and cooled to room temperature before assaying as described above. Inorganic phosphate present in the reaction mixture is converted to phosphomolybdic acid which is reduced by 1,2,4-aminonaphtholsulfonic acid to produce a blue color the intensity of which is proportional to the amount of phosphate present. The difference in the color of the heated solution and the color of the unheated solution is compared to the color given by a standard phosphate curve.

Example:

μmoles phosphate	colorimeter reading Klett units
0.0	1
2.5	29
5.0	63
7.5	94
10.0	121

Appendix 4 (Continued)

Ten μ moles of the dilithium salt of carbamyl phosphate (MW 162.89) gave a reading of 14 when it had not been previously heated and 116 when it had been heated. This indicated the carbamyl phosphate is $(116-14)/(121-1) = 102/120$ or 85.0% pure.

APPENDIX 5

Growth, Harvesting, and Drying of Neurospora crassa

Reference: Vogel, H.J. (1956), Microbiol. Genetics Bulletin
13: 42-43.

Reagents:

Trace element solution

Chemical	Grams per 100 ml.
Citric acid monohydrate	5.00
ZnSO ₄ · 7H ₂ O	5.00
Fe(NH ₄) ₂ (SO ₄) ₂ · 6H ₂ O	1.00
CuSO ₄ · 5H ₂ O	0.25
MnSO ₄ · H ₂ O	0.03
H ₃ BO ₃ (anhydrous)	0.05
Na ₂ MoO ₄ · 2H ₂ O	0.05

The chemicals are dissolved in 75 ml. H₂O and diluted to 100 ml. Chloroform may be used as a preservative.

Biotin solution

5.0 mg. biotin are dissolved in 50 ml. H₂O and stored frozen.

Minimal liquid medium

Chemicals	Amount per 1000 ml.
Sucrose	10.0 gm.
Sodium citrate · 5½H ₂ O	3.0 gm.
KH ₂ PO ₄ (anhydrous)	5.0 gm.
NH ₄ NO ₃ (anhydrous)	2.0 gm.
MgSO ₄ · 7H ₂ O	0.2 gm.
CaCl ₂ · 2H ₂ O	0.1 gm.
Trace element solution	0.10 ml.
Biotin solution	0.05 ml.

Apparatus: Vat with 320 liter capacity and stirring rotor
20 liter pyrex carboy
compressed air pump with sterile filter
International centrifuge model CM with continuous
flow attachment
Fisher Freeze Dryer
Wiley Mill

Appendix 5 (Continued)

Procedure: Wild-type *Neurospora* is grown in minimal medium in a 320-liter vat inoculated with a 16-liter suspension of conidial growth, grown for 24 hours in a serrated, sterile carboy. For pyrimidine requiring strains the medium is supplemented with 10 mg.% uridine. The vat culture is allowed to grow for 24 hours and then harvested with an International centrifuge, model CM, using a continuous flow attachment. The mycelia are washed with deionized water, spun dry, and lyophilized in a Fisher Freeze Dryer for 36 hours. The dried mycelia are then ground in a Wiley Mill with a 40-mesh screen. Five hundred grams of powder may be obtained in this way and stored dry at -20°C.

APPENDIX 6

Partial Purification of ATC and OTC

Reagents: Tris-HCl buffer 0.05 M pH 8.5 and 0.05 M, 0.10 M, 0.50 M pH 7.5. 121.14 grams of tris-(hydroxymethyl)-aminomethane (MW 121.14) is dissolved in 800 ml. H₂O. Concentrated HCl is added until the desired pH is reached. The solution is then diluted to 1000 ml. with H₂O. This makes a stock solution of 1.0 M Tris-HCl buffer and may be diluted to the desired molarity as needed.

Streptomycin sulfate, U.S.P. grade from Eli Lilly and Co., Indianapolis, Indiana.

Enzyme grade ammonium sulfate

G-75 Sephadex from Pharmacia Fine Chemical, Inc., Upsala, Sweden

H₂O adjusted to pH 8.5 with NaOH

DEAE-cellulose washed and dried as follows:

100 grams of dry DEAE-cellulose is suspended in 1000 ml. 1.0 M NaOH and stirred for one hour. The suspension is filtered through glass wool in a Büchner funnel. The DEAE is resuspended in 1000 ml. H₂O and filtered three times followed by resuspension in 1.0 N HCl. The suspension is stirred for one hour, filtered, washed and filtered as before. The DEAE is resuspended in 1.0 M NaOH and stirred five hours before filtering, washing and filtering as before. Three additional washes are added with a final wash in 95% ethanol. The DEAE cellulose is then placed in a Fisher Freeze Dryer, and dried for 36 hours before storing.

Apparatus: International centrifuge, model BD
145 ml. glass columns with a diameter to height ratio of 1:10, closed at the bottom with a sintered glass plate
Buchler fraction collector
Mixing device for producing linear concentration gradients consisting of two connected bottles of equal volumes, one containing a stirrer and a second outlet
Sargent pH Stat

Appendix 6 (Continued)

Procedure: Preparation of columns

A column of G-75 Sephadex is packed by suspending the sephadex in water adjusted to pH 8.5 with NaOH. A polyethylene bottle containing the suspension is inverted into a funnel fitted to a column which has been clamped and filled with water at pH 8.5. After three centimeters of sephadex has packed at the bottom of the column the clamp is released and the rest of the column is packed under the influence of the flow through the column.

A column of DEAE-cellulose is prepared by suspending 10.0 grams of the washed and dried material in 1000 ml. 0.05 M Tris-HCl buffer pH 7.5. The column is packed in the same manner as the sephadex column except that the column is filled with 0.05 M Tris-HCl buffer pH 7.5.

Preparation of the ATC extract:

Twenty-five grams of dry Neurospora powder are suspended in 1000 ml. of 0.05 M Tris-HCl buffer pH 8.5 and extracted for one hour at 4°C. All subsequent steps are carried out at 4°C unless otherwise noted. The suspension is centrifuged for 30 minutes at 10,000 rpm in the International centrifuge, model BD. The pellet is discarded and 2.25 grams of Streptomycin sulfate are added to the supernatant to precipitate the

Appendix 6 (Continued)

nucleoproteins. The streptomycin solution is stirred for one hour and centrifuged as above. The pellet is discarded.

The supernatant is brought to a 15% saturation point of ammonium sulfate with dry, enzyme grade, ammonium sulfate. The ammonium sulfate solution is stirred for an hour and centrifuged as before. The saturation point of the supernatant is increased in steps of 5% saturation and centrifuged as before until a saturation point of 35% is reached. The pellets are suspended in 100 ml. of 0.05 M Tris-HCl buffer pH 8.5. Eighty percent of the activity is found in the 25% saturation pellet.

The suspension of the 25% saturation pellet is recentrifuged and the supernatant applied to the G-75 sephadex column in 30 ml. volumes. The protein is washed through the column with water adjusted to pH 8.5 with NaOH. The eluate is collected in a Buchler fraction collector, 4.4 ml. per tube. The eluate pattern is shown in the attached figure, illustrating the complete dialysis of the protein.

This protein solution is fractionated on the Sargent pH Stat with 0.5 M acetic acid at 0.2 pH units. The activity is recovered from the pH 5.6 precipitate and is resuspended in 0.05 M Tris-HCl buffer pH 7.5.

Appendix 6 (Continued)

Ten mg. of protein is applied to a 10-gram DEAE-cellulose column equilibrated at pH 7.5 with 0.05 M Tris-HCl buffer. The gradient device is attached to the column and a gradient between 0.1 and 0.5 M Tris-HCl buffer pH 7.5 brings through a peak of activity which is collected in a Buchler fraction collector with 4.4 ml. per tube. ATC is not adsorbed on DEAE-cellulose in 0.10 M at pH 7.0, in 0.25 M at pH 7.5, in 0.50 at pH 8.0 and above 1.00 M at pH 8.5.

The greatest specific activity obtained using this method of purification was 161 μ moles carbamyl-aspartate per mg. protein per hour as compared to 0.2 μ moles carbamyl aspartate per mg. protein per hour for the crude extract. Starch gel electrophoresis of this protein solution gave a single band of protein as compared to one major band and six minor bands on polyacrylamide disc electrophoresis.

It was found that the fractionation procedure up to the acetic acid precipitation removed all other enzymes which utilize aspartate and carbamyl phosphate and this partial fractionation was used to prepare extracts for the kinetic examination of ATC.

Preparation of the OTC extract:

Twenty-five grams of dry Neurospora powder are

Appendix 6 (Continued)

suspended in one liter of 0.05 M Tris-HCl buffer pH 8.5 and extracted for one hour at 4°C. The suspension is centrifuged for 30 minutes at 10,000 rpm in the International centrifuge, model BD. The pellet is discarded and 2.25 grams of streptomycin sulfate are added to the supernatant to precipitate the nucleoproteins. The streptomycin solution is stirred for one hour and centrifuged as above. The pellet is discarded.

The supernatant is brought to a 40% saturation point of ammonium sulfate with dry, enzyme grade, ammonium sulfate. The ammonium sulfate solution is stirred for an hour and centrifuged as before. The saturation point of the supernatant is increased in steps of 5% and centrifuged as before until a saturation point of 65% is reached. The pellets are resuspended in 100 ml. of 0.05 M Tris-HCl buffer pH 8.5. Seventy-five percent of the activity is found in the 55% saturation pellet.

The suspension of the 55% saturation pellet is applied to the G-75 sephadex column in 30 ml. volumes. The protein is washed through the column with water adjusted to pH 8.5 with NaOH. The eluate is collected in a Buchler fraction collector, 4.4 ml. per tube. The

Appendix 6 (Continued)

eluate pattern is shown in the attached figure illustrating the complete dialysis of the protein.

This protein solution is fractionated on the Sargent pH Stat with 0.5 M acetic acid at 0.2 pH units. The activity is recovered from the pH 4.0 supernatant and is resuspended in 0.05 M Tris-HCl buffer pH 8.5.

Inactive proteins are precipitated by heating the protein solution for 90 minutes at 60°C followed by centrifugation. No activity is lost in this step.

The protein solution is cooled to 5°C. At this temperature the pH of the buffer becomes 7.5. Sufficient absolute ethanol previously cooled to -20°C was added to make the solution 40% alcohol. The suspension is centrifuged for 30 minutes at 10,000 rpm in the International centrifuge, model BD. The pellet is discarded and the supernatant is increased in alcohol content in 10% steps and centrifuged as before. The pellets are taken up in 0.05 M Tris-HCl buffer at pH 8.5. Thirty percent of the activity is found in the 60% pellet and seventy percent of the activity is found in the 70% pellet.

DEAE-cellulose may be used to purify OTC. OTC is eluted from DEAE-cellulose in 0.20 M at pH 7.0, in 0.50

Appendix 6 (Continued)

M at pH 8.0 and above 1.00 M at pH 9.0.

It was found that the fractionation procedure up to acetic acid precipitation removed all other enzymes which utilize aspartate and reduces the enzymes hydrolyzing carbamyl phosphate to a level which did not interfere with kinetic analysis of OTC.

Elution Pattern of ATC, Protein, and Buffer from G-75 Sephadex

● protein
○ ATC
□ buffer

column volume 145 ml.
void volume 35 ml.
fraction volume 4.4 ml.

APPENDIX 7

Assay for Protein

Reference: Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951), J. Biol. Chem. 193: 235-275.

Reagents: A. 4 gm. Na_2CO_3 in 100 ml. 0.2 M NaOH.

B. 20 mg. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 100 ml. of a solution containing 40 mg. of Na or K tartrate.

C. Equal volumes of A and B.

D. Folin-Ciocalteu reagent diluted to 1.0 N acid.

Apparatus: Spectronic 20 spectrophotometer or Klett-Summerson colorimeter.

Procedure: To a sample containing 5 to 100 μgm . protein add 3 ml. of C. Shake well and let stand 15 minutes. Add 0.3 ml. of D rapidly and mix thoroughly. Let stand at least 30 minutes and determine the intensity of color formed on the Spectronic-20 at 550 $\text{m}\mu$ or on the Klett-Summerson photoelectric colorimeter. Unknown solutions are compared to the color intensity given by known concentrations of bovine serum albumin.

APPENDIX 8

Starch Gel Electrophoresis

Reagents: Hydrolyzed starch from Connaught Medical Research Laboratories, Toronto, Canada.

0.02 M Borate buffer pH 8.5
50 ml. 0.2 M boric acid (MW 61.84) is brought to pH 8.5 with 0.5 M NaOH and diluted to 500 ml.

10% solution of NaCl

Protein stain Amido-Schwarz (buffalo black) in 7% acetic acid

10% glacial acetic acid solution

Apparatus: Glass mold for gel slab 9.5 x 30.5 x 0.5 cm. with opening for slot mold 3 cm. from end.

Buchler power supply.

A pair of two-compartment electrodes.

Four wicks consisting of three thicknesses of Whatman #3 filter paper 9 x 28 cm.

Procedure: Hydrolyzed starch is weighed into a vacuum flask containing buffer (10.7 gm. starch to 100 ml. buffer -- 300 ml. required to fill mold). The suspension is shaken in a boiling water bath for thirty minutes. At the end of this time the solution is fluid and clear with bubbles suspended in it. The solution is allowed to cool slightly and a vacuum applied with an aspirator to remove the air bubbles. If the solution boils under reduced pressure it should be allowed to cool longer. The solution is then quickly poured into the prepared mold and the top placed on in a manner to exclude air bubbles. The slot mold is dropped through the opening

Appendix 8 (Continued)

in the top glass plate and the gel is allowed to set for five hours. The slot mold is removed and the sample is inserted. The end glass plates are removed and the wicks are moistened in 0.02 Borate buffer pH 8.5 and connected from the ends of the gel to the second chamber of the electrodes which contains the same buffer. The two chambers of each electrode are connected using wicks moistened with buffer. The first chamber of each electrode, i.e., the chamber surrounding the metal connections, is filled with 10% NaCl solution. The voltage is turned on and the current adjusted to not more than 20 ma. During a typical run of four hours the current will vary from 17 ma to 50 ma at a constant voltage of 700 volts. At the end of this time the starch at the cathode may be partly hydrolyzed and must be cut off before fixing and staining. When the run is completed the voltage is turned off and the side and top glass plates are removed and the gel is sliced lengthwise to one-half thickness. The top half is discarded and the bottom half is fixed and stained with a solution of Amido-Schwarz in 7% acetic acid. The gel is destained in 10% acetic acid for twenty-four hours leaving blue bands of protein against the white starch. If the starch retains the blue color the Amido-Schwarz concentration in the fixing staining solution should be decreased.

APPENDIX 9

Polyacrylamide Disc Electrophoresis

Reagents: Buffer from Canalgo, Bethesda, Maryland, diluted
1:100

Lower gel A and lower gel B from Canalgo

Upper gel mixture from Canalgo

Amido-Schwarz protein stain in 7% acetic acid

14% glacial acetic acid

Apparatus: Upper and lower electrodes with buffer reservoirs
connected by $\frac{1}{8}$ -inch glass tubing containing the
gels and sample.

Buchler power supply.

Procedure: The gels and sample are prepared by mounting 63
mm. glass tubes with 5.0 mm. inner diameter perpendicu-
larly and filling from the bottom with 0.7 ml. of lower
gel composed of lower gel A, lower gel B, and aqueous
ammonium persulfate (0.04 gm. in 100 ml. H₂O) in ratio
of 1:1:2, respectively. A small amount of H₂O is
layered on top to allow the gel to set with an even
surface. The lower gel is allowed to set under the
influence of a fluorescent lamp for one hour. At the
end of this time the water is removed and 0.2 ml. of
the upper gel composed of the upper gel mixture and
H₂O in a ratio of 1:1 is added. The upper gel is
allowed to set for 30 minutes before the sample con-
sisting of 200 gamma of protein in H₂O mixed with upper

Appendix 9 (Continued)

gel mixture in a ratio of 1:1 is added to fill the tube completely. After the sample gel has set the tubes are mounted through the rubber grommets in the bottom of the upper buffer reservoir. The lower ends are placed in the lower buffer reservoir and care is taken to prevent air from being trapped in the ends of the tubes insuring equal electrical contact. After the bottom of the upper buffer reservoir has been sealed by the insertion of the gel-filled tubes, buffer is introduced and the current is turned on. The current should not exceed 5.0 ma per tube. During a typical run ten tubes are exposed to a voltage drop of 50 volts allowing a current of 20 ma. At the end of one hour a tracer dye, brom phenol blue, has passed through the entire gel and the current is turned off. The gels are removed from the tubes by submerging in water and passing a long sharp needle gently between the gel and the glass tubing. After the gel has been released from the tube wall it may be withdrawn with the needle. The gels are then stained with Amido-Schwarz in 7% acetic acid. The gels may be destained by washing in 14% glacial acetic acid for 36 hours or by mounting between the buffer reservoirs again and filling the upper reservoir with 14% glacial acetic acid. A voltage drop of 50 volts and current of 40 ma will destain within three hours.

APPENDIX 10

Determination of $V_{\max}$ with Reciprocal v_o and S plots

Reference: Michaelis, L. and M.L. Menten (1913), Biochem.
Z. 49: 333.
Lineweaver, H. and D. Burk (1934), J. Am. Chem.
Soc. 56: 658.

Theory: E - enzyme
S - substrate
P - product

$$\frac{d [ES]}{dt} = K_1 ([E] - [ES]) [S]$$

$$- \frac{d [ES]}{dt} = K_2 [ES] + K_3 [ES]$$

at steady state

$$K_1 ([E] - [ES]) [S] = K_2 [ES] + K_3 [ES]$$

or

$$\frac{[S] ([E] - [ES])}{[ES]} = \frac{K_2 + K_3}{K_1} = K_m$$

initially $v_o = K_3 [ES]$ = initial velocity at high
substrate concentrations

$$V_{\max} = K_3 [E] = \text{maximum velocity}$$

thus

$$1) \quad v_o = \frac{V_{\max} [S]}{K_m + [S]}$$

$$\text{at low } [S] \quad v_o = \frac{V_{\max}}{K_m} [S] \quad \text{first order reaction}$$

$$\text{at high } [S] \quad v_o = V_{\max} \quad \text{zero order reaction}$$

Appendix 10 (Continued)

To determine $V_{\max}$ rearrange equation 1)

$$2) \quad \frac{1}{v_o} = \frac{1}{V_{\max}} + \frac{K_m}{V_m} [S]$$

by plotting $\frac{1}{v_o}$ versus $\frac{1}{[S]}$ a graph results with

and intercept on the $\frac{1}{v_o}$ axis at $\frac{1}{V_{\max}}$.

APPENDIX 11

Determination of the Order of a Reaction

Reference: Atkinson, D.E., J.A. Hathaway, and E.C. Smith (1965), personal communication.

Theory: Order of an enzymic reaction may be defined in terms of the minimal number of substrate molecules that must be assumed to interact with each enzyme molecule to explain the observed kinetic behavior.

For an enzyme with $\underline{n}$ interacting sites

$$K_m = \frac{[E] [S]^{\underline{n}}}{[ES_{\underline{n}}]}$$

[E] = free enzyme concentration

[S] = substrate concentration

[E] = $[E_T] - [ES_{\underline{n}}]$ [ES_n] = concentration of the complex with $\underline{n}$ molecules of substrate per enzyme molecule

$$v_o = K_3 [ES_{\underline{n}}]$$

$$V_{max} = K_3 [E_T]$$

K_m = the produce of the $\underline{n}$ dissociation constants

$$K_3 [E] = V_{max} - v_o$$

[E_T] = total enzyme concentration

$$\frac{[ES_{\underline{n}}]}{[E]} = \frac{v_o}{V_{max} - v_o} = \frac{[S]^{\underline{n}}}{K_m}$$

$$\log \frac{v_o}{(V_{max} - v_o)} = \underline{n} \log [S] - \log K_m$$

Thus a plot of $\log v_o / (V_{max} - v_o)$ as a function of $\log [S]$ should give a curve of slope $\underline{n}$.