I. THE KINETICS OF THE ALPHA-CHYMOTRYPSIN CATALYSED HYDROLYSIS OF ACETYL-L-TYROSINEHYDROXAMIDE

II. GENETIC FACTORS INFLUENCING THE ACTIVITY OF TRYPTOPHANE DESMOLASE IN NEUROSPORA CRASSA

Thesis by

David Swenson Hogness

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1953

ACKNOWLEDGEMENTS

I should like to express my grateful appreciation to Drs. Carl G. Niemann and Herschel K. Mitchell, under whose direction this work was done, and my sincere thanks for their insight, interest, tolerance and enthusiasm in my work. I should also like to express my gratitude to the other members of the Chemistry and Biology Divisions who have helped to make this research pleasant and profitable.

A Predoctoral Fellowship granted by the National Institutes of Health, United States Public Health Service, has been of great assistance to me while completing these studies.

ABSTRACT

From an analysis of the pH-activity curves of the systems alpha-chymotrypsin— acetyl-L-tyrosinamide and alpha-chymotrypsin— acetyl-L-tyrosinehydroxamide it has been concluded that in the latter system the active specific substrate is acetyl-L-tyrosinehydroxamide and not acetyl-L-tyrosinehydroxamate ion. At pH 7.6 and 25° C, in aqueous solutions 0.3 M with respect to the amine component of a tris-(hydroxy-methyl)-aminomethane-hydrochloric acid buffer, the kinetics of the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydroxamide have been found to be similar to those observed previously for this enzyme and specific substrates of the acylated alpha-amino acid type at pH 7.9 ± 0.1 and 25° C in aqueous solutions 0.02 M with respect to the amine component of the same buffer system. Acetyl-D-tyrosine ethyl ester, acetyl-D-tyrosinehydrazide, acetyl-D-tyrosine-hydroxamide and acetyl-D-tyrosinamide were found to be competitive inhibitors of the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydroxamide at pH 7.6 and 25° C, under the conditions previously specified, and from these and other data it was concluded that proteins, amides, esters, hydrazides and hydroxamides are hydrolysed at the same single active site on the enzyme.

Preliminary to an examination of the effect of genetic factors on tryptophane desmolase activity in Neurospora, this enzyme was extracted from a reference strain and partially purified. The kinetics

of this tryptophane desmolase system was investigated and the apparent K_m values of indole, L-serine and pyridoxal phosphate determined. No tryptophane desmolase activity could be found in a tryptophaneless mutant, C83. The tryptophane requirement of this mutant and the lack of tryptophane desmolase activity were found to be associated with the same single gene. The reversion of C83 to wild type under the influence of ultraviolet irradiation was studied, and it was shown that the C83 mutant can backmutate. The back-mutant was found to contain a tryptophane desmolase system which could not be distinguished from the tryptophane desmolase from wild type on the basis of the kinetic criteria that were applied. A histidineless mutant, C84, was found to exert a control on the tryptophane desmolase activity of Neurospora. From a limited amount of evidence it was tentatively concluded that the histidine requirement and the alteration of the tryptophane desmolase activity are the effects of the same single gene in the C84 mutant.

TABLE OF CONTENTS

I. The Kinetics of the alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide

Introduction

The determination of the structure of protein molecules in aqueous solution is made difficult not only by the complexity of their structure but also by the lack of any really direct and general experimental method. While it is true that a great deal of knowledge has been gained by the analysis of the amino acid content of proteins and by x-ray diffraction studies of crystalline proteins, peptides, and amino acids, neither approach has revealed sufficient information to characterize the structure of protein molecules in terms of a sequence of their constituent amino acid residues. Nor does it seem likely that in the near future either approach can disclose the finer details of structure which must be known to account for the specificity that is characteristic of many protein molecules.

An indirect experimental approach to this problem which offers some information in the determination of protein configuration is the study of complex formation between proteins and small organic molecules, the structures of which are known. This method has been employed with considerable success in the problems of serological specificity of the immunochemists (1, 2), as well as in equilibrium dialysis studies on the binding of small organic ions by proteins (3-7).

Paralleling these developments, considerable work has been done in investigating the structural requirements of specific substrates and competitive inhibitors of enzymes in the hope of gaining information relative to the configuration of the active centers.

In an investigation of the structural requirements of specific substrates and inhibitors of an enzyme system, it is obviously desirable that the following requirements be met.

1. The enzyme should be highly purified and preferably in crystalline form.

2. The catalytic function of the enzyme should be solely dependent upon the configuration of the amino acid residues within the protein, i.e., no coenzyme or activator required.

3. The enzyme should be sufficiently stable in aqueous solution so that it is not appreciably inactivated during the time necessary for the determination of the kinetic data.

4. An accurate method of analysis for the concentration of the substrate or the products should be available.

The pancreatic protease, alpha-chymotrypsin, has all of the above qualifications. It can be prepared in a highly purified crystalline form, the activity of which is not dependent upon a coenzyme or activator (8, 9). It consists entirely of protein material (8), is extremely stable in aqueous solution (10), and the concentrations of its substrates or their products can be quantitatively determined by a variety of methods (9).

It has the further advantage of being commercially obtainable (Armour & Co.) in crystalline form and of a purity comparable with the material used by A. K. Balls and coworkers. (11)

As one might expect, a great deal of work has been done on the specificity of alpha-chymotrypsin. This work was initiated by Bergmann and coworkers (12-15), who first showed that synthetic peptides and peptide derivatives could be utilized in place of the much more complex protein substrates previously used. These qualitative studies were followed by the more quantitative work of Neurath (9) and Niemann (16-30) in which attempts were made to determine the relative affinity and activity effects of the various possible substrate structures. The work was also enlarged to include a systematic study of the structural requirements of inhibitors. While no attempt to give a detailed review of these studies will be made (see reference 9), a brief summary of some of the more important results is consistent with the purpose of this thesis.

The formula for a generalized substrate of alpha-chymotrypsin can be written as $L\text{-}R_1CONHCHR_2CO\dotfill R_3$, with the dotted line marking the position of the hydrolysable bond. The compounds of the D configuration have always been found to be effective competitive inhibitors. In general it would appear that compounds of the D configuration are more strongly bound to the active site than are the corresponding compounds of the L configuration, though the strength of this conclusion is modified by the difficulty in determining the enzyme-substrate dissociation

constant. This conclusion indicates that it is possible to find molecules of a structure very little different from substrate molecules (25) whose hydrolysis is not catalysed appreciably by the enzyme but which are nevertheless more strongly attracted to the active center of the enzyme than are the corresponding substrates — a result which would be predicted from Pauling's ideas of enzyme action (31).

The nature of the R_1CO- group may be varied considerably. The following R_1CO- groups have been found to be active: formyl (32), acetyl (33), chloracetyl (28), trifluoracetyl (28), benzoyl (34), glycyl, carbobenzoxyglycyl (14), carbobenzoxyglutamyl (15), nicotinyl (18, 30), isonicotinyl (32), picolinyl (32), and carboethoxyl (35). The R_2 groups which are active correspond to tryptophane, tyrosine, phenylalanine, methionine, arginine (9), and leucine (36) residues, while amides, glycinamides, glycylglycinamides, esters, hydrazides, hydroxamides (9) and thioesters (36) can constitute the hydrolysable groups (R_3) of alpha-chymotrypsin substrates. Although such a definition of the specificity of alpha-chymotrypsin substrates implies that all possible combinations of the R_1, R_2, and R_3 groups listed would result in active substrates, it should be noted that only a small fraction of these combinations have been tested.

While a majority of the investigations of the above substrates and the corresponding enantiomorphic D-inhibitors have been of a simple qualitative nature, determining only whether a given compound

is or is not active as a substrate or inhibitor, a considerable amount of quantitative work has been done to determine the relative affinity and activity effects of the various R_1 and R_2 groups (9, 21-30). Similar quantitative studies on the relative effects of variations in the R_3 group have, however, been relatively few. From kinetic studies of the hydrolysis of benzoyl-L-tyrosine ethyl ester and benzoyl-L-tyrosinamide (37) and the inhibition by acetyl-D-tyrosine ethyl ester and acetyl-D-tyrosinamide (22) it is possible to say that the ester compounds are hydrolysed much faster than the amides, and that this is probably due to the following two reasons:

1. The alpha-chymotrypsin-substrate dissociation constant for the ester has a lower value than that for the amide.

2. The alpha-chymotrypsin-substrate complex for the ester breaks down into alpha-chymotrypsin and the products of the hydrolytic reaction at a greater rate than is the case for the amide.

No information of this type was available in the case of the hydrazide and the hydroxamide, and it seemed advisable that quantitative kinetic investigations of the relative effect of these two substituents be initiated. The kinetics of the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydroxamide and the inhibition of this hydrolysis by acetyl-D-tyrosinamide, acetyl-D-tyrosine ethyl ester, acetyl-D-tyrosinehydrazide and acetyl-D-tyrosinehydroxamide have therefore been investigated.

Formulation of the Kinetics of the Enzymatic Reaction

The formulation of the kinetics of enzyme action and the derivation of the resulting rate equations has been adequately covered in a number of reviews (9, 38-40), and therefore the following treatment is given only as an aid in the interpretation of the experimental results given in the next section.

If the effect of buffer ions and molecules, the hydrogen ion activity, and the temperature are excluded from consideration by carrying out all experiments with these quantities held constant, the variables determining the reaction rate can be limited to the concentrations of the following substances: the enzyme, the substrates, the products of the reaction and any inhibitor that may be added. In the case of alpha-chymotrypsin catalytic hydrolysis we may eliminate the concentration of one of the substrates, water, from this list of variables, since in aqueous solutions it remains essentially constant. If all inhibitors are considered to react in a competitive manner, and if the reaction is considered irreversible such that the only influence of the products of the reaction is that of inhibition, the reaction can be formulated in terms of hypothetical enzyme-substrate and enzyme-inhibitor complexes as follows:

(1) $$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P1_f + P2_f$$

(2) $$E_f + P1_f \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP1_f$$

(3) $$E_f + P2_f \underset{k_7}{\overset{k_6}{\rightleftharpoons}} EP2_f$$

(4) $$E_f + I_f \underset{k_9}{\overset{k_8}{\rightleftharpoons}} EI$$ where

E_f = the free enzyme

S_f = the free substrate

$P1_f$ and $P2_f$ = free hydrolysis products

I_f = added inhibitor in free form

ES = enzyme-substrate complex

EP_1 and EP_2 = enzyme-product complexes

EI = enzyme-inhibitor complex.

Since the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosine-hydroxamide is followed by measuring the rate of disappearance of the total amount of substrate present, then the velocity measured, v, is equal to $-\frac{d\left[(ES) + (S_f)\right]}{dt}$, where the parentheses represent concentrations.

From

(5) $$-\frac{d\,(ES)}{dt} = \left[k_2 + k_3\right] \cdot (ES) - k_1 \cdot (E_f) \cdot (S_f)$$

and

$$(6) \qquad -\frac{d\,(S_f)}{dt} = k_1 \cdot (E_f) \cdot (S_f) - k_2 \cdot (ES)$$

it follows that

$$(7) \qquad v = -\frac{d\left[(ES) + (S_f)\right]}{dt} = k_3 \cdot (ES)\,.$$

An approximate solution for (ES) in terms of the total substrate concentration, (S), and total enzyme concentration, (E), — i.e., $(S) = (S_f) + (ES)$ and $(E) = (E_f) + (ES) + (EP_{1_f}) + (EP_{2_f}) + (EI)$ — is given below.

From equation (5)

$$(8) \qquad (ES) = \frac{(E_f) \cdot (S_f) - \frac{1}{k_1} \cdot \frac{d\,(ES)}{dt}}{\frac{k_2 + k_3}{k_1}}$$

and from equations (6) and (7)

$$(9) \qquad k_1 = -\frac{\left[1 + \frac{k_2}{k_3}\right] \cdot \frac{d(S_f)}{dt} + \frac{k_2}{k_3} \cdot \frac{d(ES)}{dt}}{(E_f) \cdot (S_f)}\,.$$

Thus if K_S designates the quantity $\frac{k_2 + k_3}{k_1}$, substituting k_1 of equation (9) in equation (8) gives

$$(10) \qquad (ES) = \frac{(E_f) \cdot (S_f) \left[1 + \frac{\frac{d(ES)}{dt}}{\left[1 + \frac{k_2}{k_3}\right] \cdot \frac{d(S_f)}{dt} + \frac{k_2}{k_3} \cdot \frac{d\,(ES)}{dt}}\right]}{K_S}\,.$$

With the assumption that $\frac{d(ES)}{dt} \ll \frac{d(S_f)}{dt}$ then

$$(11) \qquad (ES) \doteq \frac{(E_f) \cdot (S_f)}{K_S} = \frac{\left[(E) - (ES) - (EP_1) - (EP_2) - (EI)\right] \cdot (S_f)}{K_S}\,.$$

If it is now assumed that E_f, P_{1_f} and EP_1 are in equilibrium with dissociation constant $K_{P_1} = \frac{k_5}{k_4}$, that E_f, P_{2_f} and EP_2 are in equilibrium with dissociation constant $K_{P_2} = \frac{k_7}{k_6}$, and that E_f, I and EI are in equilibrium with dissociation constant $K_I = \frac{k_9}{k_8}$, then the following equations can be written if unit activity coefficients are assumed in all cases.

$$(12) \qquad K_{P_1} = \frac{(E_f)\cdot(P_{1_f})}{(EP_1)}$$

$$(13) \qquad K_{P_2} = \frac{(E_f)\cdot(P_{2_f})}{(EP_2)}$$

$$(14) \qquad K_I = \frac{(E_f)\cdot(I_f)}{(EI)}$$

From equations (11), (12), (13) and (14), equation (15) can be derived.

$$(15) \qquad (ES) = \frac{(E)\cdot(S_f)}{K_S\cdot\left[1 + \frac{(P_{1_f})}{K_{P_1}} + \frac{(P_{2_f})}{K_{P_2}} + \frac{(I_f)}{K_I}\right] + (S_f)}$$

From equation (7) and (15) we then have

$$(16) \qquad v = -\frac{d\left[(ES) + (S_f)\right]}{dt} = \frac{k_3(E)(S_f)}{K_S\cdot\left[1 + \frac{(P_{1_f})}{K_{P_1}} + \frac{(P_{2_f})}{K_{P_2}} + \frac{(I_f)}{K_I}\right] + (S_f)}$$

If it is now assumed that $(S_f) \gg (ES)$, $(I_f) \gg (EI)$, $(P_{1_f}) \gg (EP_1)$ and $(P_{2_f}) \gg (EP_2)$, and if $(P_1) = (P_{1_f}) + (EP_1)$, $(P_2) = (P_{2_f}) + (EP_2)$ and $(I) = (I_f) + (EI)$, then equation (16) becomes

$$(17) \qquad v = -\frac{d(S)}{dt} = \frac{k_3 \cdot (E) \cdot (S)}{K_S \cdot \left[1 + \left[(S)_o - (S)\right] \cdot \left[\frac{1}{K_{P_1}} + \frac{1}{K_{P_2}}\right] + \frac{(I)}{K_I}\right] + (S)}$$

with $(S)_o = (S)$ at time t = o. Equation (18) results if we integrate the above with respect to time.

$$(18) \quad k_3(E)\,t = K_S \cdot \left[1 + (S)_o \cdot \left[\frac{1}{K_{P_1}} + \frac{1}{K_{P_2}}\right] + \frac{(I)}{K_I}\right] \ln \frac{(S)_o}{(S)} + \left[1 - \frac{K_S}{K_{P_1}} - \frac{K_S}{K_{P_2}}\right] \cdot \left[(S)_o - (S)\right]$$

Equations (17) and (18) are the basic equations from which we may form the equations of the simpler cases in which there is no added inhibitor ((I) = o) and/or one or both of the products do not act as inhibitors ($K_{P_1} \longrightarrow \infty$ and/or $K_{P_2} \longrightarrow \infty$). Thus in the simplest case in which there is no added inhibitor and the products of the reaction do not act as inhibitors, the following equations will result:

$$(19) \qquad v = -\frac{d(S)}{dt} = \frac{k_3 \cdot (E) \cdot (S)}{K_S + (S)}$$

$$(20) \qquad k_3(E)\,t = K_S \ln \frac{(S)_o}{(S)} + (S)_o - (S).$$

At time, t = o, and with no added inhibitor, I, present, the reciprocal equation formed from equation (17) is

$$(21) \qquad \frac{1}{v_o} = \frac{K_S}{k_3(E)} \cdot \frac{1}{(S)_o} + \frac{1}{k_3(E)}.$$

Thus a plot of $\frac{1}{v_o}$ vs. $\frac{1}{(S)_o}$ should yield a straight line which determines the k_3 and K_S values (40). If the determination of K_{P_1}, K_{P_2} or K_I is

desired, they can easily be calculated from similar plots of $\frac{1}{v_o}$ vs. $\frac{1}{(S)_o}$ if a known amount of P_1, P_2 or I is present at time t = o. Thus if the determination of K_I is desired, one has simply to determine v_o at various $(S)_o$ values, in the presence of the same concentration of I. From equation (22) (derived from equation (17) for this case)

$$(22) \qquad \frac{1}{v_o} = \frac{K_S \cdot \left[1 + \frac{(I)}{K_I}\right]}{k_3 \cdot (E)} \cdot \frac{1}{(S)_o} + \frac{1}{k_3 \cdot (E)}$$

it is clear that a plot of $\frac{1}{v_o}$ vs. $\frac{1}{(S)_o}$ will yield K_I if K_S and k_3 have been determined. Similar methods involving known initial concentrations of P_2 or P_1 will yield values of K_{P_2} or K_{P_1}.

The approximation $\frac{d(ES)}{dt} \ll \frac{d(S_f)}{dt}$ requires that the initial portion of the velocity curve, during which the concentration of ES builds up from zero to its maximum or steady state value, occupy a sufficiently small time interval so as not to be observable in the extrapolation of the velocity curve to t = o for the determination of v_o. Thus v_o in the above equations is actually the velocity at the time when the approximation $\frac{d(ES)}{dt} \ll \frac{d(S_f)}{dt}$ becomes applicable, and the difference between this time and time t = o must then be so small that the amount of hydrolysis occurring in this interval is negligible, within the experimental error of the determination.

Unfortunately the determination of K_S and k_3 does not lead to a determination of the enzyme-substrate dissociation constant, $\frac{k_2}{k_1}$. It only

gives the maximum possible value of this constant. The common practice (9, 21, 41) of a comparison of K_S and k_3 values for the hydrolysis of two or more substrates by the same enzyme to give a qualitative measure of their relative affinities for the enzyme is not a valid procedure. In using this procedure it is argued that if two substrates, S_1 and S_2, of the same enzyme have K_S and k_3 values such that $(K_S)_1 < (K_S)_2$ and $(k_3)_1 > (k_3)_2$, then the corresponding enzyme-substrate dissociation constants will be in the same order as are the K_S values, i.e., $\left[\frac{k_2}{k_1}\right]_1 < \left[\frac{k_2}{k_1}\right]_2$. That this is not a valid conclusion is apparent from the fact that $\frac{k_2}{k_1} = K_S - \frac{k_3}{k_1}$ and that therefore $\frac{k_2}{k_1}$ can vary anywhere from almost zero to K_S unless some restriction is put upon the value of k_1. As the determination of K_S and k_3 only decides a minimum value of k_1 (since k_2 cannot be less than zero), it is apparent that the determination of these quantities for two substrates does not in itself determine even a qualitative relationship between their enzyme-substrate dissociation constants.

In the case of inhibition constants such as K_I and K_{P_1}, no such difficulty in interpretation is encountered. In so far as the assumptions involved in the formulation of the kinetics are approached, these constants approach the values of the true dissociation constants of the enzyme-inhibitor complexes.

Results and Discussion

While no extensive investigations have been made on the effect of pH on the alpha-chymotrypsin catalysed hydrolysis of synthetic substrates, measurement of the pH-activity curves for various substrates has indicated that the pH at which maximum activity occurs is dependent upon the nature of the substrate (22, 42, 43). Therefore, the pH-activity curve for alpha-chymotrypsin — acetyl-L-tyrosinehydroxamide has been determined in aqueous solution at 25° C. As can be seen from Fig. 1 the pH-activity curve has a rather sharp maximum at pH 7.6 which is the pH at which all subsequent experiments were carried out.

A comparison of this curve with that for the system alpha-chymotrypsin — acetyl-L-tyrosinamide (curves A and C of Fig. 1) indicates that they are identical, within the limits of experimental error, up to pH 7.6, but that at higher pH values the acetyl-L-tyrosinehydroxamide system exhibits significantly lower relative activity values than does the acetyl-L-tyrosinamide system.

Although no attempt has been made to give a quantitative explanation of the effect of pH on the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydroxamide, a possible explanation for the lower relative activity of the hydroxamide over that of the amide at higher pH values can be made. As the work of Bergmann and Fruton (12) indicates that the presence of a negative charge near the susceptible bond causes a loss of substrate activity, it could logically be assumed that the

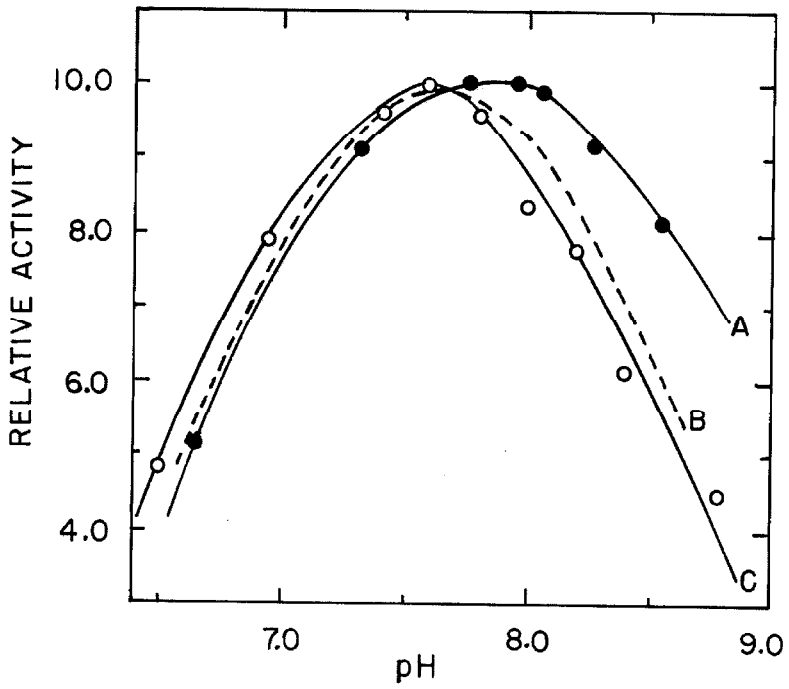


Fig. 1. pH-Activity curves for alpha-chymotrypsin.

Curve A. Acetyl-L-tyrosinamide, experimental (22).
Curve B. Acetyl-L-tyrosinehydroxamide, theoretical (see text).
Curve C. Acetyl-L-tyrosinehydroxamide, experimental (see Table II).

acetyl-L-tyrosinehydroxamate ion is inactive as a substrate for alpha-chymotrypsin. Thus, under this assumption, at higher pH values, greater percentages of the total amount of substrate added would exist in the inactive, charged form, causing a decrease in the activity over and above that observed for substrates which exhibit less ionization.

It is of course necessary to know the pK'_a values of acetyl-L-tyrosinehydroxamide to ascertain if this idea is reasonable. These values have been determined by potentiometric titration of an aqueous solution of acetyl-L-tyrosinehydroxamide with 0.01 N sodium hydroxide (pp. 59-64). It is assumed that the pK'_1 value of 9.0 relates to the hydroxamic acid group, and that the pK'_2 value of 10.2 relates to the phenolic hydroxyl group. This appears to be a reasonable assumption in view of the fact that the $pK'_{(OH)}$ values for tyrosine and glycyltyrosine have been found to be 10.07 and 10.40 respectively, while the two $pK'_{(OH)}$ values of tyrosyl tyrosine are 9.80 and 10.26 (44). Although the 9.0 value is considerably higher than the known pK'_a values for hydroxamic acids, a pK'_a value of 7.55 for acetylhydroxamide being representative of the alkylhydroxamic acids (45), it is certainly more reasonable to associate it with the hydroxamic acid group than with the phenolic group.

Using these pK'_1 and pK'_2 values, the fraction of the initial acetyl-L-tyrosinehydroxamide concentration in the neutral (or, on the basis of the above assumption, the active) form can easily be calculated

as a function of the pH. Thus from pH 6.5 to the maximum pH of 7.6 this fraction changes very slightly, dropping from 1.00 to 0.96. However, from pH 7.6 to pH 8.5, the fraction drops from 0.96 to 0.76, causing a greater loss of activity with increasing pH than might be expected of a non-ionizing substrate, such as the amide, if the assumption of the inactivity of the charged form of acetyl-L-tyrosine-hydroxamide is valid.

A graphical description of the possibility of the above assumption is given in Fig. 1, curve B, which is a theoretical curve of activity versus pH for the alpha-chymotrypsin — acetyl-L-tyrosine-hydroxamide calculated in the following manner:

It is assumed that the pH-activity curve for the acetyl-L-tyrosinamide is the same as that for the neutral form of acetyl-L-tyrosinehydroxamide in that the v_o's for both substrates may be expressed by the following equation: $v_o = f(pH) \cdot C_s \cdot (S_{act})_o$, where f(pH) is a function of pH and not dependent upon the nature or concentration of either substrate; C_s is a constant dependent upon the nature of the substrate such that $f(pH) \cdot C_s$ is the apparent first order constant (this of course implies that we are dealing with substrate concentrations sufficiently smaller than the K_S so that the rate curves are first order over the time interval required to measure v_o); and $(S_{act})_o$ is the initial

concentration of the active substrate species. In the case of acetyl-L-tyrosinamide, it is assumed that in the pH range concerned here, $(S_{act})_o = (S)_o$, the initial substrate concentration, whereas in the case of acetyl-L-tyrosinehydroxamide we assume that the charged form is inactive so that $(S_{act})_o = (S)_o \cdot \frac{[H^+]^2}{[H^+]^2 + K_1[H^+] + K_1 K_2}$. Since the activity at a particular pH is here defined as the ratio of the initial velocity at that pH to the initial velocity at the pH showing maximum initial velocity, then for acetyl-L-tyrosinamide the activity, a_A, is

$a_A(pH_i) = \frac{f(pH_i)}{f(pH_{max.})}$, where $pH_{max.}$ is the pH of maximum initial velocity and pH_i is the pH under consideration. Similarly the activity of acetyl-L-tyrosinehydroxamide, a_H, is

$$a_H(pH_i) = \frac{f(pH_i)}{f(pH_{max.})} \cdot \frac{(1 + 10^{(pH_{max.} - pK_1')} + 10^{(2pH_{max.} - pK_1' - pK_2')})}{(1 + 10^{(pH_i - pK_1')} + 10^{(2pH_i - pK_1' - pK_2')})},$$

and therefore

$$(23)\quad a_H(pH_i) = a_A(pH_i) \cdot \frac{(1 + 10^{(pH_{max.} - pK_1')} + 10^{(2pH_{max.} - pK_1' - pK_2')})}{(1 + 10^{(pH_i - pK_1')} + 10^{(2pH_i - pK_1' - pK_2')})}.$$

This treatment assumes that the difference between $f(pH)$ at the $pH_{max.}$ for acetyl-L-tyrosinamide, 7.8, and at the $pH_{max.}$ for acetyl-L-tyrosinehydroxamide, 7.6, is negligible, as indicated by a change in activity for acetyl-L-tyrosinamide of only 1% for these two pH values.

Curve B of Fig. 1 was calculated from equation (23) and curve A of Fig. 1. While it is apparent from the relatively good agreement between the experimental and theoretical pH-activity curves that the

idea of the inactivity of the charged form of the acetyl-L-tyrosinehydroxamide is reasonable, it should be considered to be tentative in view of the simplified treatment used (e.g., no consideration was taken of the probable inhibition of hydrolysis by the charged form of the substrate, or its possible hydrolysis at a reduced rate) and the assumptions involved. However, it should be pointed out that the validity of the assumption that the pH-activity curves for the neutral form of the two substrates are identical is enhanced by the observation that both the alpha-chymotrypsin — benzoyl-L-tyrosinamide and the alpha-chymotrypsin — benzoyl-L-tyrosine ethyl ester systems have similar pH-activity curves (34).

At a constant pH of 7.6 and constant temperature of 25.0°C it was assumed that equation (21) was applicable for the hydrolysis of acetyl-L-tyrosinehydroxamide. Thus the initial velocity, v_o, was determined at various substrate concentrations and plots of $\frac{1}{v_o}$ vs. $\frac{1}{(S)_o}$ were made. The data for these determinations is given in Tables III and IV and Figs. 2 and 3. The straight line curves resulting from the $\frac{1}{v_o}$ vs. $\frac{1}{(S)_o}$ plots (Fig. 3) gave mean values for K_S and k_3 of 51×10^{-3}M and 34×10^{-3}M.(min.$^{-1}$)(mg. protein-nitrogen per ml.)$^{-1}$ respectively. The complicated units of k_3 result from the uncertainty in the molecular weight of alpha-chymotrypsin and in its purity. However, if it is assumed that all of the protein in the samples used was alpha-chymotrypsin (see p. 2), that the molecular weight of alpha-chymotrypsin is 22,000 (46, 47) and that its nitrogen content is 16% (9), k_3 has the value 2.0 sec.$^{-1}$.

The analysis of $\frac{1}{v_o}$ vs. $\frac{1}{(S)_o}$ plots tells us nothing in regard to the possible inhibition by the products of the reaction. If no such inhibition occurred, a plot of $\left[K_S \ln \frac{(S)_o}{(S)} + (S)_o - (S)\right]$ versus time should yield a straight line passing through the origin and having a slope $k_3(E)$ (see equation 20). The data given in Table V and Fig. 4 indicate, by the dropping off of the points from the expected straight line after approximately 35% hydrolysis that there is inhibition by at least one of the products. This is to be expected, since it has been found that acetyl-L-tyrosine functions as a competitive inhibitor in the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinamide and has a K_{P_1} value of 0.115 ± 0.15 M (22). If equation (18) is simplified to the condition of no added inhibitor and inhibition by only one of the hydrolysis products, the following equation results:

$$(24) \qquad k_3(E)t = K_S\left[1 + \frac{(S)_o}{K_{P_1}}\right] \ln \frac{(S)_o}{(S)} + \left[1 - \frac{K_S}{K_{P_1}}\right]\left[(S)_o - (S)\right]$$

Thus if acetyl-L-tyrosine is the only inhibitor present in the system, then a plot of $\left[K_S\left[1 + \frac{(S)_o}{K_{P_1}}\right] \ln \frac{(S)_o}{(S)} + \left[1 - \frac{K_S}{K_{P_1}}\right]\left[(S)_o - (S)\right]\right]$ versus time should yield a straight line passing through the origin and having a slope equal to $k_3(E)$. From the data in Table V and Fig. 5 it can be seen that the experimental points calculated on the basis of equation (24) and assuming K_{P_1} to have the value, 0.115 M, mentioned above, do not deviate from the expected straight line. As there is no evidence to

indicate that hydroxylamine inhibits the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydroxamide, the data are therefore consistent with the kinetic formulation,

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1_f} + P_{2_f}$$

$$E_f + P_{1_f} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP_1 ,$$

if the assumptions involved in the derivation of the rate equations are valid.

The fact that the enzyme concentration was never larger than 0.031 mg. protein-nitrogen per ml. or 8.8×10^{-6} M (calculated on the same assumptions as to molecular weight, purity and nitrogen content of alpha-chymotrypsin as used previously) and that the substrate concentration was never less than 2×10^{-3} M indicates that the assumption $(S)_f \gg (ES)$ must be valid, as there is probably only one (at most two) active sites per alpha-chymotrypsin molecule (26, 42, 48-50). Similarly it must be concluded that after the initial rise of the ES concentration to its maximum or steady state value, the assumption that $\frac{d(S_f)}{dt} \gg \frac{d(ES)}{dt}$ must hold. The fact that no initial period of increasing velocity is observable (Fig. 2) indicates that the time interval required to reach the maximum (ES) value is negligible relative to the experimental error. It is also apparent from the analysis of the effect of the inhibition by the products of the hydrolysis (Table V and Figs. 4 and 5) that the concentration of acetyl-L-tyrosine must be greater than 1×10^{-3} M before it

need be considered in the formulation of the kinetics, and that therefore the assumption that $(P_{l_f}) \gg EP_l$ must also be valid in the range of (P_{l_f}) values that have an observable effect on the kinetics of the hydrolysis. Thus it would appear that the necessary assumptions involved in the derivation of the rate equation are valid under the conditions used in these experiments, and that consequently the experimental data are in accord with the kinetic formulation given in the previous paragraph. These kinetics are similar to those noted previously for alpha-chymotrypsin and other specific substrates (21, 22, 27, 28).

In order to determine the relative enzyme affinity effects of the amide, hydrazide, hydroxamide and ethyl ester groups, the inhibition of the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydroxamide by acetyl-D-tyrosinamide, acetyl-D-tyrosinehydrazide, acetyl-D-tyrosinehydroxamide and acetyl-D-tyrosine ethyl ester have been investigated. From previous observations it was expected that these four compounds would function as competitive inhibitors to this hydrolysis. Thus plots of $\frac{1}{v_o}$ against $\frac{1}{(S)_o}$ would be predicted to give straight line curves with a slope dependent upon the inhibitor concentration but with an intercept independent of this concentration (equation 22). The data given in Tables VI-IX and Figs. 6-9 show that all four inhibitors act in this manner. The K_I values calculated from these data and with the aid of equation (22) are given in Table I below, along with their corresponding $-\Delta F^o$ values for the formation of the enzyme-inhibitor complex.

Table I

Inhibitor	K_I(a)	$-\Delta F^o$(b)
acetyl-D-tyrosine ethyl ester	4.0	3270
acetyl-D-tyrosinehydrazide	6.8	2960
acetyl-D-tyrosinehydroxamide	7.7	2890
acetyl-D-tyrosinamide	11.2	2660

(a) In units of 10^{-3} M at 25°C and pH 7.6 with ca. $\pm$ 12% maximum error.
(b) In calories per mole at 25°C and pH 7.6 to the nearest 10 calories. The error corresponding to that in K_I is ca. $\pm$ 75 calories.

It is clear that the additional assumption of $(I)_f \gg (EI)$ involved in the derivation of the rate equation for competitive inhibition must be valid in all cases, since the total inhibitor concentration was never less than 5×10^{-3} M, while the enzyme concentration was 29.4×10^{-3} mg. protein-nitrogen per ml. or 8.4×10^{-6} M.

The simplest explanation of competitive inhibition kinetics is that the inhibitor and substrate compete for attachment to the same reactive site of the enzyme molecule. Therefore, if the hydrolysis of two different substrates occurs via a reaction with the same reactive site of an enzyme, a particular inhibitor acting in a competitive manner in the hydrolysis of one substrate should competitively inhibit the hydrolysis of the other substrate and exhibit the same enzyme-inhibitor dissociation constant in each case. Thus the fact that the K_I values for the acetyl-D-tyrosinamide and acetyl-D-tyrosine ethyl ester inhibition of the hydrolysis of acetyl-L-tyrosinamide ($12.0 \pm 1.0 \times 10^{-3}$ M and $3.5 \pm 0.5 \times 10^{-3}$ M respectively — ref. 22) agree, within experimental

error, with those for the inhibition of the hydrolysis of acetyl-L-tyrosine-hydroxamide, indicates that the two substrates react with the same active site on the enzyme molecule.

Similar data for the hydrolysis of the hydrazide and ethyl ester are not available. However, since the alpha-chymotrypsin catalysed hydrolysis of an asymmetric molecule is, in every case that has been investigated, competitively inhibited by its antipode, then the fact that acetyl-D-tyrosinehydrazide and acetyl-D-tyrosine ethyl ester competitively inhibit the hydrolysis of acetyl-L-tyrosinehydroxamide, would indicate that the hydrolysis of the corresponding hydrazide and ethyl ester of the L- configuration proceeds by combination with the same site as that involved in the hydroxamide hydrolysis. Similarly the fact that acetyl-D-tyrosine ethyl ester competitively inhibits the hydrolysis of acetyl-L-tyrosinamide (22), that acetyl-D-tryptophane methyl ester competitively inhibits the hydrolysis of nicotinyl-L-tryptophanamide (26) and that nicotinyl-D-tyrosine ethyl ester competitively inhibits the hydrolysis of nicotinyl-L-tyrosinamide (27) indicates that the amides and esters are hydrolysed via the same reactive site.

Balls and coworkers (51), in examining the inhibitory effects of diisopropyl fluorophosphate on the alpha-chymotrypsin catalysed protein and ester hydrolysis, have concluded that the proteinase and esterase activity of the enzyme is due to the same reactive site. It is clear that the most reasonable interpretation of the evidence given above is that the hydrolysis of proteins, amides, esters, hydrazides and

hydroxamides is catalysed by reaction with the same reactive site of alpha-chymotrypsin. Furthermore, it seems probable that there is only one such site per alpha-chymotrypsin molecule (26, 42, 48-50).

From a consideration of Table I, it is apparent that the relative order of affinity of the inhibitors to this site is ethyl ester > hydrazide > hydroxamide > amide, the ratio of the K_I values for the four compounds being 1.0: 1.7 : 1.9 : 2.8, respectively. The ratio of the K_I values for the ethyl ester (29), hydrazide (29), and amide (24) of acetyl-D-tryptophane is 0.25×10^{-3}M : 0.75×10^{-3}M : 2.7×10^{-3}M or 1.0 : 3.0 : 10.8 respectively. Thus it is clear that the degree of the effect of a change in the R_3 group upon the affinity is dependent upon the nature of the R_2 group, but that, in these two cases at least, the direction of the effect is independent of this group.

It is of interest to compare this effect of a variation in the R_3 group on the K_I values with its effect on the K_S values of the corresponding compounds of the L- configuration. The only available data upon which such a quantitative comparison can be made concern the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinamide and acetyl-L-tyrosinehydroxamide. The hydrolysis of acetyl-L-tyrosine ethyl ester has been investigated only in 30 volumes per cent methanol (9), while the hydrolysis of acetyl-L-tyrosinehydrazide has not been investigated. The K_S values for the L-amide and L-hydroxamide are 30×10^{-3}M (22) and 51×10^{-3}M respectively, and the k_3 values are

2.4×10^{-3}M · (min.)$^{-1}$ · (mg. protein-nitrogen per ml.)$^{-1}$ (22) and 34×10^{-3}M · (min.)$^{-1}$ · (mg. protein-nitrogen per ml.)$^{-1}$ respectively. The K_S ratio of L-amide to L-hydroxamide is thus 0.59 as compared to the K_I ratio of 1.45 for the corresponding D isomers. With the present data it is impossible to conclude whether the inversion of the ratio is due to the large change in k_3 (i.e., k_3 cannot be neglected relative to k_2) or actually represents a difference in the effect of a change of the R_3 group on the affinity of the L and D compounds.

A change in the R_1 group of tryptophanamide from the free amino to the acetamido- to the nicotinamido- yields the same ratio for the K_{P_1} and K_S values for the L compounds as for the K_I values of the D compounds (25), and a change in the R_1 group of tyrosinamide from the acetamido- to the nicotinamido- yields the same K_S and K_I ratios for the L and D compounds respectively (27). As has been pointed out by Huang and Niemann (25), if this regularity is not fortuitous, the simplest explanation is that a change in R_1 causes the same change in the K_I value of the D compound and the $\frac{k_2}{k_1}$ ratio of the L compound, and that k_3 is always sufficiently small in relation to k_2 as to be a negligible factor in the value of K_S. If this interpretation is adopted, then the K_S value of acetyl-L-tyrosinamide approximates the $\frac{k_2}{k_1}$ value of that compound, and if it is assumed that a change in R_3 of amide to hydroxamide in acetyltyrosine produces the same effect on the affinity of the L and D isomers, then the k_2 of the acetyl-L-tyrosinehydroxamide hydrolysis

must be of the same order of magnitude as the k_3 value. If indeed it is further assumed that the ratio of the K_I values for the amide and hydroxamide of acetyl-D-tyrosine is equal to the ratio of the $\frac{k_2}{k_1}$ values for the amide and hydroxamide of acetyl-L-tyrosine, then the k_1 and k_2 values for acetyl-L-tyrosine hydroxamide must be 66 $M^{-1} \cdot$ sec.$^{-1}$ and 1.4 sec.$^{-1}$ respectively. It should be pointed out that the interpretation of the K_S for acetyl-L-tyrosinamide hydrolysis given above is the opposite of that given by Kaufman and Neurath (33), who conclude from the fact that the $\frac{1}{K_S}$ value of acetyl-L-tyrosinamide hydrolysis varies linearly with increasing methanol concentration while the k_3 value is unchanged, that k_2 must be small relative to k_3 and that therefore K_S approximates $\frac{k_3}{k_1}$. However, as Huang and Niemann (21) point out, their data can be equally well interpreted on the assumption that K_S approximates $\frac{k_2}{k_1}$.

It is not possible with the available data to reach a sound conclusion as to the relative sizes of k_2 and k_3 in the hydroxamide or the amide hydrolysis. However, an investigation of the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydrazide and acetyl-L-tyrosine ethyl ester might serve as a partial test for the interpretation that $k_2 >> k_3$ in the amide hydrolysis. The k_3 value for the ethyl ester hydrolysis would be expected to be much greater than that for the amide (or even hydroxamide) (9), whereas the k_3 value for the hydrazide hydrolysis should be of the same order of magnitude as that of the amide (17, 29). Thus the interpretation that $k_2 >> k_3$ for the amide and

that in all cases a variation in R_3 causes the same variation in the dissociation constants of the L and D forms, leads to the prediction that the hydrazide to amide K_I and K_S ratios would be the same, but that the ethyl ester or hydroxamide to amide or hydrazide K_S ratios would be appreciably larger than the corresponding K_I ratios for the D isomers. An investigation of the acetyl-L-tyrosinehydrazide hydrolysis is now in progress by others of Dr. Niemann's group.

Experimental (52, 53)

Acetyl-D-tyrosine ethyl ester (I). I, m.p. 96-97° C, $[\alpha]_D^{25}$ = -24.8° (c. 7% in ethanol) was prepared from acetyl-DL-tyrosine ethyl ester (54) as directed by Thomas, MacAllister and Niemann (22); lit. (22), m.p. = 95-97° C, $[\alpha]_D^{25}$ = -24.8° (c. 7% in ethanol).

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.2; H, 6.8; N, 5.6.

Acetyl-D-tyrosinamide (II). II, m. p. 225-226° C, $[\alpha]_D^{25}$ = -51.1° C (c. 0.8% in water) was prepared by ammonolysis of I (22); lit. (22), m.p. 225-226° C, $[\alpha]_D^{25}$ = -49.4° (c. 0.9% in water).

Anal. Calcd. for $C_{11}H_{14}O_3N_2$ (222): C, 59.4; H, 6.3; N, 12.6. Found: C, 59.5; H, 6.3; N, 12.6.

Acetyl-D-tyrosinehydrazide (III). A solution of 6.0 g. of I in 15 ml. of absolute ethanol was slowly added to a refluxing solution of 2.5 ml. of hydrazine hydrate in 5 ml. of absolute ethanol and this reaction mixture allowed to reflux for two hours. The precipitate which formed, after standing for three days at 4° C, was recovered, recrystallized twice from water, and dried in vacuo over phosphorus pentoxide to yield 4.1 g. of III, m. p. 236-236.5° C, $[\alpha]_D^{25}$ = -44.4° (c. 0.4% in water).

Anal. Calcd. for $C_{11}H_{15}O_3N_3$ (237): C, 55.7; H, 6.4; N, 17.7. Found: C, 55.6; H, 6.4; N, 17.6.

Acetyl-D-tyrosinehydroxamide (IV). A solution of 11.0 g. of I in 22 ml. of 3.5 M hydroxylamine in methanol was cooled to 0° C, 41 ml. of 1.3 N methanolic sodium methoxide added and the reaction mixture allowed to stand at 4° C for five days. The crystalline precipitate which formed was recovered, washed with methanol and dried in vacuo over phosphorus pentoxide to give 7.5 g. of the sodium acetyl-L-tyrosine-hydroxamate (V), m.p. 182-183° C with decomposition. Evaporation of the mother liquors to 40 ml. and addition of 20 ml. of 2.6 M hydroxylamine in methanol gave a second crop of 2.5 g. of V, m.p. 179-180° C with decomposition. To 7.5 g. of V and 3.6 g. of sodium bisulfate, previously dried in vacuo over phosphorus pentoxide for nine days to remove water (the molecular weight of this sodium bisulfate was 124 as determined by titration with sodium hydroxide solution), was added 180 ml. of dry, absolute ethanol. This reaction mixture was closed from air and shaken for four and one-half hours at 0° C. An extremely fine white precipitate of sodium sulfate formed which was removed and the filtrate evaporated in vacuo to yield 6.4 g. of crystalline IV, m.p. 141-142° C with decomposition. This material was recrystallized three times from water to give IV, m.p. 143-144° C with decomposition, $[\alpha]_D^{25}$ - 38.5° (c. 5% in water).

Anal. Calcd. for $C_{11}H_{14}O_4N_2$ (238): C, 55.5; H, 5.9; N, 11.8. Found: C, 55.6; H, 5.8; N, 11.7.

Acetyl-L-tyrosine ethyl ester (VI). Nine hundred fifty ml. of 2 N sodium hydroxide and 120 ml. of acetic anhydride were added alternately in eight equal portions to a solution of 86 g. of L-tyrosine in 144 ml. of water and 445 ml. of 2 N sodium hydroxide, the temperature of the reaction mixture being kept below 10° C. This solution was kept at room temperature for four and one-half hours and then 484 ml. of 6 N H_2SO_4 was added. This solution, after standing for two days at 4° C was filtered to remove a small amount of inorganic material. It was then evaporated in vacuo to a thick syrup (55), taken up in 1150 ml. of absolute ethanol, filtered, evaporated, the residue dissolved in 1150 ml. of absolute ethanol and the solution again evaporated. The residue was taken up in 575 ml. of absolute ethanol, saturated with anhydrous hydrogen chloride and refluxed for two hours. The reaction mixture was evaporated in vacuo to a thick syrup which upon treatment with 140 ml. of M sodium carbonate crystallized and yielded after recrystallization from water and drying in vacuo over phosphorus pentoxide at 58° C, 60 g. of VI, m.p. 95-96° C. A portion of this material was twice recrystallized from aqueous ethanol to yield VI, m.p. 96-97° C, $[\alpha]_D^{25}$ + 24.6° (c. 7% in ethanol); lit. (16, 22), m.p. 96-97° C, $[\alpha]_D^{25}$ + 24.7°.

Anal. Calcd. for $C_{13}H_{17}O_4N$ (251): C, 62.1; H, 6.8; N, 5.6. Found: C, 62.2; H, 6.8; N, 5.5.

Acetyl-L-tyrosinehydroxamide (VII). VII, m.p. 143-144° C, $[\alpha]_D^{25}$ + 38.3° (c. 5% in water) was prepared from VI in the same manner and in approximately the same yield as was IV from I, except that isopropyl alcohol as well as water was used in recrystallization.

Anal. Calcd. for $C_{11}H_{14}O_4N_2$ (238): C, 55.5; H, 5.9; N, 11.8. Found: C, 55.5; H, 6.0; N, 11.6.

Buffer solutions: Technical tris-(hydroxymethyl)-aminomethane (Commercial Solvents) was recrystallized twice from aqueous methanol to give a product, large colorless rods, m.p. 169-169.5°. A stock solution, 1.53 formal with respect to the amine component, was prepared by the addition of sufficient 3 N hydrochloric acid to an aqueous solution of the amine to give a solution of pH 7.62 at 25° C. This stock solution was used in all studies conducted at pH 7.6, since it was found that in the presence of enzyme, substrate and inhibitor, a 2.0:10.2 dilution of this stock solution yielded a reaction mixture, 0.300 formal in the amine component and of pH 7.60 ± 0.02 at 25° C. A maximum pH change of only 0.05 pH units was noted when as much as 0.015 moles/liter of the substrate was hydrolysed. Other stock solutions 1.5 formal with respect to the amine component were prepared in the same manner for studies at other pH values in the determination of the pH-activity curves.

Ferric chloride solutions: A standard ferric chloride solution (Solution A) was prepared as follows: 15.0 g. of reagent grade anhydrous

ferric chloride was dissolved in 500 ml. of 1.29 N hydrochloric acid and diluted to one liter with absolute methanol. The ferric chloride solution used in the colorimetric analysis of acetyl-L-tyrosinehydroxamide (Solution B) was prepared by dilution of 100 ml. of the Solution A with 500 ml. of absolute methanol and sufficient water to make a total volume of one liter. When kept in the dark, Solution A was stable over a period of five months. Solution B was freshly prepared from Solution A every few weeks, and no instability was observed. Stability of these solutions was measured by the intensity of the color formed with acetyl-L-tyrosinehydroxamide. Since this intensity of color is sufficiently independent of the concentration of the ferric chloride that a $\pm$ 10% variation in this concentration does not cause an appreciable difference in the colorimeter reading for all concentrations of acetyl-L-tyrosinehydroxamide used in this investigation, it was possible, in spite of the deliquescence of the ferric chloride, to make up standard ferric chloride solutions which gave identical calibration curves in the colorimetric analysis of acetyl-L-tyrosinehydroxamide.

Enzyme solutions: Crystalline alpha-chymotrypsin (Armour, Lot No. 90402), containing magnesium sulfate, was used in all of the experiments. Enzyme stock solutions were prepared daily and kept at 4° C except for not more than four periods during the twenty-four hour interval when they were brought to 25° C for withdrawal of a 0.2 ml. aliquot. The solutions maintained a constant enzyme activity over this

interval; in fact the activity remained constant over a 48-hour interval when kept at 4° C. The protein-nitrogen content of the enzyme solution was determined by the Kjeldahl method after precipitation with trichloroacetic acid.

Enzymatic reactions: The substrate and buffer, or substrate, inhibitor and buffer, in solution form were added to a 10-ml. G.S. volumetric flask and the flask filled with water to the 10-ml. mark. This flask was then placed in a 25.00 ± 0.05° bath for at least 30 minutes. At minus 10 seconds from zero time, 0.2 ml. of enzyme solution was added and at zero time the flask was stoppered and gently inverted approximately 10 times. The flask was then replaced in the bath and 0.2 ml. aliquots were removed at convenient intervals and delivered into calibrated colorimeter tubes containing 10.0 ml. of ferric chloride solution B. The contents of the tubes were shaken vigorously and the intensity of the color determined with a Klett-Summerson photoelectric colorimeter equipped with a green filter (filter no. 54 -- transmission range of ca. 500-570 millimicrons). A standard calibration curve was prepared from solutions containing various amounts of acetyl-L-tyrosinehydroxamide in tris-(hydroxymethyl)-aminomethane 0.300 formal in respect to the amine component. It was found that for concentrations of acetyl-L-tyrosinehydroxamide up to 0.050 molar in the original solution the colorimeter readings were directly proportional to the concentration of the hydroxamide. The proportionality constant

was found to be independent of the presence of alpha-chymotrypsin, acetyl-L-tyrosine, hydroxylamine, acetyl-D-tyrosine ethyl ester, acetyl-D-tyrosinamide, and acetyl-D-tyrosinehydrazide, in amounts equal to or greater than the maximum amounts involved in the enzymatic reactions. The same proportionality constant is observed with acetyl-D-tyrosinehydroxamide, and this constant is independent of the hydrogen ion activity over the pH range 6.5 to 9.1. The color formed, whether from the addition of aliquots from the enzyme reaction mixture or from the solutions used for calibration, was stable over a period of two hours after mixing. All readings were taken within this time interval and were reproducible to within $\pm$ 2%.

As the initial portion of the rate curves (20-30% hydrolysis) was always observed to be first order, values of $\log_{10} R$ (R = colorimeter reading) were plotted against time (typical curves are shown in Fig. 2) and the initial velocity at zero time determined from the apparent initial first order rate constant and the initial substrate concentration. It should be pointed out that it is only necessary to know that the colorimeter readings are directly proportional to the acetyl-L-tyrosine-hydroxamide concentration, since the value of the proportionality constant is not a factor in the determination of the apparent first order constant.

The following data have been determined according to the procedure outlined in this section. The legend of symbols is as follows:

t = time in minutes

(E) = alpha-chymotrypsin concentration in mg. protein-nitrogen per ml.

(S) = substrate concentration in moles per liter

$(S)_o$ = initial substrate concentration in moles per liter

R = colorimeter reading

c = proportionality constant of substrate concentration to colorimeter reading in moles per liter, i.e., $(S) = c \cdot R$

v_o = velocity of reaction at $t = o$ in moles per liter per minute

a = relative activity

Table II

The Dependence of the alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide on pH at 25° C.

$(S)_o = 9.8 \times 10^{-3}$ molar.

(E) = 0.0306 mg. protein-nitrogen per ml.

0.294 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer.

c = 7.5×10^{-5} molar.

pH	t	R	Log R	$v_o \times 10^4$	a	pH	t	R	Log R	$v_o \times 10^4$	a
6.5*	1.2	128	2.107	0.85	0.48	7.59	1.1	130	2.114	1.76	1.00
	6.0	121	2.083				4.7	120	2.079		
	11.0	116	2.064				10.3	109	2.037		
	16.0	112	2.049				15.0	100	2.000		
	22.5	105	2.021				21.0	90	1.954		
	26.0	102	2.009				25.0	84	1.924		
	31.0	99	1.996				30.0	76	1.881		
	36.2	94	1.973				35.1	70	1.845		
	46.0	87	1.940				45.0	58	1.763		
	56.0	80	1.903				55.0	49	1.690		
6.93	1.2	128	2.107	1.39	0.79	7.80	1.1	129	2.111	1.68	0.96
	6.2	117	2.068				6.1	115	2.061		
	12.5	107	2.029				11.0	107	2.029		
	18.0	100	2.000				16.0	98	1.991		
	23.0	95	1.978				21.0	91	1.959		
	28.0	87	1.940				26.0	84	1.924		
	33.0	82	1.914				31.0	76	1.881		
	38.0	76	1.881				36.0	69	1.839		
	44.0	69	1.839				46.0	59	1.771		
	48.0	65	1.806				56.0	50	1.699		
7.40	1.0	128	2.107	1.69	0.96	7.99	1.2	128	2.107	1.47	0.84
	6.0	117	2.068				6.0	119	2.076		
	10.5	109	2.037				11.2	109	2.037		
	15.0	101	2.004				16.0	103	2.013		
	20.5	93	1.968				21.0	94	1.973		
	25.5	85	1.929				26.0	89	1.949		
	30.5	76	1.881				31.0	81	1.908		
	35.5	70	1.845				36.0	76	1.881		
	40.5	66	1.820				46.0	65	1.813		
	45.5	60	1.778				56.0	56	1.748		

Table II, cont.

pH	t	R	Log R	$v_o \times 10^4$	a	pH	t	R	Log R	$v_o \times 10^4$	a
8.20	1.0	130	2.114	1.37	0.78	8.78	1.1	128	2.107	0.79	0.45
	6.0	120	2.079				5.8	122	2.086		
	11.0	113	2.053				10.9	117	2.068		
	16.0	103	2.013				15.8	115	2.061		
	21.0	98	1.991				20.8	108	2.033		
	26.0	90	1.954				25.8	104	2.017		
	31.0	85	1.929				30.8	102	2.009		
	36.5	79	1.898				35.8	96	1.982		
	46.0	69	1.839				45.8	90	1.954		
	56.0	61	1.785				55.8	84	1.924		
8.39	1.2	130	2.114	1.13	0.61	9.07	1.0	129	2.111	0.26	0.15
	7.0	120	2.079				5.0	127	2.104		
	12.0	113	2.053				9.8	125	2.097		
	17.0	107	2.029				15.0	125	2.097		
	23.0	100	2.000				20.0	123	2.090		
	27.0	97	1.987				25.0	120	2.079		
	32.0	93	1.964				30.0	120	2.079		
	37.0	86	1.934				35.0	118	2.072		
	47.0	77	1.886				45.0	115	2.061		
	57.0	70	1.845				55.0	111	2.045		

*The pH dropped from 6.58 to 6.42 during the course of the reaction. At all other pH values the buffer held the pH constant to within $\pm$ 0.02 pH units.

Table III

The alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide at pH 7.6 and 25.0° C.

(E) = 0.0209 mg. protein-nitrogen per ml.

0.300 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6.

c = 7.5×10^{-5} molar.

K_S = 51.6×10^{-3} molar.

k_3 = 33.7×10^{-3} molar·(min.)$^{-1}$·(mg. protein-nitrogen per ml.)$^{-1}$

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$	$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
40.0	1.0	525	2.720	3.12	40.0	1.0	520	2.716	3.04
	4.2	510	2.708			6.0	495	2.695	
	8.2	490	2.690			12.0	477	2.678	
	12.0	475	2.677			18.0	445	2.648	
	16.0	470	2.672			24.0	440	2.644	
	20.0	450	2.653			30.0	412	2.615	
	23.0	415	2.618			36.0	399	2.601	
30.0	1.1	385	2.586	2.48	30.0	1.0	395	2.597	2.70
	4.0	380	2.580			6.0	378	2.578	
	7.7	365	2.562			12.0	348	2.542	
	12.0	360	2.556			24.0	322	2.508	
	16.0	347	2.540			30.0	298	2.474	
	20.0	340	2.532			36.0	287	2.458	
	26.0	318	2.502						
20.0	1.0	267	2.426	1.97	20.0	1.0	267	2.426	1.96
	6.0	244	2.387			6.0	254	2.405	
	12.0	238	2.377			12.0	237	2.375	
	18.0	226	2.354			18.0	227	2.356	
	24.0	211	2.324			24.0	214	2.330	
	30.0	199	2.299			30.0	201	2.303	
	36.0	195	2.290			36.0	191	2.281	
10.0	1.0	131	2.117	1.16	10.0	1.0	133	2.124	1.15
	6.0	124	2.093			6.0	126	2.100	
	12.0	111	2.045			12.0	119	2.076	
	18.0	104	2.017			18.0	108	2.033	
	24.0	98	1.991			24.0	102	2.009	
	30.0	92	1.964			30.0	95	1.978	
	36.0	87.5	1.942			36.0	90	1.954	

Table III, cont.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$	$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
5.0	1.0	67	1.826	0.615	5.0	1.1	65	1.813	0.611
	6.0	60	1.778			6.0	62	1.792	
	12.0	57	1.756			12.0	57	1.756	
	18.0	55	1.740			18.0	53	1.724	
	24.0	50	1.699			24.0	50	1.699	
	30.0	47	1.672			30.0	46.5	1.668	
	36.0	43	1.634			36.0	43	1.634	

Note: Solutions of acetyl-L-tyrosinehydroxamide buffered at pH 7.6 do not show any change in acetyl-L-tyrosinehydroxamide concentration when kept at 25.0° C for at least three days in the absence of alpha-chymotrypsin.

Table IV

The alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide at pH 7.6 and 25.0° C.

(E) = 0.0294 mg. protein-nitrogen per ml.
0.300 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6.
c = 7.4×10^{-5} molar.

K_S = 51.0×10^{-3} molar.
k_3 = 34.7×10^{-3} molar·(min.)$^{-1}$·(mg. protein-nitrogen per ml.)$^{-1}$.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$	$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
35.0	1.0	470	2.672	4.09	16.0	1.0	210	2.322	2.45
	6.0	448	2.651			6.0	195	2.290	
	12.0	400	2.602			18.0	166	2.220	
	18.0	390	2.591			24.0	148	2.170	
	24.0	354	2.549			30.0	138	2.140	
	30.0	335	2.525			36.0	120	2.079	
	37.0	303	2.481						
25.0	1.0	335	2.525	3.37	13.0	1.0	174	2.240	2.10
	6.0	305	2.484			6.0	160	2.204	
	12.0	276	2.441			12.0	147	2.167	
	18.0	262	2.418			18.0	132	2.121	
	24.0	240	2.380			24.0	120	2.079	
	30.0	223	2.348			30.0	108	2.033	
	36.0	205	2.312			36.0	100	2.000	
20.0	1.0	268	2.428	2.86	11.0	1.0	149	2.173	1.84
	6.0	248	2.394			6.0	135	2.130	
	12.0	232	2.366			12.0	121	2.083	
	18.0	209	2.320			18.0	111	2.045	
	24.0	192	2.283			24.0	100	2.000	
	36.0	163	2.212			30.0	91	1.959	
						36.0	83	1.919	

Table IV, cont.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
9.00	1.0	120	2.079	1.53
	6.0	108	2.033	
	12.0	99	1.996	
	18.0	90	1.954	
	24.0	80	1.903	
	30.0	73	1.863	
	36.0	65	1.813	
7.00	1.0	95	1.978	1.23
	6.0	89	1.949	
	12.0	80	1.903	
	18.0	71	1.851	
	24.0	64	1.806	
	30.0	57	1.756	
	36.0	53.5	1.728	
5.00	1.0	67	1.826	0.88[5]
	6.0	61	1.785	
	12.0	56	1.748	
	18.0	49	1.690	
	24.0	45	1.653	
	30.0	40	1.602	
	36.0	36	1.556	

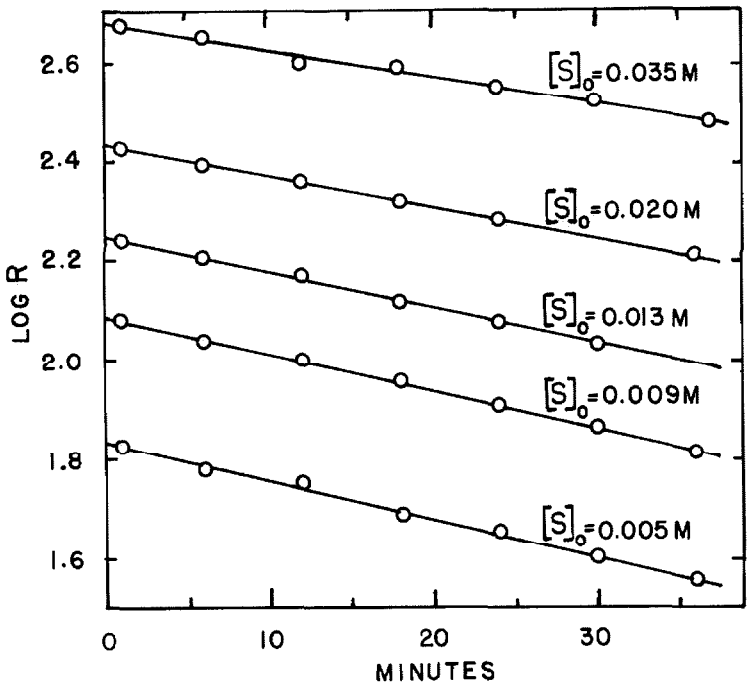


Fig. 2. Logarithm of the colorimeter reading versus time for the alpha-chymotrypsin catalysed hydrolysis of acetyl-L-tyrosinehydroxamide. (E) = 0.294 mg. protein-nitrogen per ml.

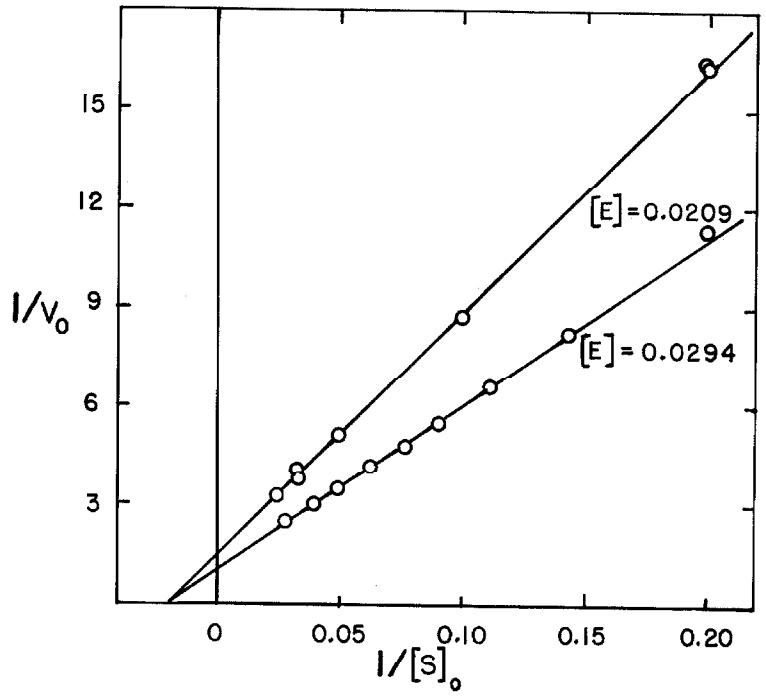


Fig. 3. $\frac{1}{v_o}$ versus $\frac{1}{(S)_o}$ plots for the system alpha-chymotrypsin — acetyl-L-tyrosinehydroxamide at pH 7.6 and 25° C. $(S)_o$ in units of 10^{-3} M; v_o in units of 10^{-3} M. min.$^{-1}$; (E) in units of mg. protein-nitrogen per ml.

Table V

Inhibition of the alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide by the Products of the Reaction.

$(S)_o$ = 20.0×10^{-3} molar.
(E) = 0.0209 mg. protein-nitrogen per ml.
0.300 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6 at 25.0° C.
c = $7.4^1 \times 10^{-5}$.

t	R	$(S) \times 10^3$	% hydrolysis	$((S)_o - (S)) \times 10^{-3}$	$\text{Log} \frac{(S)_o}{(S)}$	$F(S) \times 10^3$	$F^*(S)$
0.0	270	20.0_0	0.0	0.00	0.0000	0.0	0.0
1.0	268	19.8^7	0.6	0.1^3	0.0030	0.5	0.5
5.0	258	19.1^2	4.4	0.8^8	0.0195	3.2	3.2
15.0	234	17.3^4	13.3	2.6^6	0.0614	9.9	10.0
25.0	210	15.5^6	22.2	4.4^4	0.1096	17.4	17.7
40.0	179	13.2^7	33.6	6.7^3	0.1784	27.8	28.5
55.0	150	11.1^1	44.4	8.8^9	0.2550	39.0	40.3
70.0	131	9.7^2	51.4	10.2^8	0.3134	47.3	49.1
85.0	110	8.1^5	59.2	11.8^5	0.3902	57.9	60.8
100.0	92	6.8^2	65.9	13.1^8	0.4673	68.4	72.2
120.0	76	5.6^3	71.8	14.3^7	0.5511	79.5	84.5

$$F(S) = 2.303\ K_S \log \frac{(S)_o}{(S)} + (S)_o - (S)$$

$$F^*(S) = 2.303\ K_S \left(1 + \frac{(S)_o}{K_{P_1}} \log \frac{(S)_o}{(S)} + \left(1 - \frac{K_S}{K_{P_1}}\right)((S)_o - (S))\right.$$

K_S = 0.0513 molar.
k_3 = 0.0342 molar·(min.)$^{-1}$·(mg. protein-nitrogen per ml.)$^{-1}$
-- see Tables III and IV.

K_{P_1} = 0.115 molar.
-- see reference 22.

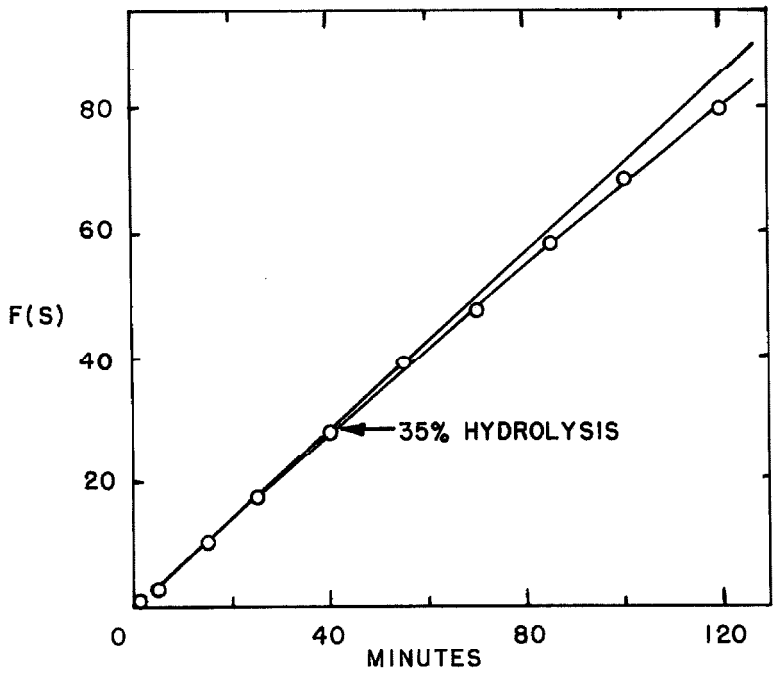


Fig. 4. F(S) in units of 10^{-3} M; acetyl-L-tyrosinehydroxamide, $(S)_o = 20.0 \times 10^{-3}$ M; (E) = 0.0209 mg. protein-nitrogen per ml.

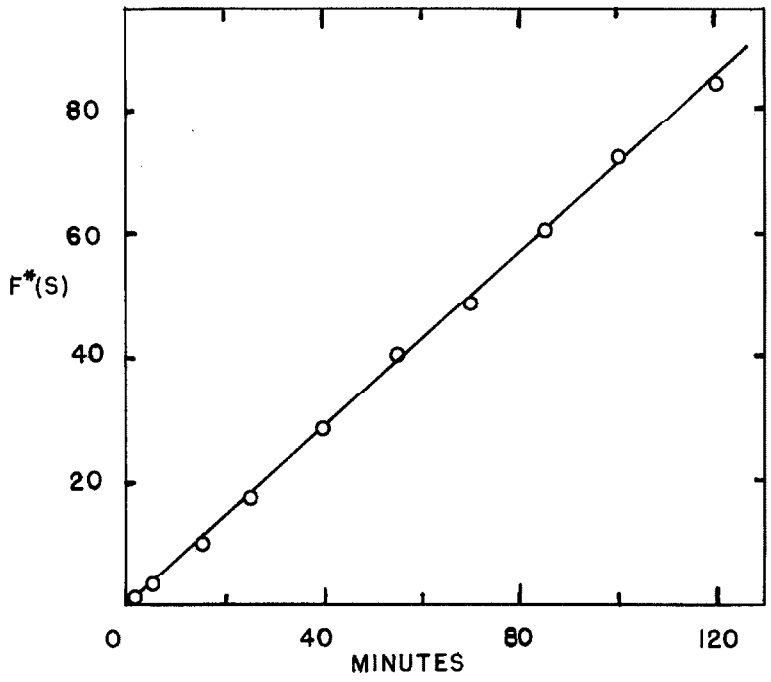


Fig. 5. $F^*(S)$ in units of 10^{-3} M; acetyl-L-tyrosine-hydroxamide, $(S)_o = 20.0 \times 10^{-3}$ M; (E) = 0.0209 mg. protein-nitrogen per ml.

Table VI

Inhibition of the alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide by Acetyl-D-tyrosine Ethyl Ester at pH 7.6 and 25.0° C.

(E) = 0.0294 mg. protein-nitrogen per ml.

(I) = 5.00×10^{-3} molar.

0.300 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6 at 25.0° C.

c = 7.5×10^{-5} molar.

$K_I = 4.0 \times 10^{-3}$ molar.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$	$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
35.0	1.0	455	2.658	2.56	20.0	1.0	261	2.417	1.60
	8.0	430	2.634			8.0	254	2.405	
	16.0	410	2.613			16.0	236	2.373	
	24.0	385	2.586			24.0	222	2.346	
	32.0	363	2.560			32.0	204	2.310	
	40.0	340	2.532			40.0	191	2.281	
	48.0	326	2.513			48.0	183	2.262	
31.3	1.0	415	2.618	2.33	16.2	1.0	217	2.336	1.34
	8.0	385	2.586			8.0	206	2.314	
	16.0	375	2.574			16.0	195	2.290	
	24.0	355	2.550			24.0	182	2.260	
	32.0	330	2.518			32.0	166	2.220	
	40.0	312	2.494			40.0	156	2.193	
	48.0	286	2.456			48.0	143	2.155	
27.5	1.0	368	2.566	2.07	12.5	1.0	164	2.215	1.06
	8.0	350	2.544			8.0	154	2.188	
	16.0	323	2.509			16.0	146	2.164	
	24.0	304	2.483			24.0	132	2.121	
	32.0	291	2.464			32.0	128	2.107	
	40.0	274	2.438			40.0	118	2.072	
	48.0	259	2.413			48.0	111	2.045	
23.8	1.0	310	2.491	1.85	8.78	1.0	116	2.064	0.761
	8.0	296	2.471			8.0	111	2.045	
	16.0	281	2.449			16.0	103	2.013	
	24.0	261	2.417			24.0	97	1.987	
	32.0	249	2.396			32.0	89	1.949	
	40.4	228	2.358			40.0	83	1.919	
	48.0	217	2.336			48.0	79	1.898	

Table VI, cont.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
5.00	1.0	67.5	1.829	0.446
	8.0	64	1.806	
	16.0	60	1.778	
	24.0	55	1.740	
	32.0	51	1.708	
	40.0	48	1.681	
	48.0	43.5	1.638	

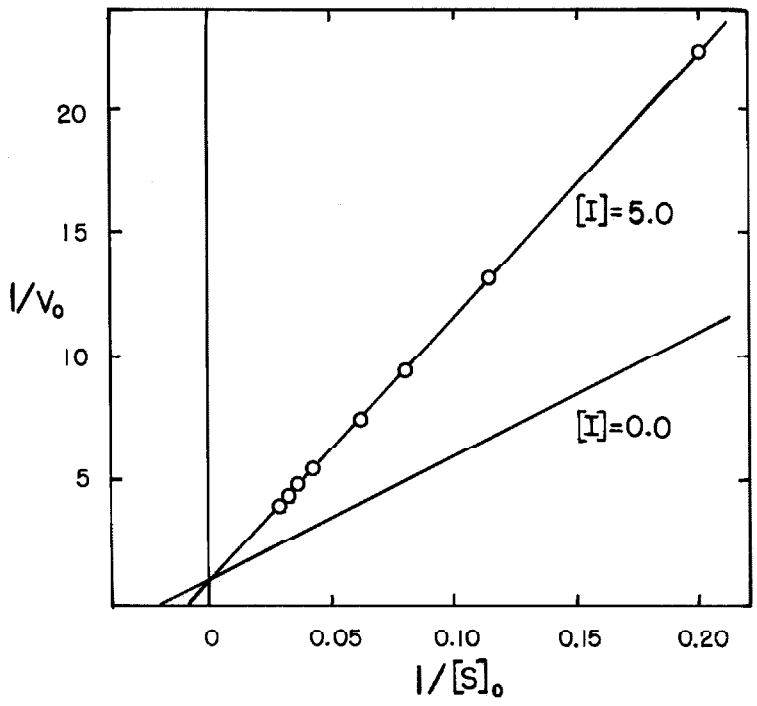


Fig. 6. Inhibition of hydrolysis of acetyl-L-tyrosine-hydroxamide by acetyl-D-tyrosine ethyl ester; v_o in units of 10^{-3} M·(min.)$^{-1}$; $(S)_o$ and (I) in units of 10^{-3} M; (E) = 0.0294 mg. protein-nitrogen per ml.

Table VII

Inhibition of the alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide by Acetyl-D-tyrosine-hydrazide at pH 7.6 and 25.0° C.

(E) = 0.0294 mg. protein-nitrogen per ml.

(I) = 7.51×10^{-3} molar.

0.300 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6 at 25.0° C.

c = 7.45×10^{-5} molar.

$K_I = 6.8 \times 10^{-3}$ molar.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$	$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
35.0	1.0	459	2.662	2.52	23.8	1.0	327	2.517	1.93
	8.0	444	2.647			8.0	308	2.489	
	16.0	410	2.613			16.0	287	2.458	
	24.0	382	2.582			24.0	270	2.431	
	32.0	370	2.568			32.0	254	2.405	
	40.0	347	2.540			40.0	238	2.377	
	48.0	330	2.518			48.0	220	2.342	
31.3	1.0	417	2.620	2.35	20.0	1.0	265	2.423	1.65
	8.0	396	2.598			8.0	252	2.401	
	16.0	366	2.564			16.0	228	2.358	
	24.0	355	2.550			24.0	218	2.338	
	32.0	325	2.512			32.0	206	2.314	
	40.0	315	2.498			40.0	192	2.283	
	48.0	295	2.470			48.0	187	2.272	
27.5	1.0	370	2.568	2.11	16.2	1.0	218	2.338	1.38
	8.0	345	2.538			8.0	202	2.305	
	16.0	327	2.514			16.0	190	2.279	
	24.0	308	2.489			24.0	180	2.255	
	32.0	290	2.462			32.3	167	2.223	
	40.0	272	2.435			40.0	158	2.199	
	48.0	257	2.410			48.0	145	2.161	

Table VII, cont.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
12.5	1.0	167	2.223	1.07
	9.0	152	2.182	
	18.0	143	2.155	
	28.0	133	2.124	
	38.0	122.5	2.088	
	48.0	112	2.049	
8.76	1.0	116.5	2.066	0.745
	9.0	109	2.037	
	17.0	105	2.021	
	26.0	97	1.987	
	37.0	87	1.940	
	48.0	80	1.903	
5.02	1.0	67	1.826	0.474
	8.0	62	1.792	
	16.0	58	1.763	
	25.0	54	1.732	
	32.0	50	1.699	
	40.0	46	1.663	
	48.0	42	1.623	

A solution — 10.0×10^{-3} molar in acetyl-D-tyrosinehydrazide, 0.150 mg. protein-nitrogen per ml. in alpha-chymotrypsin, 0.0306 formal in tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6 — showed no hydrolysis of the hydrazide over a period of six hours at 25.0° C. The formal potentiometric titration method (21) was used for the determination of the activity.

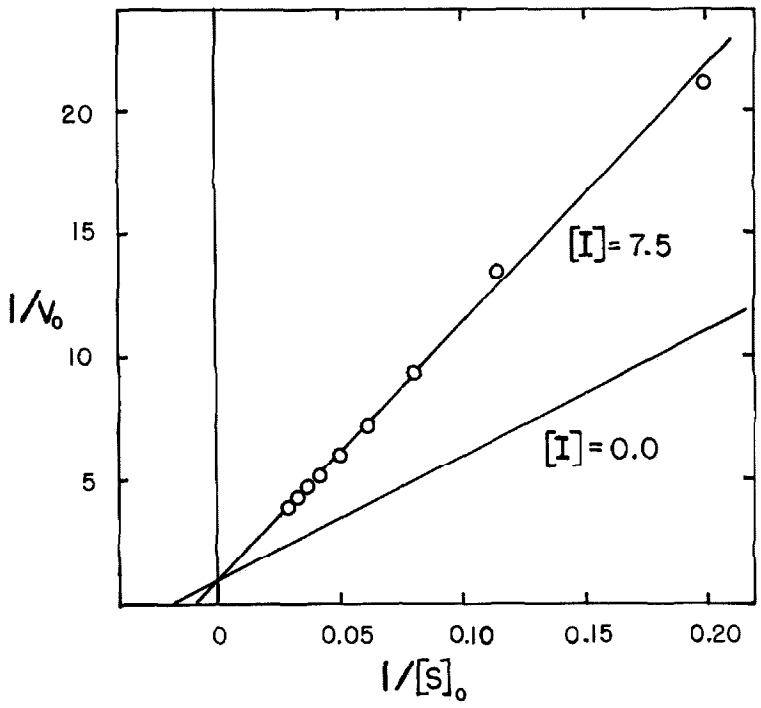


Fig. 7. Inhibition of hydrolysis of acetyl-L-tyrosine-hydroxamide by acetyl-D-tyrosinehydrazide; v_o in units of 10^{-3} M·min.$^{-1}$; $(S)_o$ and (I) in units of 10^{-3} M; (E) = 0.0294 mg. protein-nitrogen per ml.

Table VIII

Inhibition of the alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide by Acetyl-D-tyrosine-hydroxamide at pH 7.6 and 25.0° C.

(E) = 0.0294 mg. protein-nitrogen per ml.
(I) = 7.50×10^{-3} molar (Colorimeter reading of 100, R').
0.300 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6 at 25.0° C.
c = 7.5×10^{-5} molar.

$K_I = 7.7 \times 10^{-3}$ molar.

$(S)_o \times 10^3$	t	R	R - R'	Log (R - R')	$v_o \times 10^4$
35.0	1.1	560	460	2.663	2.67
	4.0	540	440	2.644	
	8.0	543	443	2.646	
	12.0	520	420	2.623	
	16.0	510	410	2.613	
	21.0	497	397	2.599	
	24.0	490	390	2.591	
	28.0	475	375	2.574	
	32.0	462	362	2.559	
30.0	1.2	490	390	2.591	2.42
	8.0	473	373	2.572	
	16.0	448	348	2.542	
	24.2	424	324	2.510	
	32.0	407	307	2.487	
	40.0	390	290	2.462	
	48.1	371	271	2.433	
	56.0	353	253	2.403	
25.6	1.0	435	335	2.525	2.08
	8.0	415	315	2.498	
	16.0	399	299	2.476	
	24.0	381	281	2.449	
	32.0	362	262	2.418	
	40.0	350	250	2.398	
	48.0	330	230	2.362	

Table VIII, cont.

$(S)_o \times 10^3$	t	R	R - R'	Log (R - R')	$v_o \times 10^4$
21.2	1.0	376	276	2.441	1.83
	8.0	362	262	2.418	
	16.0	345	245	2.389	
	24.0	325	225	2.352	
	32.0	312	212	2.326	
	40.0	297	197	2.294	
	48.0	283	183	2.262	
16.8	1.2	320	220	2.342	1.47
	8.1	306	206	2.314	
	16.0	293	193	2.286	
	24.0	281	181	2.258	
	32.1	269	169	2.228	
	40.0	258	158	2.199	
	48.0	245	145	2.161	
12.4	1.1	264	164	2.215	1.15
	8.0	253	153	2.185	
	16.0	239	139	2.143	
	24.0	232	132	2.121	
	32.0	222	122	2.086	
	40.0	214	114	2.057	
	48.0	204	104	2.017	
8.00	1.1	206	106	2.025	0.774
	8.0	199	99	1.996	
	16.0	190	90	1.954	
	24.0	185	85	1.929	
	32.0	178	78	1.892	
	40.0	171	71	1.845	
	48.0	166	66	1.820	
	50.0	165	65	1.813	

Table VIII, cont.

$(S)_o \times 10^3$	t	R	R - R'	Log (R - R')	$v_o \times 10^4$
7.00	1.1	192	92	1.964	0.681
	4.1	188	88	1.944	
	8.0	185	85	1.929	
	12.1	181	81	1.908	
	16.0	179	79	1.898	
	20.1	175	75	1.875	
	25.0	172	72	1.857	
	28.0	169	69	1.839	
	32.0	167	67	1.826	
	36.0	164	64	1.806	

A solution — 20.0×10^{-3} molar in acetyl-D-tyrosinehydroxamide, 0.0294 mg. protein-nitrogen per ml. in alpha-chymotrypsin, 0.300 formal in tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6 — showed no hydrolysis (i.e., maintained same colorimeter reading) over a period of twenty-two hours at 25.0° C.

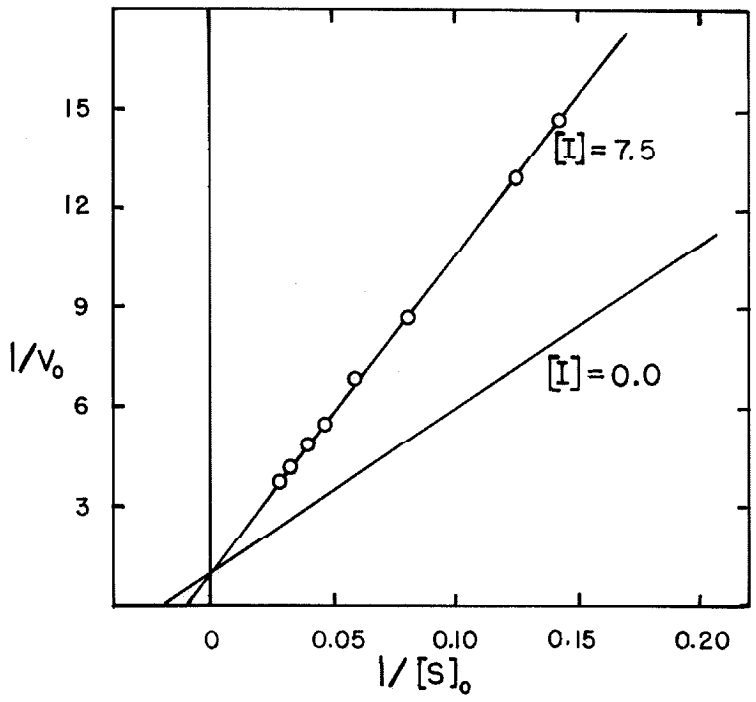


Fig. 8. Inhibition of hydrolysis of acetyl-L-tyrosinehydroxamide by acetyl-D-tyrosinehydroxamide; v_o in units of 10^{-3} M·min.$^{-1}$; $(S)_o$ and (I) in units of 10^{-3} M; (E) = 0.0294 mg. protein-nitrogen per ml.

Table IX

Inhibition of alpha-Chymotrypsin Catalysed Hydrolysis of Acetyl-L-tyrosinehydroxamide by Acetyl-D-tyrosinamide at pH 7.6 and 25.0° C.

(E) = 0.0294 mg. protein-nitrogen per ml.
(I) = 10.0×10^{-3} molar.
0.300 formal tris-(hydroxymethyl)-aminomethane — hydrochloric acid buffer, pH 7.6 at 25.0° C.
c = 7.5×10^{-5} molar.

K_I = 11.2.

$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$	$(S)_o \times 10^3$	t	R	Log R	$v_o \times 10^4$
35.0	1.0	469	2.671	2.80	20.0	6.2	260	2.415	1.82
	6.2	445	2.648			12.0	241	2.382	
	12.0	427	2.630			18.0	232	2.366	
	18.0	408	2.611			24.0	219	2.340	
	24.0	390	2.591			30.0	206	2.314	
	30.0	370	2.568			36.0	194	2.288	
	36.0	355	2.550						
31.3	1.2	425	2.628	2.59	16.2	6.0	209	2.320	1.50
	6.0	408	2.611			12.0	202	2.305	
	12.0	382	2.582			18.0	187	2.272	
	18.0	356	2.551			24.0	179	2.253	
	24.0	342	2.534			30.0	170	2.230	
	30.0	329	2.517			36.0	160	2.204	
	36.0	320	2.505						
27.5	1.0	368	2.566	2.32	12.5	1.0	162	2.210	1.18
	5.8	351	2.546			6.6	150	2.176	
	11.8	340	2.532			12.0	148	2.170	
	17.8	323	2.509			18.0	138	2.140	
	23.8	303	2.481			24.0	129	2.111	
	29.8	288	2.459			30.0	123	2.090	
	35.8	272	2.435			36.0	118	2.072	
23.8	6.0	304	2.483	2.08	8.76	1.0	118	2.072	0.887
	12.0	290	2.462			6.0	113	2.053	
	18.4	273	2.436			12.0	108	2.033	
	24.0	258	2.412			18.0	99	1.996	
	30.0	252	2.401			24.0	92	1.964	
	36.0	236	2.373			30.0	88	1.944	
						36.0	84	1.924	

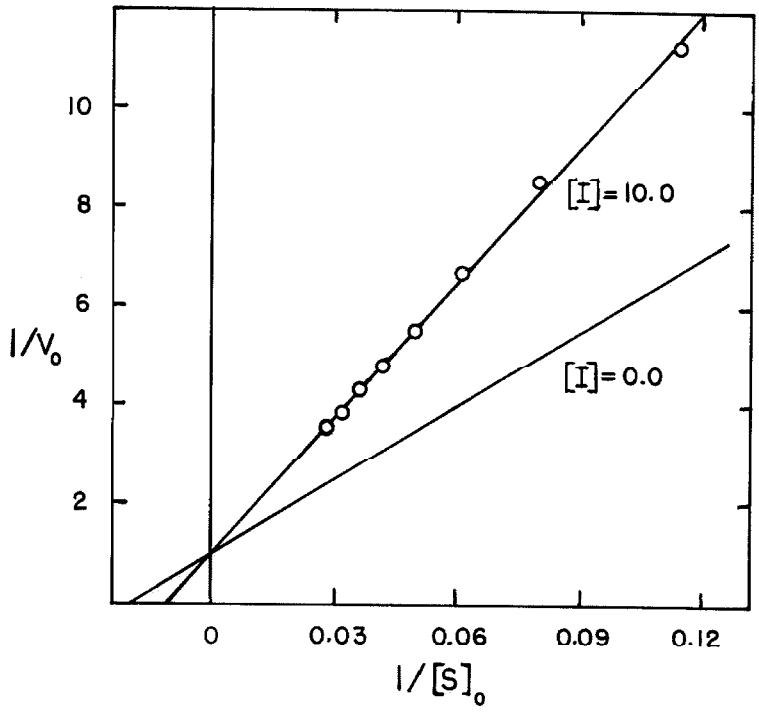


Fig. 9. Inhibition of hydrolysis of acetyl-L-tyrosine-hydroxamide by acetyl-D-tyrosinamide; v_o in units of 10^{-3} M·min.$^{-1}$; $(S)_o$ and (I) in units of 10^{-3} M; (E) = 0.0294 mg. protein-nitrogen per ml.

Apparent dissociation constants of acetyl-L-tyrosine hydroxamic acid.

A 2.00 ml. aliquot of a 0.01002 molar solution of acetyl-L-tyrosinehydroxamide was titrated with a 0.01075 N sodium hydroxide solution and the titration followed with a Beckman Model G pH meter. The treatment of these data is essentially that of Simms (56) in which the corrected equivalents of base, b', are plotted against the pH as in Fig. 11. A summary of this treatment is given below.

$b' = \frac{b + h - oh}{c}$, where b = concentration of strong base added (in this case the concentration of sodium ion), h = concentration of the hydrogen ion, oh = concentration of hydroxyl ion, and c = total concentration of the acid being titrated. Since h is negligible compared to b - oh in the pH range of this titration, then $b' = \frac{b - \frac{10^{pH - 14}}{\gamma_{OH^-}}}{c}$, and specifically if we let y equal the number of ml. of 0.01075 added to the 2.00 ml. of 0.01002 molar acid solution, then $b' = 0.536y - \frac{10^{pH-12.00} + y \cdot 10^{pH-12.30}}{\gamma_{OH^-}}$. The γ_{OH^-} values used are those listed by Lewis and Randall (57).

For the case of two monovalent acids, having apparent dissociation constants G_1' and G_2', $b' = \frac{[H^+](G_1' + G_2') + 2G_1'G_2'}{[H^+]^2 + [H^+](G_1' + G_2') + G_1'G_2'} = \alpha_1 + \alpha_2$, where $\alpha_1 = \frac{G_1'}{[H^+] + G_1'}$ and $\alpha_2 = \frac{G_2'}{[H^+] + G_2'}$. If the titration data involving b' as a function of pH for a divalent acid are treated according to the above equation for two monovalent acids, then the G_1' and G_2' calculated

from these data are called the titration constants of the divalent acid. These have been shown by Simms (56) to be related to the two apparent dissociation constants of the divalent acid, K_1' and K_2', by the equations, $K_1' = G_1' + G_2'$ and $\frac{1}{K_2'} = \frac{1}{G_1'} + \frac{1}{G_2'}$, where $K_1' = \frac{[H^+] \cdot m}{n}$, and $K_2' = \frac{[H^+]d}{m}$, with n representing the concentration of the undissociated acid, m representing the concentration of that form of the acid with one hydrogen ion removed, and d representing the concentration of that form of the acid with both hydrogens removed.

The calculation of G_1' and G_2' was performed as follows:

1) From the plot of b' vs. pH (Fig. 10) the approximate $p\,G_2'$ value was calculated. Thus when $pH = p\,G_2' = x + z$, with $p\,G_1' = x$, then $b' = \frac{3 + 10^{-z}}{2 + 2 \cdot 10^{-z}}$ and it is apparent that the larger the z value, the closer the b' value at which the $pH = p\,G_2'$ is to 1.5. z was estimated to be 1.2, giving a value of b' of 1.44 and, from the plot, an approximate $p\,G_2'$ value of 10.2.

2) Using this approximated value of $p\,G_2'$, α_2 values were calculated for pH values in the first buffer range (i.e., for values of b' near 0.5). Subtracting these α_2 values from their corresponding b' values, values of α_1 were calculated and from these, the corresponding $p\,G_1'$ values.

3) The average $p\,G_1'$ value was then used to calculate α_1 values at each pH in the second buffer range (i.e., for values of b' near

1.5). As in step two, the α_1 values were used to calculate the α_2 values and hence the pG_2' values.

Table XI summarizes the results of the above type calculations which lead to a pG_1' of 9.02 and a pG_2' of 10.19. The corresponding pK_1' and pK_2' values are thus 8.99 and 10.22 or 9.0 and 10.2. Assuming these values for pK_1' and pK_2', the theoretical curve representing b' as a function of pH has been drawn in Fig. 10. It is believed that the large deviation of the experimental points from this curve at pH values greater than 10.5 is due to the fact that a given error in the pH determination creates a larger and larger error in b' as the pH approaches a value of 12, and that at pH values above 10.5 the pH meter becomes inaccurate. A sodium ion correction was made according to the directions of the manufacturer (56), but this is admittedly only an approximation for pH values above 10.5. Theoretical curves for pK_a' values that differ from those above by $\pm$ 0.1 pK units indicate that the values, $pK_1' = 9.0$ and $pK_2' = 10.2$, can be considered accurate to within $\pm$ 0.1 pK units. A duplicate experiment gave experimental data that agree with those given in Table X to within $\pm$ 0.03 pH units and thus pK' values to within $\pm$ 0.1 pK units.

Table X

Potentiometric Titration of an Acetyl-L-tyrosinehydroxamide Solution.

Initial solution: 2.00 ml. of a 10.02×10^{-3} molar aqueous solution of acetyl-L-tyrosinehydroxamide. The water used in making the solution was boiled distilled water of pH 6.98.

Sodium hydroxide solution: Carbonate free 0.01075 N aqueous solution of sodium hydroxide.

Temperature = $25.0 \pm .3^{\circ}$ C.

ml. of NaOH solution	Total volume (ml.)	pH	b'	ml. of NaOH solution	Total volume (ml.)	pH	b'
0.000	2.000	6.38	0.000	2.298	4.298	9.92	1.214
0.100	2.100	7.75	0.054	2.398	4.398	9.98	1.266
0.200	2.200	8.06	0.107	2.501	4.501	10.04	1.316
0.232	2.232	8.14	0.124	2.597	4.597	10.10	1.362
0.271	2.271	8.21	0.145	2.702	4.702	10.16	1.413
0.302	2.302	8.28	0.162	2.798	4.798	10.22	1.459
0.352	2.352	8.35	0.189	2.903	4.903	10.28	1.507
0.402	2.402	8.41	0.216	2.998	4.998	10.34	1.551
0.503	2.503	8.52	0.270	3.098	5.098	10.40	1.594
0.600	2.600	8.62	0.321	3.198	5.198	10.45	1.639
0.706	2.706	8.72	0.377	3.298	5.298	10.50	1.681
0.804	2.804	8.78	0.430	3.498	5.498	10.59	1.763
0.900	2.900	8.90	0.482	3.704	5.704	10.70	1.835
0.951	2.951	8.95	0.509	3.897	5.897	10.82	1.885
1.036	3.036	9.02	0.554	3.997	5.997	10.86	1.907
1.103	3.103	9.07	0.590	4.098	6.098	10.92	1.923
1.203	3.203	9.14	0.644	4.293	6.293	11.00	1.961
1.300	3.300	9.22	0.695	4.498	6.498	11.08	1.984
1.402	3.402	9.31	0.750	4.696	6.696	11.16	1.984
1.501	3.501	9.39	0.802	5.004	7.004	11.27	1.964
1.604	3.604	9.44	0.856	5.309	7.309	11.34	1.970
1.707	3.707	9.50	0.910	5.615	7.615	11.40	1.968
1.802	3.802	9.58	0.960	6.502	8.502	11.54	1.866
1.896	3.896	9.65	1.008				
1.998	3.998	9.71	1.061				
2.098	4.098	9.81	1.113				
2.200	4.200	9.86	1.165				

Table XI

pK_1' and pK_2' for Acetyl-L-tyrosinehydroxamide.

$pK_1' = 9.0$
$pK_2' = 10.2$

pH	b'	α_2*	α_1	pG_1'	α_1**	α_2	pG_2'
8.72	0.377	0.032	0.345	9.00			
8.78	0.430	0.037	0.393	8.97			
8.90	0.482	0.048	0.434	9.02			
8.95	0.509	0.053	0.456	9.03			
9.02	0.554	0.062	0.492	9.03			
9.07	0.590	0.069	0.521	9.03			
9.14	0.644	0.080	0.564	9.03			
9.22	0.695	0.095	0.600	9.04			
			av. =	9.02			
9.98	1.266				0.901	0.365	10.22
10.04	1.316				0.913	0.403	10.21
10.10	1.362				0.934	0.428	10.22
10.16	1.413				0.933	0.480	10.20
10.22	1.459				0.941	0.518	10.19
10.28	1.507				0.948	0.559	10.18
10.34	1.551				0.954	0.597	10.17
10.40	1.594				0.960	0.634	10.16
						av. =	10.19

$$pK_1' = pG_1' - \log\left(1 + \frac{G_2'}{G_1'}\right) = 8.99 \doteq 9.0$$

$$pK_2' = pG_2' + \log\left(1 + \frac{G_2'}{G_1'}\right) = 10.22 \doteq 10.2$$

*Calculated from $\alpha_2 = \frac{G_2'}{[H^+] + G_2'}$ using approximate pG_2' of 10.2.

**Calculated from $\alpha_1 = \frac{G_1'}{[H^+] + G_2'}$ using average pG_2' value of 9.02.

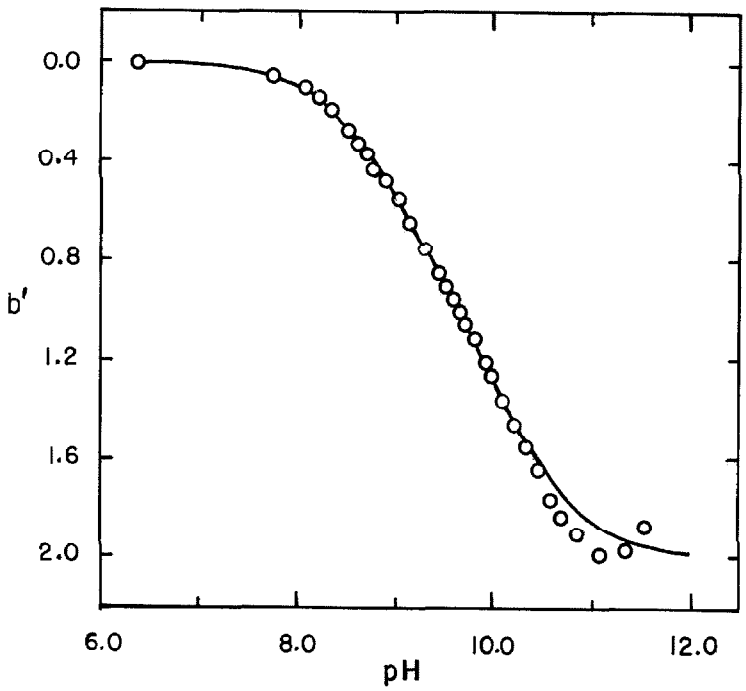


Fig. 10. Titration data for acetyl-L-tyrosinehydroxamide and the titration curve constructed on the basis of the constants: $pK_1' = 8.99$ and $pK_2' = 10.22$.

References

1. Landsteiner, K., "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1945.

2. Pressman, D., and Pauling, L., J. Am. Chem. Soc. 71, 2893 (1949) and preceding papers of this series (loc. cit.).

3. Karush, F., and Sonnenberg, M., J. Am. Chem. Soc. 71, 1369 (1949).

4. Klotz, I. M., Walker, F. M., and Pivan, R. B., J. Am. Chem. Soc. 68, 1486 (1946).

5. Klotz, I. M., and Urquhart, J. M., J. Am. Chem. Soc. 173, 21 (1949).

6. Klotz, I. M., and Urquhart, J. M., J. Am. Chem. Soc. 71, 847, 1597 (1949).

7. Teresi, J. D., and Luck, J. M., J. Biol. Chem. 174, 653 (1948).

8. Northrup, J. H., Kunitz, M., and Herriot, R. M., "Crystalline Enzymes," second edition, Columbia University Press, New York, 1948.

9. Neurath, H. and Schwert, G. W., Chem. Rev. 46, 69 (1950).

10. Thomas, D. W., Thesis, Calif. Inst. of Tech., 1951.

11. Huang, H. T., unpublished results.

12. Bergmann, M., and Fruton, J. S., J. Biol. Chem. 118, 405 (1937).

13. Bergmann, M., and Fruton, J. S., J. Biol. Chem. 124, 321 (1938).

14. Fruton, J. S., and Bergmann, M., J. Biol. Chem. 145, 253 (1942).

15. Bergmann, M., and Fruton, J. S., Adv. in Enzymol. 1, 63 (1941).

16. MacAllister, R. V., Harmon, K. M., and Niemann, C., J. Biol. Chem. 177, 767 (1949).

17. MacAllister, R. V., and Niemann, C., J. Am. Chem. Soc. 71, 3854 (1949).

18. Iselin, B. M., Huang, H. T., MacAllister, R. V., and Niemann, C., J. Am. Chem. Soc. 72, 1729 (1950).

19. Iselin, B. M., and Niemann, C., J. Biol. Chem. 182, 821 (1950).

20. Iselin, B. M., Huang, H. T., and Niemann, C., J. Biol. Chem. 183, 403 (1950).

21. Huang, H. T., and Niemann, C., J. Am. Chem. Soc. 73, 1541 (1951).

22. Thomas, D. W., MacAllister, R. V., and Niemann, C., J. Am. Chem. Soc. 73, 1548 (1951).

23. Foster, R. S., and Niemann, C., J. Am. Chem. Soc. 73, 1552 (1951).

24. Huang, H. T., and Niemann, C., J. Am. Chem. Soc. 73, 1555 (1951).

25. Huang, H. T., and Niemann, C., J. Am. Chem. Soc. 73, 3223 (1951).

26. Huang, H. T., and Niemann, C., J. Am. Chem. Soc. 73, 3228 (1951).

27. Huang, H. T., MacAllister, R. V., Thomas, D. W., and Niemann, C., J. Am. Chem. Soc. 73, 3231 (1951).

28. Shine, H. J., and Niemann, C., J. Am. Chem. Soc. 74, 97 (1952).

29. Huang, H. T., and Niemann, C., J. Am. Chem. Soc. 74, 101 (1952).

30. Huang, H. T., Foster, R. J., and Niemann, C., J. Am. Chem. Soc. 74, 105 (1952).

31. Pauling, L., Am. Scientist 36, 51 (1948).

32. MacAllister, R. V., Thesis, Calif. Inst. of Tech., 1949.

33. Kaufman, S., and Neurath, H., J. Biol. Chem. 180, 181 (1949).

34. Kaufman, S., Neurath, H., and Schwert, G. W., J. Biol. Chem. 177, 793 (1949).

35. Manning, D., and Niemann, C., unpublished results.

36. Goldenberg, H., Goldenberg, V., and McLaren, A. D., Biochim. Biophys. Acta 7, 110 (1951).

37. Kaufman, S., Neurath, H., and Schwert, G. W., J. Biol. Chem. 177, 793 (1949).

38. Haldane, J. B. S., "Enzymes," Longmans, Green and Company, London, New York, Toronto, 1930.

39. Wilson, P. H., in "Respiratory Enzymes," by H. A. Lardy, p. 16 ff. Burgess Publishing Company, Minneapolis, 1949.

40. Lineweaver, H., and Burk, D., J. Am. Chem. Soc. 56, 658 (1934).

41. Snoke, J. E., and Neurath, H., Arch. Biochem. 21, 351 (1949).

42. Jansen, E. F., Fellows-Nutting, M. D., Jang, R., and Balls, A. K., J. Biol. Chem. 185, 209 (1950).

43. Foster, R. J., Thesis, Calif. Inst. of Tech., 1952.

44. Cohn, E. J., and Edsall, J. T., "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions." Reinhold Publishing Corporation, New York, 1943.

45. Oliveri-Mandala, E., Gazz. chim. ital. 40, I, 102 (1910).

46. Schwert, G. W., and Kaufman, S., J. Biol. Chem. 190, 807 (1951).

47. Smith, E. L., Brown, D. M., and Kaskowski, M., J. Biol. Chem. 191, 639 (1951).

48. Jansen, E. F., Fellows-Nutting, M. D., and Balls, A. K., J. Biol. Chem. 179, 201 (1949).

49. Hartley, B. S., and Kilby, B. A., Biochem. J. 50, 672 (1952).

50. Jansen, E. F., Curl, A. L., and Balls, A. K., J. Biol. Chem. 190, 557 (1951).

51. Jansen, E. F., Fellows-Nutting, M. D., Jang, R., and Balls, A.K., J. Biol. Chem. 179, 189 (1949).

52. All melting point values are corrected.

53. Microanalyses by Dr. A. Elek.

54. Niemann, C., and McCasland, G. E., J. Am. Chem. Soc. 66, 1870 (1944).

55. du Vigneaud, V., and Meyer, C. E., J. Biol. Chem. 98, 295 (1932).

56. Simms, H., J. Am. Chem. Soc. 48, 1239 (1926).

57. Lewis, G. N., and Randall, M., "Thermodynamics and Free Energy of Chemical Substances," p. 382. McGraw-Hill Book Company, Inc., New York and London, 1923.

58. Beckman Bulletin 85-A.

II. Genetic Factors Influencing the Activity of Tryptophane Desmolase in Neurospora crassa

Introduction

The assumption that physiological reactions are subject to genetic control has been inherent in the thinking of most geneticists for many years and has received substantial support from numerous biochemical and genetical investigations (1). In many instances it has been possible to show that as a result of a single gene change a block in a single metabolic reaction has occurred. As many metabolic reactions have been shown to be enzyme-catalysed, these observations have led to the hypothesis that a single gene is concerned in a primary way with the formation of only one enzyme. The evidence supporting such a hypothesis is largely of an indirect nature, our direct knowledge of any relationship between genes and enzymes being very limited indeed. The hypothesis can thus best be regarded as a working one which serves to focus our attention on the possibility of a direct relationship between enzymes and genes and which may help in planning experiments designed to define more clearly such a relationship. That at least has been the view adopted in regard to the investigations described in this section of the thesis — investigations which were initiated in the hope of increasing our direct knowledge of enzyme-gene relationships.

The enzyme system utilized in these investigations is the tryptophane desmolase of Neurospora crassa, the enzyme which catalyses the formation of L-tryptophane from indole and L-serine. In 1944 Tatum and Bonner (2) reported on some experiments with whole mycelia of Neurospora which strongly indicated that L-tryptophane was synthesized via the coupling of indole and L-serine. These experiments led to the more conclusive work of Umbreit, Wood and Gunsalus (3), who showed that cell-free extracts of Neurospora could catalyse the synthesis of tryptophane from indole and DL-serine. These workers also reported good evidence that pyridoxal phosphate was required as a cofactor in this catalysis. More recently Mitchell and Lein (4), Gordon and Mitchell (5), Nason et al.(6), and Yanofsky (7, 8) have confirmed and extended this work.

The effect of a genetic change on tryptophane desmolase activity in Neurospora has also been reported. Mitchell and Lein (4) reported that cell-free preparations from a mutant of Neurospora (strain C83) which is characterized by a tryptophane requirement that cannot be satisfied by indole, do not contain measurable tryptophane desmolase activity. A later report (5) gave evidence for tryptophane desmolase activity in this mutant but has been retracted (9). In repeated experiments to be described later in this thesis no tryptophane desmolase activity could be found in the C83 strain. This lack of activity has been confirmed by Yanofsky (8).

Yanofsky (8) has also been unable to measure any tryptophane desmolase activity in another mutant, strain S1952, which is also characterized by a tryptophane requirement which cannot be satisfied by indole. The available evidence (8) indicates that the C83 and S1952 mutant genes are allelic. Yanofsky has described a suppressor which partially suppresses the tryptophane requirement of S1952 but does not suppress the requirement of C83. Furthermore, he found that tryptophane desmolase activity could be detected in extracts of S1952-su but not in the extracts of C83-su, and that this tryptophane desmolase from S1952-su could not be distinguished from that derived from wild type strains on the basis of kinetic criteria.

Many biochemical mutants of Neurospora are capable of back-mutations (10) — cases in which a single gene change at the mutant locus has resulted in the mutant's becoming wild type in so far as its growth requirements are concerned. This ability of a mutant to backmutate furnishes strong evidence that the mutant does not represent a deficiency in the chromosomal material. Hence if the C83 mutant were capable of backmutation, this fact would indicate that the mutation did not arise as the result of a deficiency.

The question of whether or not the C83 mutant represents a deficiency has a bearing on the suppressor experiments of Yanofsky. Thus one possible interpretation of the results of these experiments is that the suppressor gene in some manner (such as by shifting the balance

among the various reactions which occur within the cell) allows the S1952 mutant gene to carry on, at least qualitatively, the normal functions of the wild type allele. If the C83 mutant should represent a deficiency, then it would be impossible under any shift of conditions within the cell (other than the restoration of the deleted chromosomal material) for the C83 mutant gene to carry on the function of the wild type allele. Hence the suppressor would not be able to eliminate the L-tryptophane requirement of the C83 mutant.

The above discussion indicates one reason why experiments designed to test the ability of the C83 mutant to backmutate were initiated. A second reason for undertaking such experiments is that if the backmutation were found to occur, it would provide another situation in which the effect of a single gene change on a specific enzyme system (tryptophane desmolase) could be studied.

It has been possible in this work to demonstrate the backmutation of the C83 mutant. Furthermore, such a backmutated strain was found to contain tryptophane desmolase activity. In view of these facts a study of the kinetics of the tryptophane desmolase systems from both the backmutated strain and a wild type strain was performed to see if any differences in the two enzyme systems could be detected — differences that could conceivably have arisen in the mutation-backmutation process.

Mitchell (11) has found that cell-free extracts of several mutant strains of Neurospora (tryptophaneless — 10575; nicotinicless — 3416;

histidineless — C84; and succinicless — B1318) have specific tryptophane desmolase activities that are two to three times those found in wild type (8a, 25A, and Abbott 4A) extracts. One of these mutant strains, histidineless — C84, is considered here in more detail. The C84 mutant has a histidine requirement that cannot be satisfied by any other substance that has been tested (12). From data concerning the accumulation of various imidazoles by a series of histidine mutants (12) it seems probable that one result of the C84 mutation is a block in a single reaction leading to the formation of histidine. Thus it is probable that the C84 mutant gene has caused a rather drastic alteration of the activity of some enzyme system associated with the synthesis of histidine. The fact that extracts of the C84 mutant strain contain higher than normal (or wild type) tryptophane desmolase activity certainly indicates that here we may have a case in which a single gene change has affected the activity of more than one enzyme.

It is of course possible that some other gene (or genes) in the C84 strain is responsible for this alteration in tryptophane desmolase activity. An attempt has therefore been made to determine whether or not the C84 gene does influence the activity of the tryptophane desmolase system. A cross of the C84 mutant to a wild type strain, 8a (12), was made and the progeny investigated for tryptophane desmolase activity. Thus if the C84 gene is primarily responsible for the alteration of enzyme activity, it would be expected that all of the histidine-dependent

progeny (C84) would have tryptophane desmolase activities different from those of the wild type parent, whereas the wild type progeny should have the normal parent wild type activity. If some other gene is responsible for the alteration of enzyme activity, it would be expected that some of the wild type progeny would have altered tryptophane desmolase activity and that some of the histidine-dependent progeny would have the normal activity of the wild type parent. The results of these experiments and of those dealing with the backmutation of the C83 mutant are given in the following pages.

The Partial Purification and Properties of Tryptophane Desmolase

Preceding any analysis of the effect of gene change on tryptophane desmolase activity, the properties of this enzyme system obtained from some reference strain must be defined.

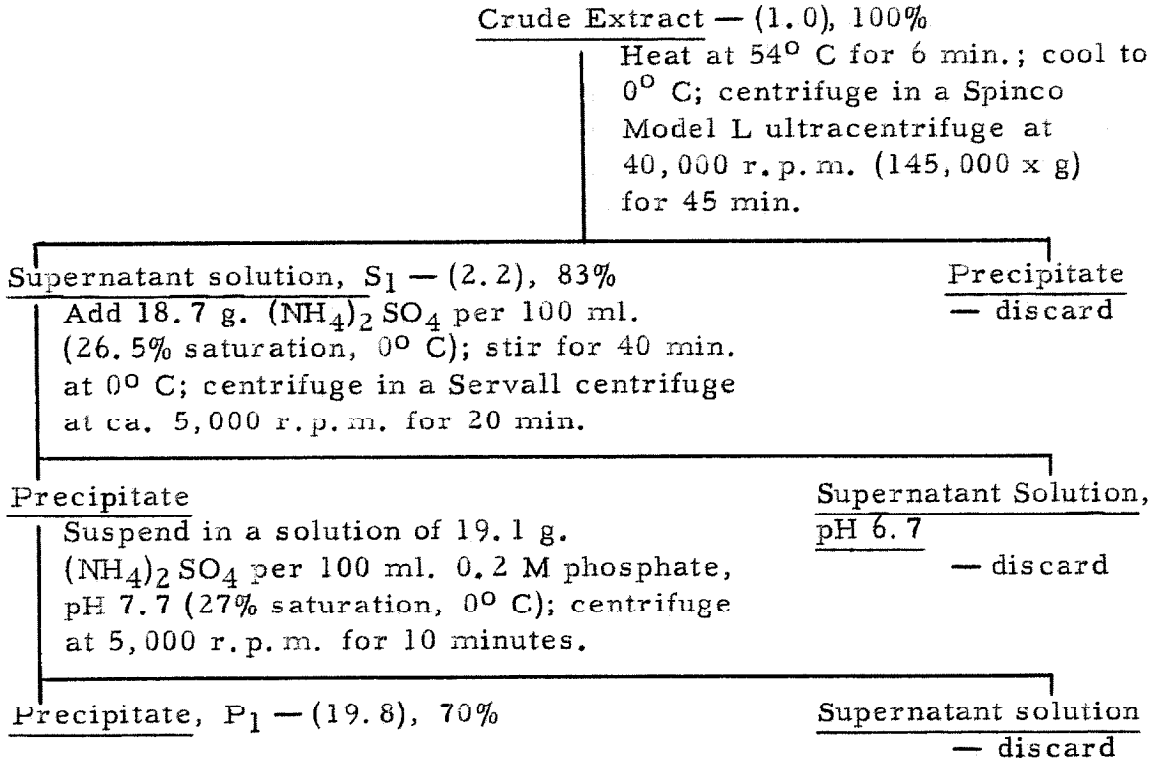
In these studies the reference strain used was a double mutant (kindly supplied by Dr. T. H. Pittenger) of albino 15300 and C102. The C102 mutant is phenotypically wild at 25° C but "colonial" at temperatures above 31° C (13). This particular double mutant was chosen as the reference strain because the 15300 and C102 mutants are used as markers in the backmutation studies of C83 which will be described later.

Purification — A partial purification of the crude extract from this strain was accomplished by a heat treatment that was followed by ammonium sulfate precipitation. The details of the purification procedure are shown in Table I. A 15- to 23-fold purification was generally obtained by this procedure.

The procedure of heating at 54° C for six minutes accomplished the following two objects: (1) it increased the purification factor and (2) it increased the stability of the enzyme preparations. The relative stability at 3° C of heated and unheated preparations comparable to S_1 of Table I is shown in Table II. Heating preparations at 54° C for longer periods of time than six minutes increases the stability somewhat but is not profitable because of the high loss of activity during the heat

Table I

Purification Scheme for Crude Tryptophane Desmolase Extracts of C102-15300.



The purification — specific activity (initial rate of indole disappearance in $M \cdot (min.)^{-1}$ (mg. protein-nitrogen per ml.)$^{-1}$) relative to that of the crude extract — obtained in a representative run is indicated in parentheses and is followed by the per cent total activity recovered. Unless otherwise indicated, all operations carried out at 0-4° C. See Experimental section for preparation of crude extract.

treatment. It seems probable that the increase in stability due to heating arises from a denaturation of enzyme systems, such as proteases, which can destroy the tryptophane desmolase activity.

All enzyme extracts, as well as the mycelium, can be kept in a frozen

Table II

Stability of Heated and Unheated Preparations, S_1

Hours at 3° C	Per Cent Total Activity of Crude Extract	
	Heated at 54° C for 6 min.	Not heated
0	85%	101%
24	71	42
48	37	11
72	29	4
96	21	< 1

state at -16° C for at least two weeks without any loss of tryptophane desmolase activity.

No protein is precipitated from fraction S_1 below 20 per cent ammonium sulfate saturation (0° C). Furthermore, the per cent total activity precipitated and the per cent total protein-nitrogen precipitated are both proportional to the percentage ammonium sulfate saturation between 20% and 26.5% saturation (0° C). Hence preliminary fractionation below 26.5% saturation does not increase the purification. The specific activity of the partially purified preparation, P_1, could not be appreciably increased by further ammonium sulfate fractionation at pH 6.6 or pH 7.4; by calcium phosphate absorption at pH 7 or pH 8; or by precipitation resulting from increasing the hydrogen ion activity.

Stoichiometry — In the presence of the tryptophane desmolase fraction P_1 of Table I the rate of indole disappearance and the rate of tryptophane appearance are within experimental error (Fig. 1).

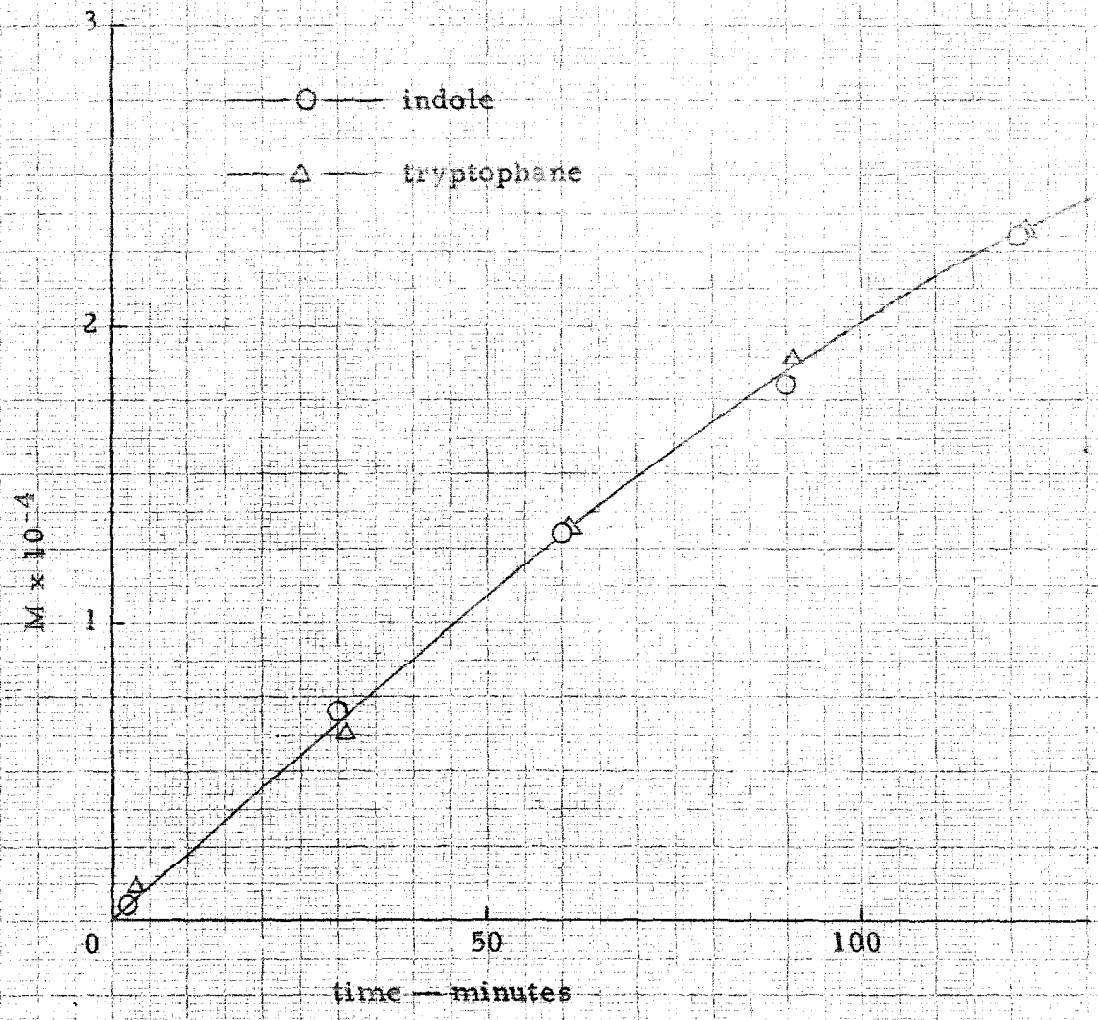


Fig. 1. Indole disappearance and tryptophane appearance as a function of time. The ordinate represents the concentration of the tryptophane formed or the difference between the initial concentration of indole and the concentration at time, t.

Because of the greater ease and accuracy of the indole determination over that of the tryptophane, the activity of the tryptophane desmolase was generally determined by measuring the disappearance of indole. The rate of L-serine disappearance was found to be 2.2 times that of indole. It is probable that this higher rate of L-serine disappearance is due to the presence of enzyme systems other than tryptophane desmolase which attack L-serine (e.g., serine deaminase (7, 14)).

Indole disappearance cannot be effected by the tryptophane desmolase fraction P_1 unless both L-serine and pyridoxal phosphate are present. It is evident from the results presented in Table III that D-serine is effectively inert in the tryptophane desmolase system, functioning neither as a substrate nor as an inhibitor. The small rate of indole disappearance in the presence of D-serine can be explained on the basis of a 1% impurity of L-serine in the D-serine sample.

These results confirm the work of Tatum and Bonner (2) on whole mycelia in which they observed that on a molar basis the activity of DL-serine was one half that of L-serine in so far as the rate of indole uptake was concerned. They also observed that biological assay with Neurospora strain 10575 of the tryptophane produced during the reaction gave results that were in good agreement with a chemical determination of the tryptophane. Since 10575 cannot utilize D-tryptophane for growth, they concluded that the product was L-tryptophane.

The reaction catalysed by tryptophane desmolase is evidently

Table III

The Effect of D-Serine on Tryptophane Desmolase Catalysis
(Strain C102-15300)

Initial conc. of L-serine, M	Initial conc. of D-serine, M	$v_o \times 10^6$
0	0	0.00
16×10^{-3}	0	3.26
0	16×10^{-3}	0.31
7.7×10^{-3}	0	2.50
7.7×10^{-3}	80×10^{-3}	2.52
80×10^{-3}	0	4.58
80×10^{-3}	80×10^{-3}	4.68

v_o is the initial velocity of indole disappearance in $M \cdot min.^{-1}$ at pH 7.6 and 37.0° C. Initial concentrations are indole, 4.74×10^{-4} M; pyridoxal phosphate, 4.6×10^{-5} M; 0.2 M phosphate buffer; and 18 μg. of protein-nitrogen/ml.

irreversible. No indole could be detected four hours after an active preparation of tryptophane desmolase was added to a solution of L-tryptophane and pyridoxal phosphate.

pH Optimum — The dependence of tryptophane desmolase activity on the hydrogen ion activity was determined over the range of pH values of 6.5—8.4. The results of this determination are given in Fig. 8. It is apparent that the pH optimum is approximately 7.6; consequently, all further activity measurements were carried out at this pH value. This curve of relative activity vs. pH is similar to that obtained by Yanofsky (7) for tryptophane desmolase from the wild type

strain Em-5256, and to that obtained by Mitchell and Gordon (15) for the enzyme from wild type strain 8a. The data of Yanofsky indicate that the optimum pH is approximately 7.8, while those of Mitchell and Gordon indicate that the optimum is at a pH value of 7.7. Considering the nature of the curves and their accuracy, all of these optimal values — 7.6, 7.7 and 7.8 — may be considered within the range of experimental error.

Kinetics — From a consideration of the purity obtained for the tryptophane desmolase of Neurospora it is apparent that a kinetic study of the tryptophane desmolase catalysed reaction cannot be as quantitative and complete as the kinetic work on α-chymotrypsin catalysed reactions described in Part I of this thesis. In fact the primary motive behind this study of tryptophane desmolase kinetics was to establish conditions that would enable the comparison of two tryptophane desmolase systems isolated from different strains of Neurospora through the use of empirical kinetic "constants." Thus no particular attempt has been made to interpret the mechanism of the reaction on the bases of the kinetic data obtained.

The formulation of the kinetics developed in the following paragraphs should therefore be regarded as speculation, based mainly upon what is known of enzyme reactions in general (see p. 6, Part I of this thesis and the references given there), that may give us some idea of what the empirical "constants" represent and to what extent they may

be dependent upon the variables of the reaction.

It is assumed that the overall reaction that is catalysed by the tryptophane desmolase system is the following:

$$\text{L-serine} + \text{indole} \xrightarrow[\text{pyridoxal phosphate}]{\text{tryptophane desmolase}} \text{L-tryptophane} + \text{water}$$

Thus the total concentration of the apoenzyme and the coenzyme (pyridoxal phosphate) are assumed not to change during the reaction. This then represents an enzyme catalysed reaction involving two substrates and a coenzyme. Although some papers have appeared which analyse the kinetics of the simpler two substrate or one substrate—one coenzyme reactions (16, 17, 18), no analysis of the kinetics of the two substrate—one coenzyme reaction has been reported. The basic concept that the enzyme and substrates (and coenzyme, if present) must unite to form an intermediate complex in order that catalysis can occur is inherent in the treatment of both the single substrate system (see Part I of this thesis) and the two substrate or one substrate—one coenzyme systems (16, 17, 18). This concept is also used in the following formulation of the kinetics of the tryptophane desmolase system.

It is assumed that the indole, L-serine and pyridoxal phosphate can unite with the enzyme at individual specific sites on the enzyme surface. It is further assumed that each molecule can unite at its specific site whether or not the other sites are occupied by their respective molecules, but that the affinity of each molecule to its site

may be influenced by the presence or absence of the other molecules on their sites. The only enzyme complex which is assumed to be capable of breaking down to form L-tryptophane is the enzyme — indole — L-serine — pyridoxal phosphate complex. The following mechanism is based upon these primary assumptions:

$$E_f + C_f \overset{K_1}{\rightleftharpoons} EC \qquad ES_1 + S_{2_f} \overset{K_7}{\rightleftharpoons} ES_1S_2$$

$$E_f + S_{1_f} \overset{K_2}{\rightleftharpoons} ES_1 \qquad ES_2 + C_f \overset{K_8}{\rightleftharpoons} ECS_2$$

$$E_f + S_{2_f} \overset{K_3}{\rightleftharpoons} ES_2 \qquad ES_2 + S_{1_f} \overset{K_9}{\rightleftharpoons} ES_1S_2$$

$$EC + S_{1_f} \overset{K_4}{\rightleftharpoons} ECS_1 \qquad ECS_1 + S_{2_f} \overset{K_{10}}{\rightleftharpoons} ECS_1S_2$$

$$EC + S_{2_f} \overset{K_5}{\rightleftharpoons} ECS_2 \qquad ECS_2 + S_{1_f} \overset{K_{11}}{\rightleftharpoons} ECS_1S_2$$

$$ES_1 + C_f \overset{K_6}{\rightleftharpoons} ECS_1 \qquad ES_1S_2 + C_f \overset{K_{12}}{\rightleftharpoons} ECS_1S_2$$

$$ECS_1S_2 \xrightarrow{k} E_f + C_f + P_{1_f} + P_{2_f}$$

where E_f = free enzyme,

C_f = free pyridoxal phosphate,

S_{1_f} = free indole,

S_{2_f} = free L-serine,

P_{1_f} = free L-tryptophane,

P_{2_f} = free water,

the K's represent the dissociation constants for the various enzyme complexes, and k represents the first order reaction constant for

the irreversible breakdown of ECS_1S_2 into the products of the reaction. Since only the initial reaction velocities will be considered in this treatment, the possible competitive inhibition by the product, L-tryptophane, has not been included in the above mechanism.

The total concentrations of the enzyme (E), the indole (S_1), the L-serine (S_2), and the pyridoxal phosphate (C) are given by the following equations:

$$(E) = (E_f) + (ES_1) + (ES_2) + (EC) + (ECS_1) + (ECS_2) + (ES_1S_2) + (ECS_1S_2),$$

$$(S_1) = (S_{1_f}) + (ES_1) + (ECS_1) + (ES_1S_2) + (ECS_1S_2),$$

$$(S_2) = (S_{2_f}) + (ES_2) + (ECS_2) + (ES_1S_2) + (ECS_1S_2), \quad \text{and}$$

$$(C) = (C_f) + (EC) + (ECS_1) + (ECS_2) + (ECS_1S_2),$$

where the parentheses indicate concentrations.

In the kinetic experiments to be described in the following pages, the initial reaction velocity was obtained from the change of total indole concentration (S_1) with time. Hence we desire an expression for $\frac{d(S_1)}{dt}$ that contains the quantities (E), (S_1), (S_2) and (C). From a consideration of the above mechanism it is apparent that

$$(1) \qquad -\frac{d(S_1)}{dt} = -\frac{d(S_2)}{dt} = \frac{d(P1)}{dt} = k(ECS_1S_2).$$

If it is assumed that all of the enzyme complexes are in equilibrium with their respective components and that all components of the reaction have unit activity coefficients, then $K_1 = \frac{(E_f)(C_f)}{(EC)}$, $K_2 = \frac{(E_f)(S1_f)}{(ES_1)}$,

$K_3 = \frac{(E_f)(S_{2_f})}{(ES_2)}$, etc. These dissociation constants are then related to each other by the following set of equations:

(2) $K_1K_4 = K_2K_6$

(3) $K_1K_5 = K_3K_8$

(4) $K_2K_7 = K_3K_9$

(5) $K_8K_{11} = K_9K_{12}$

(6) $K_4K_{10} = K_5K_{11}$.

Using these dissociation constants, (ECS_1S_2) can be given in terms of (E_f), (C_f), (S_{1_f}), (S_{2_f}) and seven of the twelve dissociation constants (equation 7).

(7) $$(ECS_1S_2) = (E)(C_f)(S_{1_f})(S_{2_f}) / \left[K_3K_8K_{11} + K_5K_{11}(C_f) + K_7K_{12}(S_{1_f}) + K_8K_{11}(S_{2_f}) + K_{10}(C_f)(S_{1_f}) + K_{11}(C_f)(S_{2_f}) + K_{12}(S_{1_f})(S_{2_f}) + (C_f)(S_{1_f})(S_{2_f})\right]$$

It is apparent that the particular dissociation constants used in equation 7 may be varied in accordance with equations 2-6.

If it is now assumed that $(C) >> (E)$, that $(S_1) >> (E)$, and that $(S_2) >> (E)$, then (C_f), (S_{1_f}) and (S_{2_f}) can be approximated by (C), (S_1) and (S_2) respectively. With the use of these approximations, equation 8 can be derived from equations 1 and 7.

(8) $$v_o = \left[-\frac{d(S_1)}{dt}\right]_o = k(E)(C)(S_1)_o(S_2)_o / \left[K_3K_8K_{11} + K_5K_{11}(C) + K_7K_{12}(S_1)_o + K_8K_{11}(S_2)_o + K_{10}(C)(S_1)_o + K_{11}(C)(S_2)_o + K_{12}(S_1)_o(S_2)_o + (C)(S_1)_o(S_2)_o\right]$$

The subscript zeros represent the time, t = o. The reciprocal of equation 8 is given below.

$$(9) \qquad \frac{1}{v_o} = \frac{K_{11}\beta}{k(E)} \cdot \frac{1}{(S_1)_o} + \frac{\gamma}{k(E)}$$

$$\text{with } \beta = 1 + \frac{K_3K_8}{(S_2)_o(C)} + \frac{K_5}{(S_2)_o} + \frac{K_8}{(C)}$$

$$\text{and } \gamma = 1 + \frac{K_7K_{12}}{(S_2)_o(C)} + \frac{K_{10}}{(S_2)_o} + \frac{K_{12}}{(C)}$$

A plot of $\frac{1}{v_o}$ versus $\frac{1}{(S_1)_o}$ should then yield a straight line if (E), (C) and $(S_2)_o$ are held constant. The slope of such a line would be $\frac{K_{11}\beta}{k(E)}$ and its intercept with the $\frac{1}{v_o}$ axis (at $\frac{1}{(S_1)_o}$ = o) would be $\frac{\gamma}{k(E)}$. If the slope is divided by the intercept, we get a quantity which is independent of the enzyme concentration, namely $\frac{K_{11}\beta}{\gamma}$. This quantity we shall call the apparent Michaelis constant for indole, K_{mS_1}.

This method of determining the apparent Michaelis constant is identical with the procedure originally devised by Lineweaver and Burk (19) for the determination of the Michaelis constant of a single substrate reaction (also see Part I of this thesis). However, in the case of a single substrate the Michaelis constant is a definite kinetic constant, whereas for the case at hand it is apparent that unless some relationship between the dissociation constants independent of equations 2-6 is assumed, then K_{mS_1} is dependent upon (C) and $(S_2)_o$.

The apparent Michaelis constants for L-serine and pyridoxal

phosphate, $K_{m_{S_2}}$ and K_{m_C} respectively, are similarly dependent upon the concentrations of indole and pyridoxal phosphate ($K_{m_{S_2}}$) or of indole and L-serine (K_{m_C}). Thus $K_{m_{S_2}} = K_{10}\delta/\epsilon$ and $K_{m_C} = K_{12}\theta/\lambda$,

where $$\delta = 1 + \frac{K_1K_4}{(C)(S_1)_o} + \frac{K_4}{(S_1)_o} + \frac{K_6}{(C)},$$

$$\epsilon = 1 + \frac{K_9K_{12}}{(S_1)_o(C)} + \frac{K_{11}}{(S_1)_o} + \frac{K_{12}}{(C)},$$

$$\theta = 1 + \frac{K_3K_9}{(S_2)_o(S_1)_o} + \frac{K_9}{(S_1)_o} + \frac{K_7}{(S_2)_o},$$

and $$\lambda = 1 + \frac{K_5K_{11}}{(S_2)_o(S_1)_o} + \frac{K_{11}}{(S_1)_o} + \frac{K_{10}}{(S_2)_o}.$$

If, in the determination of one apparent K_m — say $K_{m_{S_1}}$ — (C) and $(S_2)_o$ are large relative to their apparent K_m's, one might say that $K_{m_{S_1}}$ is then approximately equal to K_{11}. Certainly as (C) and $(S_2)_o$ approach infinity, $K_{m_{S_1}}$ approaches K_{11}. However this approximate interpretation, when applied to all of the apparent Michaelis constants, assumes that all of the dissociation constants associated with S_{2_f} (i.e., K_3, K_5, K_7 and K_{10}) are of the same order of magnitude; that all those associated with C_f (i.e., K_1, K_6, K_8 and K_{12}) are of the same order of magnitude; and that all those associated with S_{1_f} (i.e., K_2, K_4, K_9 and K_{11}) are of the same order of magnitude.

A more drastic assumption would be that $K_1 = K_6 = K_8 = K_{12} = K_C$; that $K_2 = K_4 = K_9 = K_{11} = K_{S_1}$; and that $K_3 = K_5 = K_7 = K_{10} = K_{S_2}$. This assumption implies that each substrate and the coenzyme has a specific site on the enzyme surface and that its affinity for this site is independent

of the presence or absence of the other substrates or the coenzyme on their respective sites. If this assumption is made, then the rate equation 8 becomes

$$(10) \qquad v_o = \frac{k(E)(C)(S_1)_o(S_2)_o}{\left[K_C + C\right]\left[K_{S_1} + (S_1)_o\right]\left[K_{S_2} + (S_2)_o\right]},$$

and the reciprocal equation 9 becomes

$$(11) \qquad \frac{1}{v_o} = \frac{K_{S_1}}{k(E)} \cdot \left(\frac{K_C}{(C)} + 1\right) \cdot \left(\frac{K_{S_2}}{(S_2)_o} + 1\right) \cdot \frac{1}{(S_1)_o}$$

$$+ \frac{1}{k(E)} \cdot \left(\frac{K_C}{(C)} + 1\right) \cdot \left(\frac{K_{S_2}}{(S_2)_o} + 1\right).$$

In this case then $K_{m_{S_1}} = K_{S_1}$, $K_{m_{S_2}} = K_{S_2}$, and $K_{m_C} = K_C$. Thus the apparent Michaelis constants would represent the enzyme-substrate (or enzyme-coenzyme) dissociation constants.

In any case it is evident that the determination of an apparent K_m value under only one condition of the concentrations of the other substrates (or substrate and coenzyme) cannot tell us which set of approximating assumptions can validly be applied and thus cannot give any assurance about the proper interpretation of the apparent K_m value. However, such single determinations of apparent K_m values can reasonably be expected to give us empirical kinetic "constants" which are characteristic of the enzyme system under specifically stated conditions of the concentrations of the other components of the reaction. Thus it should be possible to pick up any large differences in substrate-enzyme

or pyridoxal phosphate-enzyme affinities by a comparison of apparent K_m values of two tryptophane desmolase preparations from different sources.

Preliminary to an investigation of the apparent K_m values of indole, L-serine and pyridoxal phosphate for the partially purified tryptophane desmolase, P_1, from the C102-15300 strain, an investigation of the dependence of the initial velocities on the enzyme concentration was performed. It can be seen from Fig. 2 that the dependence is linear over the range of enzyme concentrations investigated.

This linear dependence of v_o on enzyme concentration is what would be predicted from equation 8 and is indicative that for this experiment the assumption that $(S_1)_o$, $(S_2)_o$ and (C) are much greater than (E) is valid. A non-linear dependence of v_o on (E) would be expected if the assumption did not hold (17, 20, 21). It should also be noted that crude extracts of tryptophane desmolase from C102-15300, from wild type 8a and from the histidineless strain C84 show this linear relationship between v_o and the concentration of the enzyme. Thus in all these strains it is possible to assay for the enzyme in crude extracts by measuring the initial velocity of the reaction at standard values of the pH, temperature, and of the indole, serine and pyridoxal phosphate concentrations (see Experimental).

The determination of the apparent K_m values for indole, L-serine and pyridoxal phosphate was made as indicated on page 86. Typical $\frac{1}{v_o}$

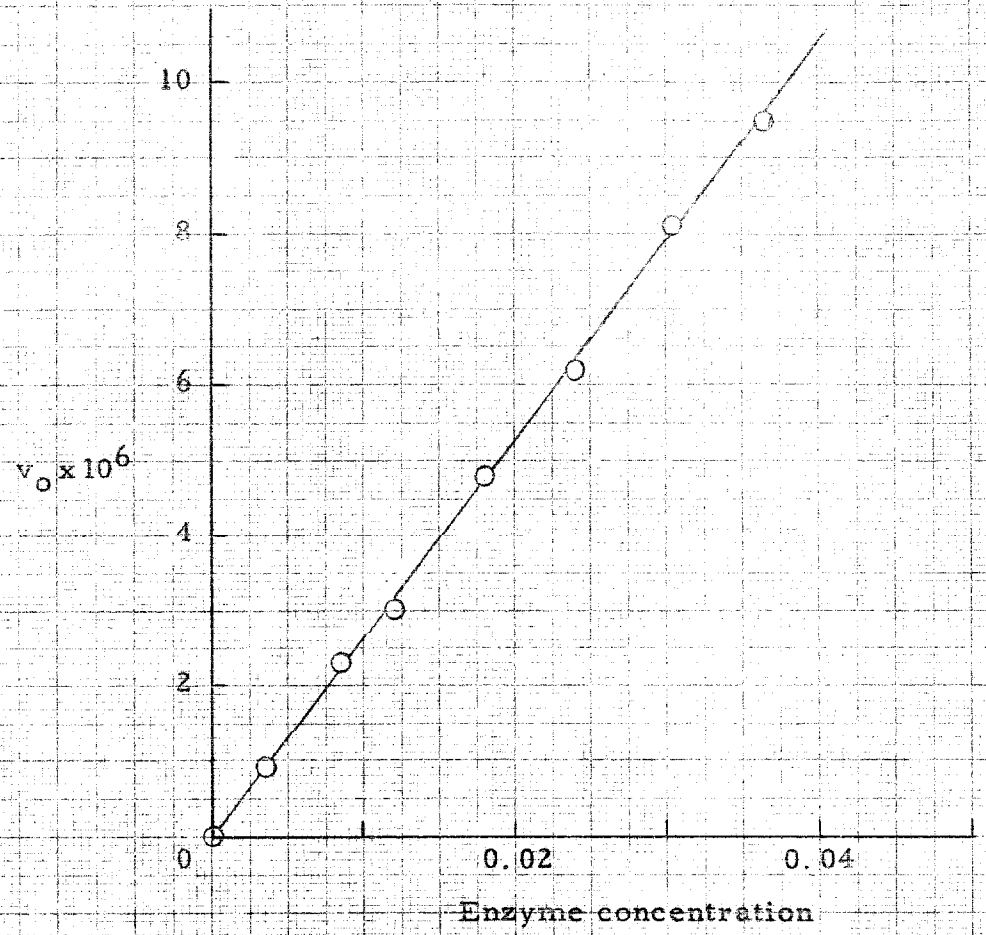


Fig. 2. Dependence of v_o upon enzyme concentration at 37.0° C and pH 7.6 for fraction P_1 from C102-15300. v_o is given in $M \cdot min.^{-1}$ and enzyme concentration in mg. protein-nitrogen per ml. Initial concentrations are indole, 4.74×10^{-4} M; DL-serine, 0.171 M; and pyridoxal phosphate, 4.6×10^{-5} M.

versus $\frac{1}{(S)_o}$ plots for these three components are given in Figs. 3-5 and the apparent K_m values are given in Table IV.

Table IV

Apparent K_m Values for Tryptophane Desmolase from Strain C102-15300 at 37.0° C in 0.2 M Phosphate Buffer, pH 7.6.

Substrate or Coenzyme	K_m	Initial Concentrations		
		Indole, $(S_1)_o$	L-Serine, $(S_2)_o$	Pyridoxal Phosphate, (C)
Indole	2.3×10^{-5}M	0.47×10^{-4}M to 4.74×10^{-4}M	8.0×10^{-2}M	4.6×10^{-5}M
L-Serine	6.3×10^{-3}M	4.74×10^{-3}M	3.8×10^{-3}M to 8.0×10^{-2}M	4.6×10^{-5}M
Pyridoxal phosphate	3.4×10^{-6}M	4.74×10^{-3}M	8.0×10^{-2}M	0.39×10^{-5}M to 4.6×10^{-5}M

Yanofsky (7, 8) has reported two values for the apparent K_m of L-serine with tryptophane desmolase extracts of a wild type strain. A value of 6×10^{-3}M was found at pH 7.8 and 32° C, but the initial concentrations of indole and pyridoxal phosphate are not given except he states that they are "in excess" (7). A value of 3.4×10^{-3}M was found at 37° C and presumably at pH 7.8 with initial concentrations of indole and pyridoxal phosphate of 3×10^{-4}M and 5.2×10^{-5}M respectively (8). The latter value was determined under conditions

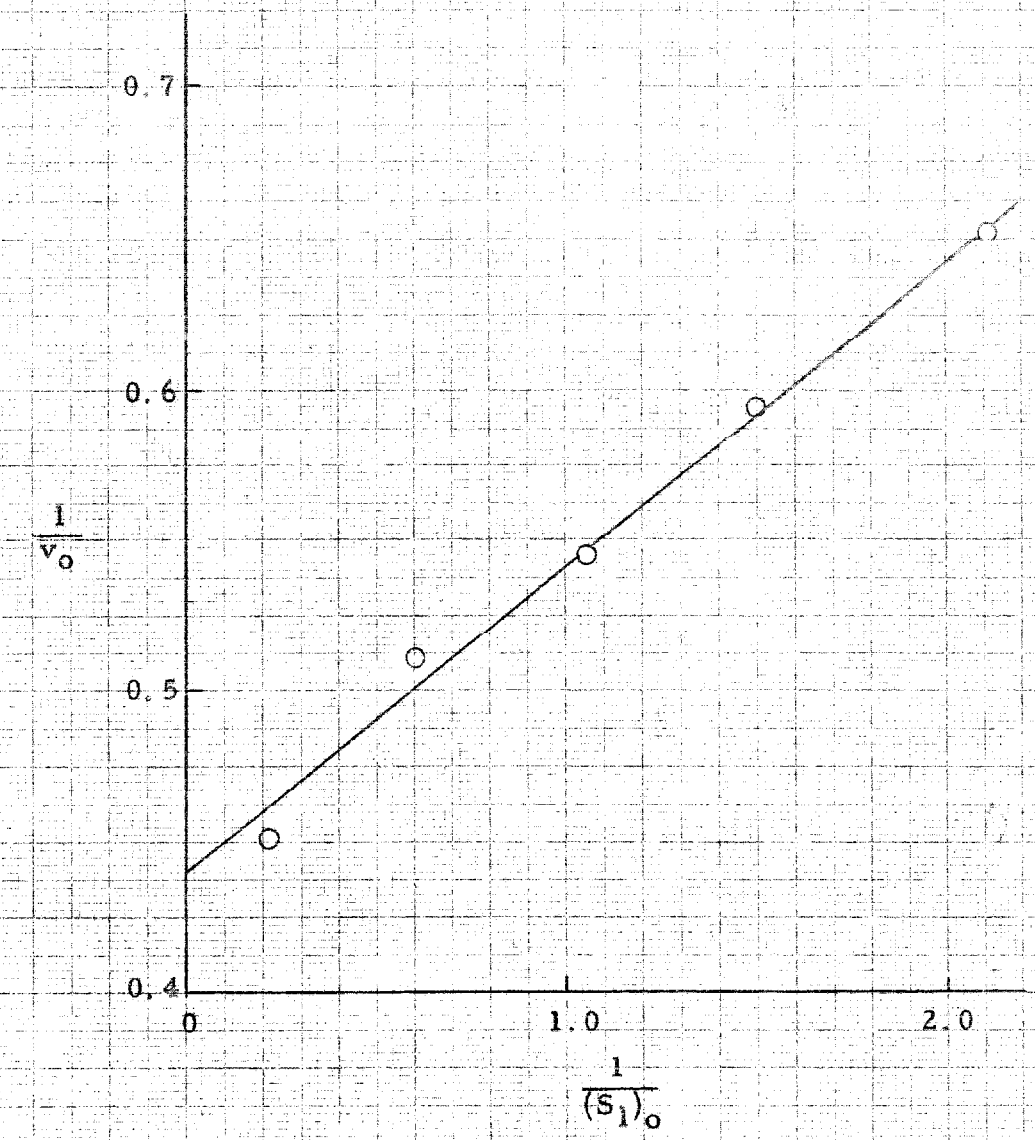


Fig. 3. $\frac{1}{v_o}$ versus $\frac{1}{(S_1)_o}$ plot for indole and tryptophane desmolase from C102-15300. v_o is in units of M·min.$^{-1}$ x 10^{-6} and initial indole concentrations $(S_1)_o$ in units of M x 10^{-4}. Other conditions are given in Table IV.

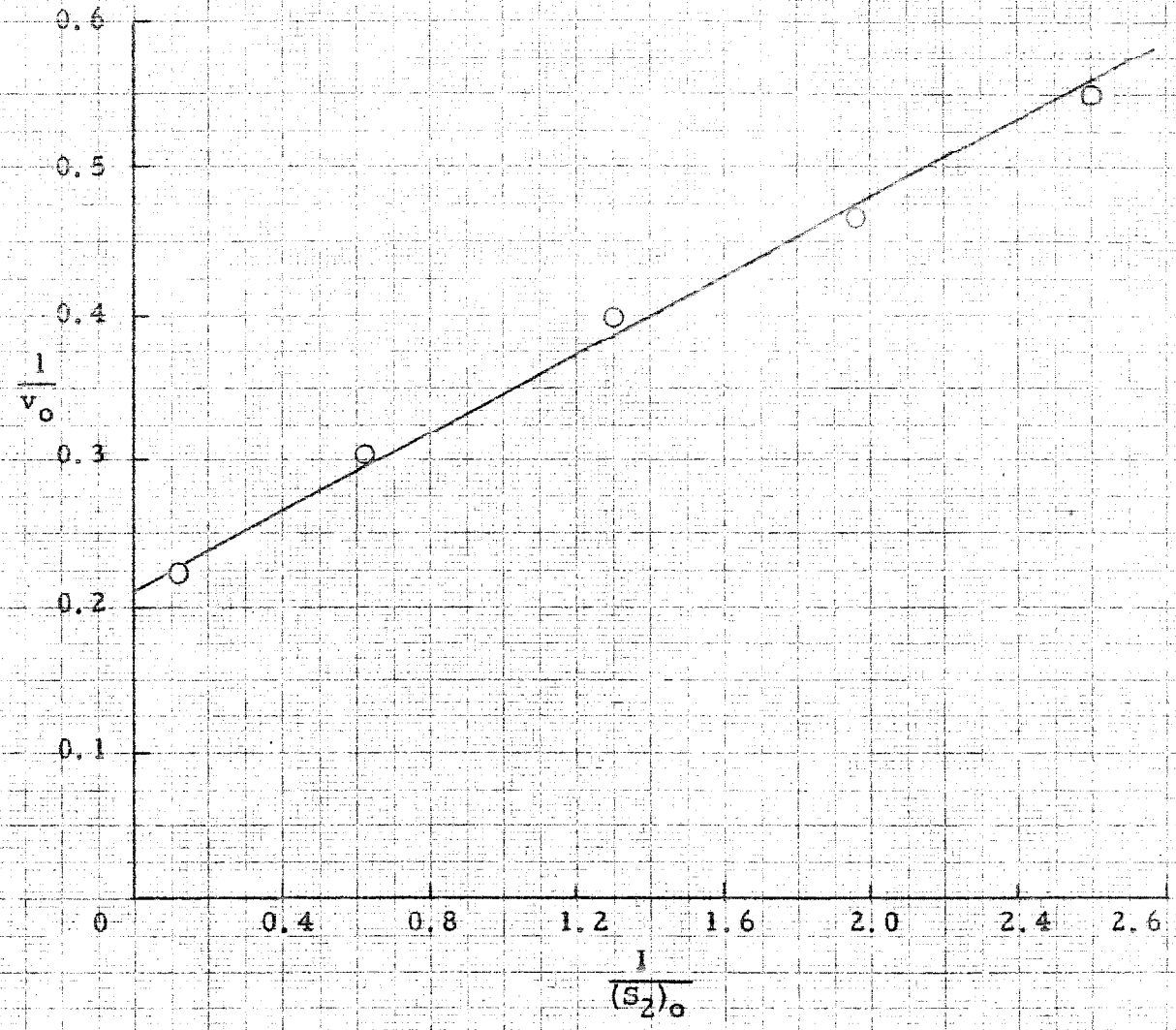


Fig. 4. $\frac{1}{v_o}$ versus $\frac{1}{(S_2)_o}$ plot for L-serine and tryptophane desmolase from C102-15300. v_o is in units of M · min.$^{-1}$ x 10^{-6} and initial L-serine concentrations $(S_2)_o$ in units of M x 10^{-2}. Other conditions are given in Table IV.

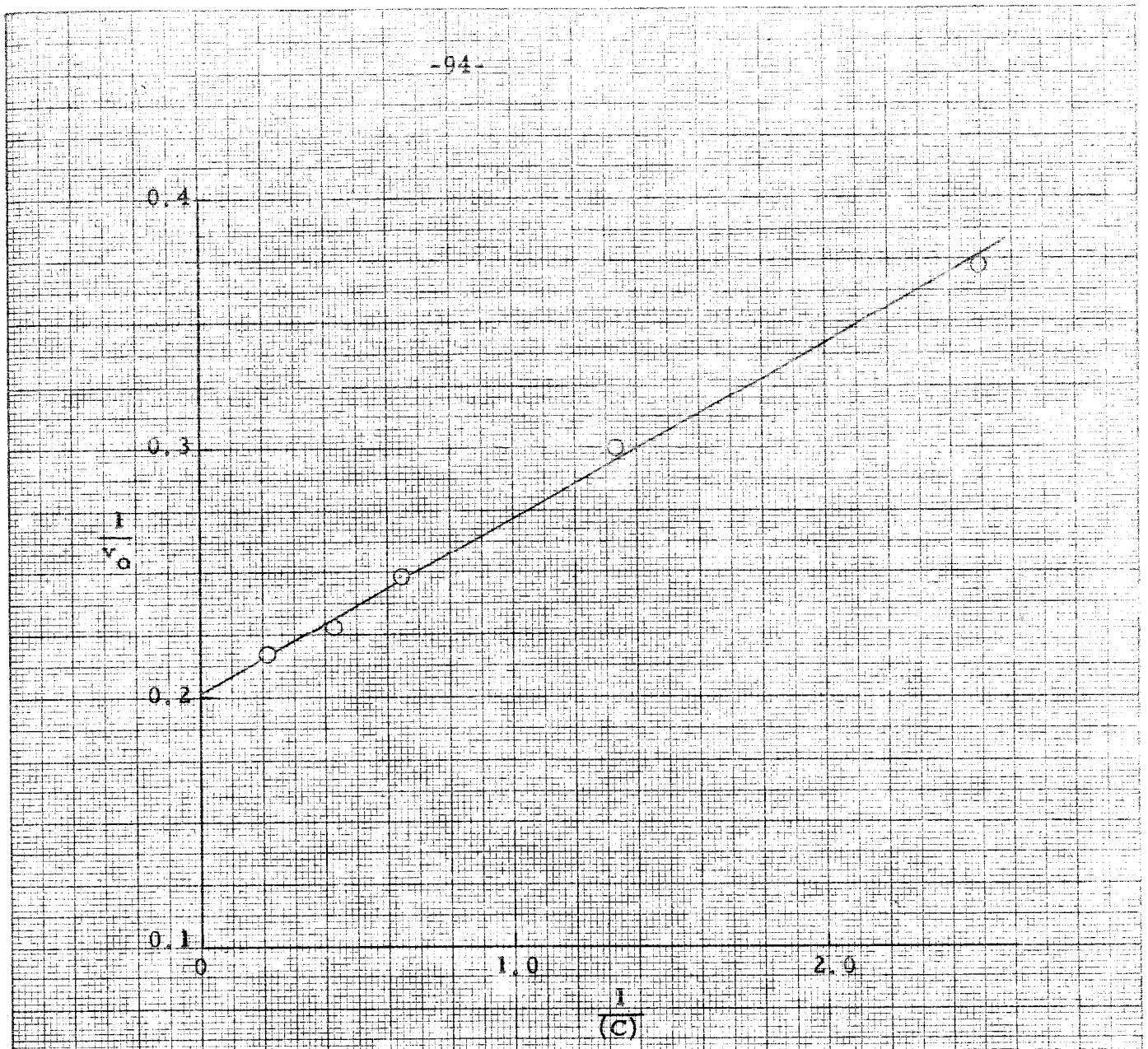


Fig. 5. $\frac{1}{v_o}$ versus $\frac{1}{(C)}$ plot for pyridoxal phosphate and tryptophane desmolase from C102-15300. v_o in units of $M \cdot min.^{-1} \times 10^{-6}$ and pyridoxal phosphate concentrations (C) in units of $M \times 10^{-5}$. Other conditions are given in Table IV.

that are more comparable to those used here. It is hard to tell whether the difference in the two values is significant in view of the fact that the conditions are somewhat different in the two cases and the accuracy of Yanofsky's work is not known. A very approximate K_m value for pyridoxal phosphate of 3×10^{-6} M is also reported by Yanofsky (8). This was determined at 37° C and pH 7.8 and at initial concentrations of indole and DL-serine of 3×10^{-4} M and 6×10^{-2} M respectively. This value is comparable to that found here though the conditions are slightly different.

It should be noted that in the K_m determinations performed here, the concentration of the substrate or coenzyme which is held constant is from thirteen to twenty-one times its apparent K_m value (for L-serine it is 13; for pyridoxal phosphate, 14; and for indole, 21). Thus if we utilize either of the approximations outlined in the formulation of the kinetics, we can conclude that both pyridoxal phosphate and indole have a much greater affinity for the tryptophane desmolase than does the L-serine. It is also probable that pyridoxal phosphate has a greater affinity than does indole, though the differences in K_m values are not so pronounced. It should also be noted that the possible error in the estimation of the purity of the calcium pyridoxal phosphate utilized in these experiments (see Experimental) makes the apparent K_m value for pyridoxal phosphate as expressed in moles per liter subject to a larger error than is the case for the apparent K_m values for indole and L-serine.

It was found that L-tryptophane inhibits the reaction catalysed by the tryptophane desmolase from strain C102-15300. At pH 7.6 and 37° C, a concentration of 2×10^{-3} M L-tryptophane causes the initial velocity to drop to 71% of that of the control when the initial concentrations of indole, DL-serine and pyridoxal phosphate are 4.74×10^{-4}M, 0.160 M and 4.6×10^{-5} M respectively.

Tryptophane Desmolase Activity in the Mutant, C83

Attempts by Gordon and Mitchell to confirm their original findings of tryptophane desmolase activity in extracts of the C83 mutant (5) have been unsuccessful (9). In further experiments designed to detect the presence of tryptophane desmolase activity in C83, the author has also been unable to confirm the original findings of Gordon and Mitchell. The results of these experiments are summarized in Table V. These particular experiments were performed on preparations from the triple mutant of C83, C102 and 15300.

The assay conditions employed in these experiments are capable of detecting approximately 1/200 of the activity present in the C102-15300 strain or in the 8a wild type. It should also be noted that there is no inhibition of active wild type extracts by the inactive C83 extracts, a further indication that the lack of activity is not due to the presence of some inhibitor.

While it is obviously difficult to prove the absence of tryptophane desmolase in C83 mycelium, these results, coupled with the later experiments of Gordon and Mitchell (9) and the experiments of Yanofsky (8), make it very improbable that the active form of the enzyme exists in C83 under the conditions of growth that have been employed. This conclusion is further enhanced by the isotope experiments of Partridge, Bonner and Yanofsky (22) in which C83 was grown on N^{15} ring labeled L-tryptophane. After maximum growth had been obtained,

Table V

Tryptophane Desmolase Activity in Preparations from the Triple Mutant of C83, C102 and 15300.

Age of Mycelium,* Days	Type of Preparation	Tryptophane Desmolase Activity
2, 3, 4 & 5	Crude extract	0
2, 3, 4 & 5	Dialysed crude extract†	0
2 & 4	Partially purified preparation, P_1 of Table I.**	0
2 & 4	Dialysed P_1†	0
2 & 4	Precipitates resulting from S_1 (Table I)** by the action of $(NH_4)_2 SO_4$ at 25, 40, 60 and 80% saturation, 0° C.	0
3 & 5	Whole washed mycelium	0

* Grown at 25° C in minimal medium containing 100 µg. L-tryptophane per ml.

** The crude extract was not heated as is indicated in Table I. In the preparation of P_1, all fractions indicated in Table I were tested for activity and negative results obtained.

† Dialysed for 4, 6, 10, 14 and 24 hours against 0.2 M phosphate, pH 7.7 at 3° C.

tryptophane was isolated from the mycelium and was found to have the same N^{15} content as the tryptophane that was fed. These facts indicate that significant amounts of tryptophane were not synthesized by C83 under the conditions employed.

In order to determine whether or not the two characteristics of the C83 mutant, of L-tryptophane requirement for growth and lack of

tryptophane desmolase in extracts, are the result of the same single gene change, the mutant C83A was crossed to wild type 8a and crude extracts from the progeny were examined for tryptophane desmolase activity. Asci from this cross were dissected, and after germination the ascospores were transferred singly or as spore pairs to minimal agar medium supplemented with L-tryptophane. Growth on minimal medium with and without the addition of L-tryptophane was then tested and the progeny classified as C83 or wild type. These strains were then grown for four days at 25° C in minimal medium supplemented with 100 μg. L-tryptophane per ml. and crude extracts of the resulting cultures tested for tryptophane desmolase activity. The results from nineteen asci are presented in Table VI.

It is apparent from these data that the tryptophane requirement always has associated with it a lack of tryptophane desmolase activity in the crude extracts. Furthermore, except for one ascus (8-1), all of the wild type are seen to possess an activity comparable to that of the parent wild type 8a. The deviation of these values from 1.00 is not significantly different from the deviation of six values of the specific activity of 8a extracts from their average value.

Cultures of the wild spore pair of ascus 8-1 that showed no tryptophane desmolase activity in crude extracts (spore pair 8-1-3) were reinvestigated. When extracts from four-day-old cultures of spore pair 8-1-3 grown at 25° C in minimal medium supplemented with

Table VI

Tryptophane Desmolase Activity in Crude Extracts from the Progeny of C83A x 8a.

Ascus number	Relative Specific Activity of Tryptophane Desmolase*			
	C83	C83	Wild type	Wild type
1-1	0	0,0 (0)	1.04	0.90, 0.86 (0.88)
1-2	0,0 (0)	0,0 (0)	1.01	0.93, 0.96 (0.94)
1-3	0,0	--	1.00	1.28, 1.32 (1.30)
2-1	0	0	1.16	0.96
3-1	0	0	0.96	0.96
4-1	0	0	1.03	0.98
5-1	0	0	1.10	0.97
6-1	0	0	1.21	0.99
6-2	0	0	1.10	0.79
6-3	0	0	1.17	1.08
6-4	0	0	1.14	1.09
7-1	0	0	1.04	1.23
8-1	0	0	0	1.27
8-2	0	0	1.20	1.15
8-4	0	0	0.99	0.72
9-1	0	0	0.91	0.94
9-2	0	0	0.95	1.18
9-3	0	0	0.98	1.04
9-4	0	0	1.10	0.96

*The specific activity (initial rate of indole disappearance in $M \cdot (min.)^{-1} \cdot (mg.\ protein\text{-}nitrogen\ per\ ml.)^{-1}$) relative to the

average specific activity of the parent 8a when grown for the same time (4 days) under the same conditions (25° C and in minimal medium supplemented with 100 g. L-tryptophane per ml.). Average of six specific activity values for 8a extracts is 1.69×10^{-5} M·(min.)$^{-1}$·(mg. protein-nitrogen per ml.)$^{-1}$.

When two values are given for a single spore pair, they represent the values obtained for each member of the pair. The average of the two values is given in parentheses. The single values for the first four asci are for cultures from single ascospores. The rest of the single values are for cultures from a spore pair.

100 µg. L-tryptophane per ml. were retested in six separate experiments, the following relative specific activity values were found: 0.00, 0.00, 0.00, 0.77, 0.99 and 0.87. When grown under the same conditions except with no L-tryptophane present, the following relative specific activity values were found: 1.03, 1.35, 1.37 and 0.96. A variability in the amount of growth achieved in four days was also noted. In view of the possibility that contamination of this wild type (8-1-3a) with C83 to form a heterocaryon might be the cause of this anomalous behavior, a cross of this wild type (8-1-3a) with wild type P1400-4A was made and germinating ascospores observed at random. An examination of 4327 germinated ascospores revealed only 22 that gave a germination pattern that could be considered different from wild type. These possible mutants were isolated and tested for growth on minimal medium. All of them grew on minimal medium though somewhat more slowly than the parent wild types. These data effectively eliminate the heterocaryon possibility.

Extracts with zero activity were obtained only from cultures of 8-1-3a grown in the presence of L-tryptophane and never from

cultures grown in its absence. Therefore, the tryptophane desmolase activity of extracts from cultures of 8-1-3a grown on different concentrations of L-tryptophane for varying amounts of time was determined. The results are presented in Fig. 6 along with similar data for extracts from 8a grown in the absence and in the presence of L-tryptophane.

It would appear that the absence of enzyme activity in 8-1-3a extracts is due to the presence of L-tryptophane in the growth medium. The variability in the observance of absence of activity at 96 hours could then be caused by a small shift in time of the point at which the rapid drop off in specific activity that is caused by L-tryptophane occurs. That the lack of activity in extracts from 8-1-3a grown in the presence of 100 μg. L-tryptophane per ml. is not due to the presence of an inhibitor is indicated by mixing experiments. When inactive extracts from 8-1-3a are mixed with active extracts, the measured activity is that which would be predicted if one assumes that the inactive extracts are inert, exerting neither an activating nor an inhibiting influence on the active extracts.

It is impractical to test all of the wild type progeny of the cross of C83A with 8a in a similar manner. Certainly it is possible that some of them are affected by L-tryptophane in the growth medium in the same manner as is 8-1-3a. However, considering the variability observed in the activity of extracts from 8-1-3a at 96 hours' growth (i.e., zero activity observed in four out of eight experiments) it is not likely that

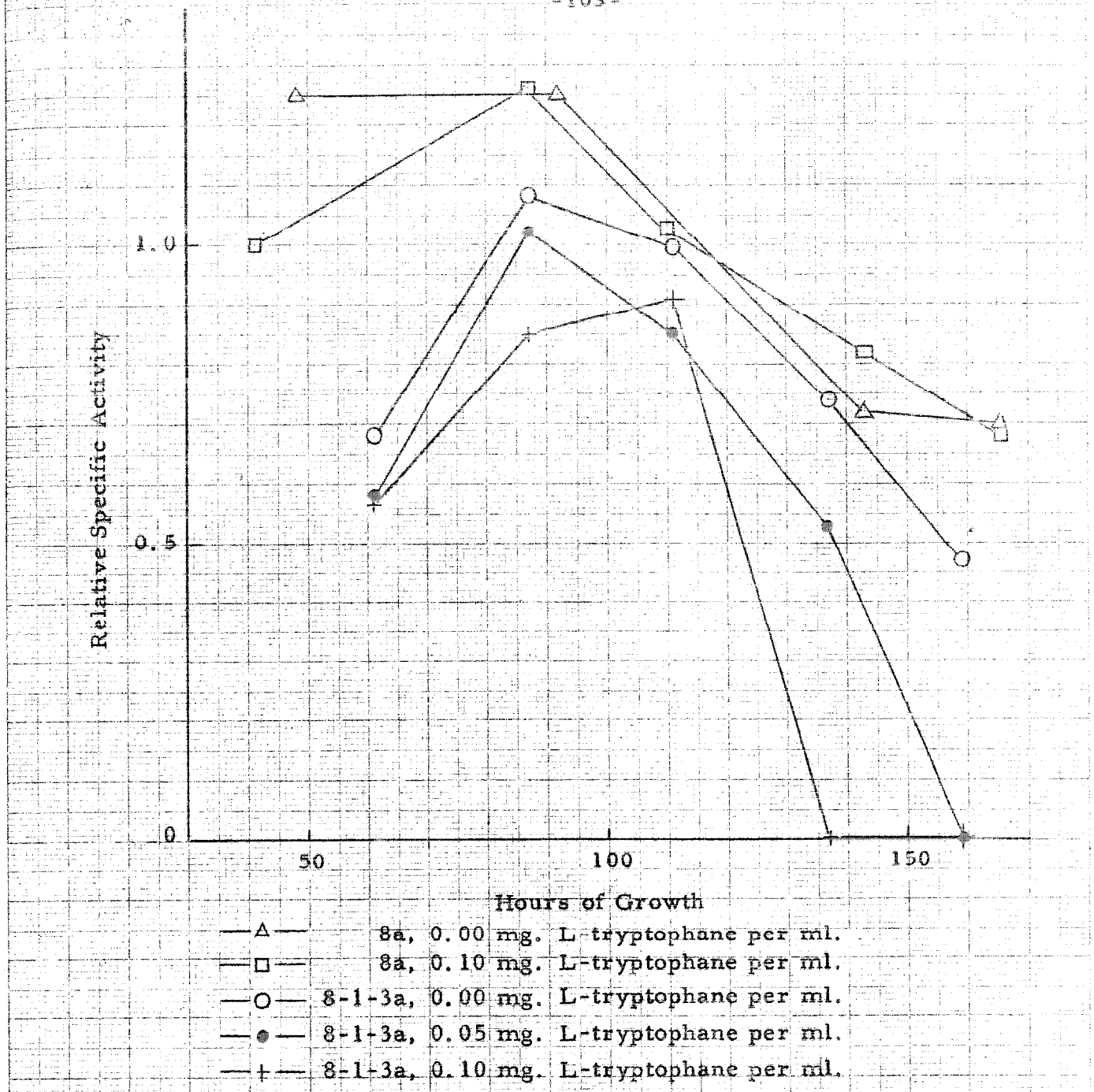


Fig. 6. Variation in the relative specific activity in crude extracts of 8-1-3a and 8a as a function of time of growth and the L-tryptophane concentration of the growth medium.

more than four or five of the wild type spore pairs are the same as 8-1-3a in their response to L-tryptophane in the growth medium.

The present data concerning the nature of this suppression of tryptophane desmolase activity in 8-1-3a extracts by the presence of L-tryptophane in the growth medium are so meager that without building an edifice of speculation one can only regard them as an interesting phenomenon. In the field of speculation, the possibilities are numerous. It is conceivable that C83A actually represents a double mutant, one gene of which is primarily responsible for the lack of tryptophane desmolase activity (and consequently for the L-tryptophane requirement), and the other gene primarily responsible for suppression of tryptophane desmolase activity when grown on L-tryptophane. The double mutant would not be distinguishable from the single mutant carrying the gene responsible for lack of tryptophane desmolase activity by the methods that have been applied, i.e., a test for L-tryptophane requirement and the lack of tryptophane desmolase activity. The single mutant carrying the gene responsible for suppression of tryptophane desmolase activity when grown on L-tryptophane would be strain 8-1-3a which would have arisen as a result of a cross over between the two genes of the double mutant. However, it is of course also possible that the 8-1-3a strain could have arisen by spontaneous mutation, or that this tryptophane suppression effect is not of genetic origin. In regard to this latter possibility it should be mentioned that the tryptophane suppression effect

has not been observed in the 8a wild type even after keeping the strain on minimal supplemented with 100 μg. L-tryptophane per ml. for a period of six months. In any case it is apparent that one cannot say which of the above possibilities is correct on the basis of the present data.

In concluding this section on tryptophane desmolase activity in the C83 mutant, it is possible to say that all of the evidence accumulated at this date indicates that C83 strains do not contain measurable tryptophane desmolase activity, and that the tryptophane requirement of C83 and this lack of tryptophane desmolase activity are end effects produced by the action of the same single gene.

The Backmutation of C83

Reversion of nutritional mutants of Neurospora to the ability to grow on minimal media has been observed in several cases. Thus the reversion of an adenineless strain has been used as a technique for measuring the mutagenic activity of a variety of mutagenic agents (23-25). Perhaps the most complete and quantitative studies of reversion in Neurospora have been undertaken by Giles (10, 26). The majority of data that he presents concern the reversion, either spontaneously or by the action of ultraviolet of x-ray irradiation, of methionineless and inositolless mutants. The idea that reversions caused by single gene changes are the result of backmutation or of suppressor action is equivalent to the fact that the gene change must occur at a locus indistinguishable from the mutant locus by cross-over tests (backmutation) or at some other locus (suppressor action). Both types of reversions have been observed. Only reversions that could be traced to suppressor action were found with the methionineless mutant. In the case of the inositolless mutants both types of genetic reversion were found, though the vast majority were due to backmutation.

As was mentioned earlier, Yanofsky (8) has found that a spontaneous reversion of the tryptophaneless mutant S1952 was due to suppressor action. The only known difference between the C83 and S1952 genes is that they respond differently to the action of the

suppressor gene. The tryptophane requirement and the lack of tryptophane desmolase activity of C83 could not be suppressed by the suppressor gene, whereas both characteristics are partially suppressed in the case of S1952.

It was of interest to investigate the possible reversion of C83 and to determine the nature of this reversion if it could be found. Ultraviolet irradiation was used to induce the reversions.

A triple mutant of C83, C102 and 15300 was employed in these investigations. The C102 and 15300 mutants were used as markers in order that any possible contamination could be detected. The "colonial" C102 marker has the added advantage of making viable conidial counts relatively easy, since it grows in colonial form at temperatures above 31° C. Thus if a viable conidial count is desired, conidia of the triple mutant are plated out on L-tryptophane supplemented minimal agar and allowed to grow at 33° C for three days. The colonies can then be counted to determine the number of viable conidia. All conidial concentration assays were carried out in this manner and thus represent viable conidial concentrations and not total conidial concentrations.

A suspension of C83-C102-15300 macroconidia in distilled water, filtered through glass wool, and containing 3.1×10^{6} viable conidia per ml. was divided in half. One half was subjected to ultraviolet irradiation from a Model UV-30 American Sterilizer Company sterilizing lamp for 2.0 minutes at a distance of 10 cm. The other

half was not irradiated and was used as the control. The irradiation lowered the conidial count to 1.6×10^6 viable conidia per ml., representing a survival of 52% of the conidia. Thirty 125-ml. flasks were then inoculated with 0.1 ml. of the irradiated suspension (1.6×10^5 conidia per flask) and allowed to stand at 25° C. Similarly twenty-eight 125-ml. flasks containing 20 ml. of minimal medium were inoculated with 0.1 ml. of the control suspension (3.1×10 conidia per flask) and allowed to stand at 25°C. These flasks were observed for growth over a period of fifteen days. The results are presented in Table VII.

Table VII

Reversion of C83-C102-15300 Macroconidia.

Suspension	Total no. of flasks	Flasks showing growth
Non-irradiated control	28	0
Irradiated	30	27

Thus no reversions were noted in a total of 8.7×10^7 viable conidia not subject to ultra-violet irradiation, whereas at least 27 reversions occurred in 5.3×10^7 viable conidia that were subject to irradiation. All of the reverted cultures continued to grow when transferred to minimal medium. They were indistinguishable from wild type in the amount of growth achieved in four days on minimal medium.

There is thus no doubt that the C83 mutant can revert. A cross of a reverted strain, C83re-C102-15300a, to C83-C102-15300A was made, the resulting asci were dissected, and the nature of the individual ascospores determined. Considering only one ascus from each perithecium, out of a total of twenty such asci nine showed a spore pattern of four tryptophane-independent to four tryptophane-dependent. In the remaining eleven asci all eight spores were tryptophane-dependent. In each case all eight spores were found to carry the albino 15300 and "colonial" C102 markers. An examination of 2402 random spores germinating on minimal agar revealed 614 (or 26%) which had a wild type germination pattern and 1788 (or 74%) which had a germinating pattern identical with the C83 mutant.

Since the macroconidia which were irradiated generally contain more than one nucleus, it is to be expected that the original reverted cultures would be heterocaryotic, containing both tryptophane-independent and tryptophane-dependent nuclei. Thus one would expect the two types of asci found, the $4T^+:4T^-$ asci resulting from the tryptophane-independent nuclei and the $8T^-$ asci from the tryptophane-dependent nuclei (T^+ = tryptophane-independent, T^- = tryptophane-dependent). The reversion is then of genetic origin.

It was then necessary to determine whether this genetic reversion resulted from a backmutation or from the action of a suppressor gene. This can best be established by a cross of a homocaryotic culture

of the reverted strain (i.e., a culture from one of the T^+ spores in a $4T^+ : 4T^-$ ascus of the preceding cross) to wild type. If the reversion is caused by a backmutation, all of the progeny should be T^+. If, however, the reversion is due to the presence of a suppressor, some of the progeny should be T^- or C83. In this latter case we should expect to find three types of asci ($8T^+$, $4T^+ : 4T^-$, and $6T^+ : 2T^-$), or, if the suppressor were so closely linked to the C83 mutant gene that four strand double crossovers did not occur, two types of asci ($8T^+$, and $6T^+ : 2T^-$).

In a cross of wild type P1347 (2a) with a homocaryotic reverted strain (C83re-C102-15300 (1-5A)) forty asci were dissected and all showed eight spores with wild type germination patterns on minimal agar. In an examination of 8,791 random ascospores germinating on minimal medium, only three showed germination patterns that were not wild type. These three possible mutant spores were isolated and transferred to minimal agar supplemented with 100 μg of L-tryptophane per ml. These three germinated spores did not show any growth on this medium over a period of fifteen days, and hence are not C83 mutants. These experiments indicate that the reversion arose as the result of backmutation and not by the formation of a suppressor gene.

No appreciable differences in growth characteristics between the backmutated strain C83re-C102-15300 and the comparable strain, C102-15300, could be demonstrated (Fig. 7).

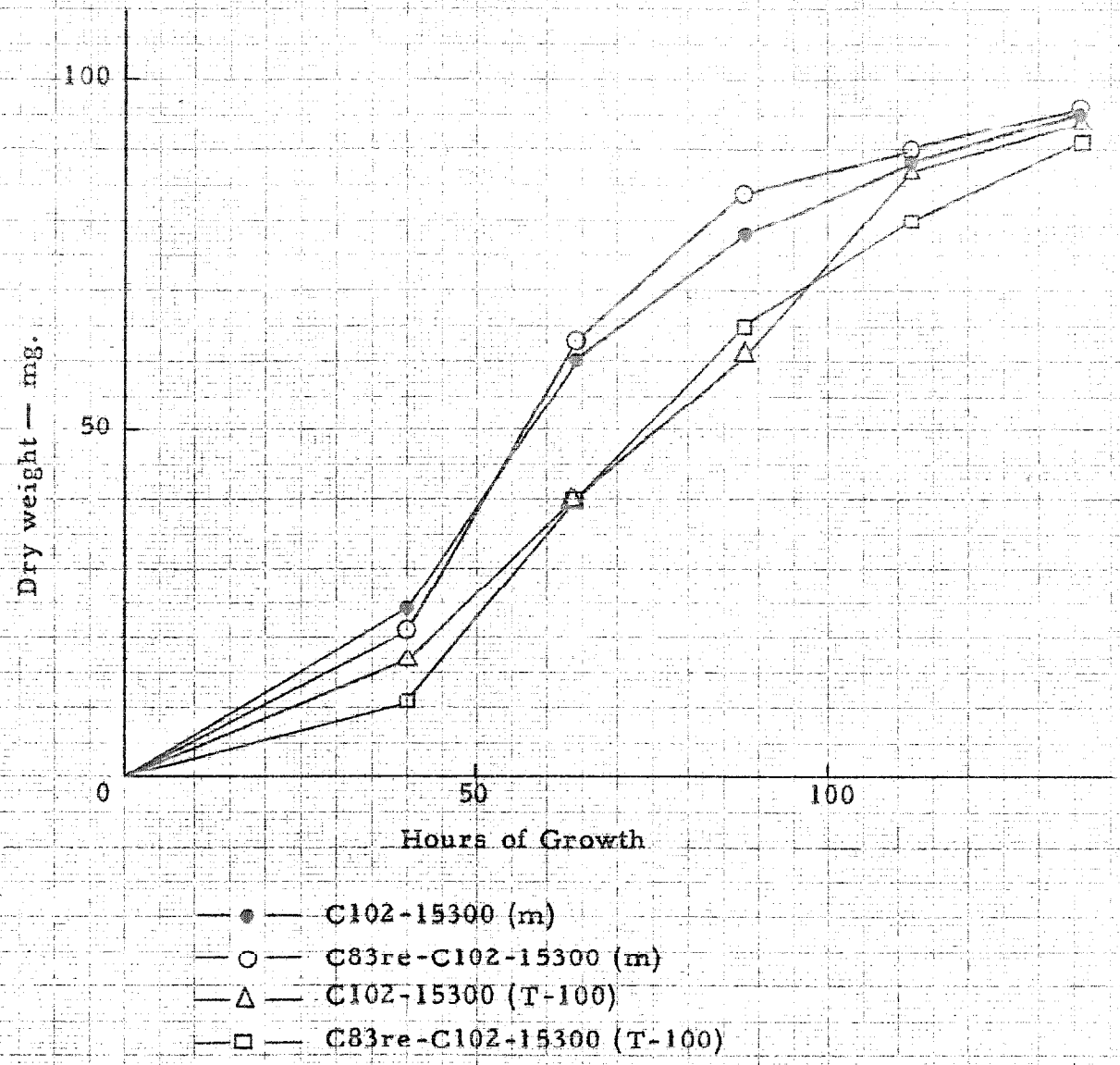


Fig. 7. Amount of growth at 25° C in 125-ml. Erlenmeyer flasks containing 20 ml. minimal medium (m) or 20 ml. minimal medium supplemented with 0.10 mg. L-tryptophane per ml. (T-100) as a function of time.

Since the backmutated strain will grow well without the addition of L-tryptophane to the medium, it should contain tryptophane desmolase activity. To investigate this possibility, crude extracts of C83re-C102-15300 were prepared and purified according to the scheme outlined in Table I. Tryptophane desmolase activity was found in the crude extracts although its specific activity was only 40% of that of crude extracts from C102-15300 — both strains having been grown under the same conditions (see Experimental). The purification observed for these extracts was not significantly different from that obtained with extracts from C102-15300.

The properties of the partially purified tryptophane desmolase extract of C83re-C102-15300 were then examined. As was found to be the case with the C102-15300 preparations, the rate of indole disappearance and of tryptophane appearance was the same, but the rate of L-serine disappearance was 2.6 times that of indole (a value of 2.2 was found for the C102-15300 preparations). No indole disappearance was observed unless both L-serine and pyridoxal phosphate were present. The action of D-serine was tested and the results are expressed in Table VIII.

A comparison of these data with those presented in Table III indicates that with regard to the influence of D-serine there is no significant difference between the two enzyme systems.

The pH-activity curve for the reverted preparations is not

Table VIII

The Effect of D-Serine on Tryptophane Desmolase Catalysis
(Strain C83re-C102-15300)

Initial concentration of L-serine, M.	Initial concentration of D-serine, M.	$v_o \times 10^6$
0	0	0.00
16×10^{-3}	0	1.24
0	16×10^{-3}	0.06
80×10^{-3}	0	1.52
80×10^{-3}	80×10^{-3}	1.48

v_o in $M \cdot min.^{-1}$ at pH 7.6 and 37.0°C. Initial concentrations are: indole, 4.74×10^{-4} M; pyridoxal phosphate, 4.6×10^{-5} M; 0.2 M phosphate buffer; and 20 μg. of protein-nitrogen per ml.

significantly different from that obtained with the reference material (Fig. 8).

Utilizing the partially purified preparations of the backmutated strain, the apparent K_m values for indole, L-serine and pyridoxal phosphate were determined (Figs. 9-11). The conditions under which these values were found are the same as those utilized for the K_m determinations with extracts from C102-15300 — Table IV. For purposes of comparison both sets of values are given in Table IX. The inhibition produced by L-tryptophane is also given in Table IX. The K_m values and the per cent inhibition by L-tryptophane determined with the tryptophane desmolase from the backmutated strain do not differ significantly from the corresponding values obtained with the preparations from the reference strain.

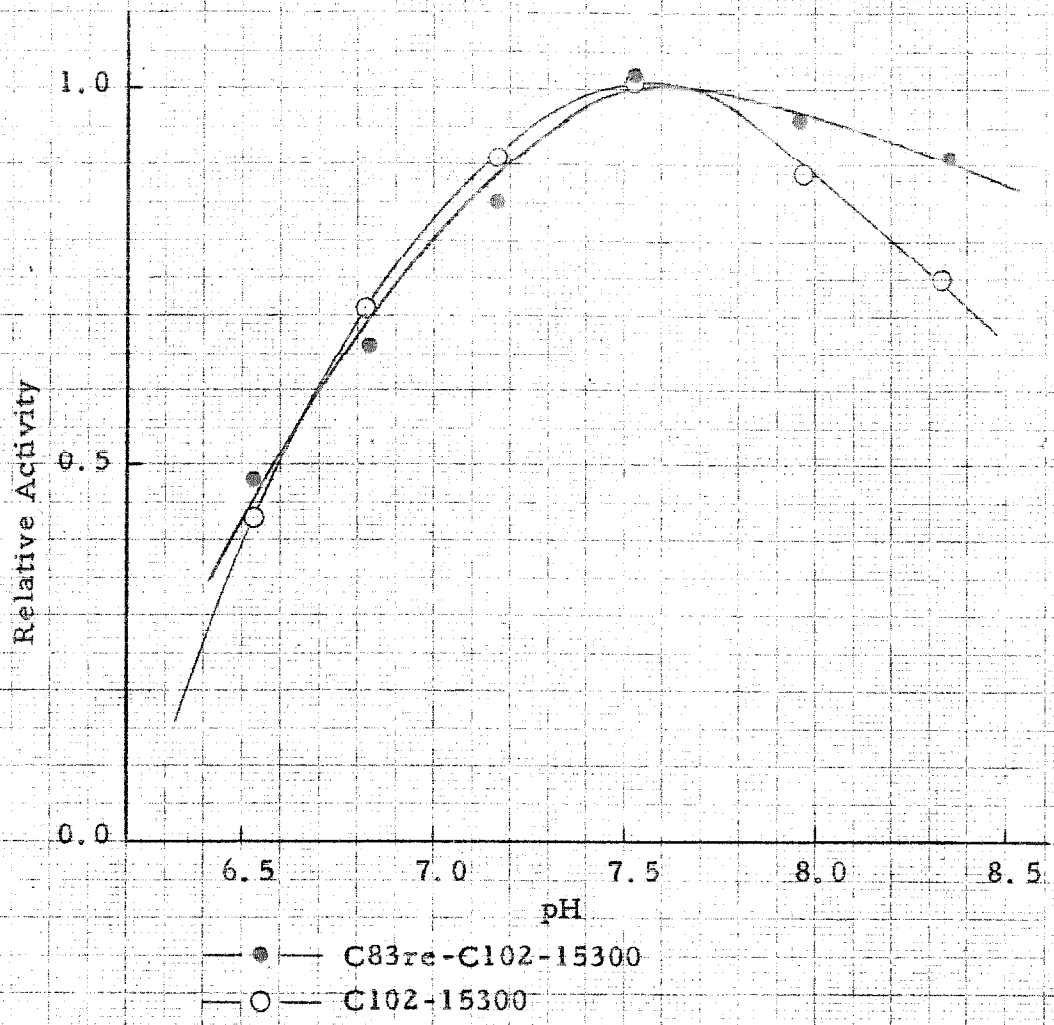


Fig. 8. Relative activity of tryptophane desmolase from C83re-C102-15300 and C102-15300 at 37.0° C as a function of the pH. Initial concentrations are indole, 4.74×10^{-4} M; DL-serine, 0.171 M; and pyridoxal phosphate, 4.7×10^{-5} M.

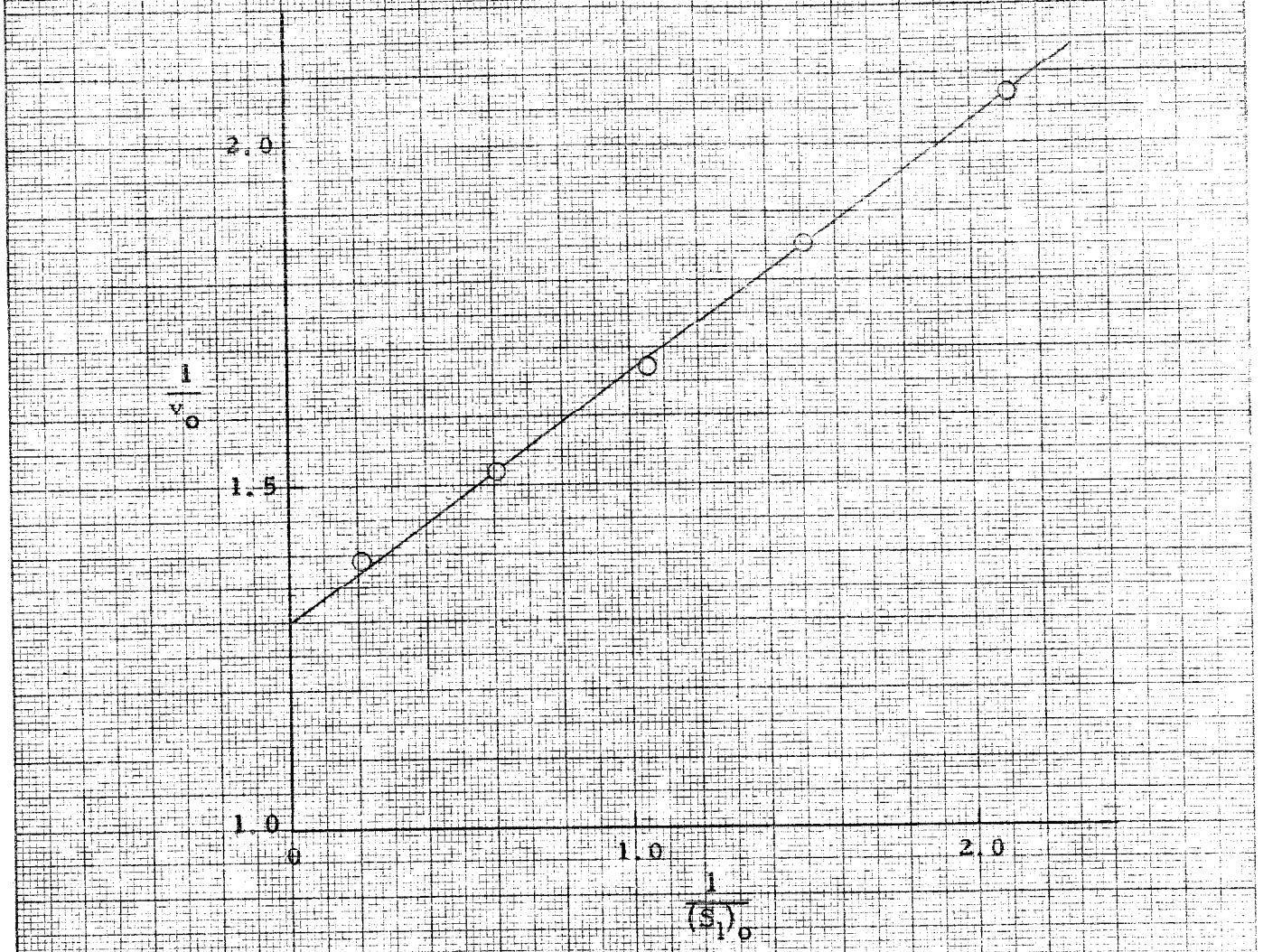


Fig. 9. $\frac{1}{v_o}$ versus $\frac{1}{(S_1)_o}$ plot for indole and tryptophane desmolase from C83re-C102-15300. v_o is in units of M·min.$^{-1}$ x 10^{-6} and initial indole concentrations $(S_1)_o$ in units of M x 10^{-4}. Other conditions are the same as for Fig. 3.

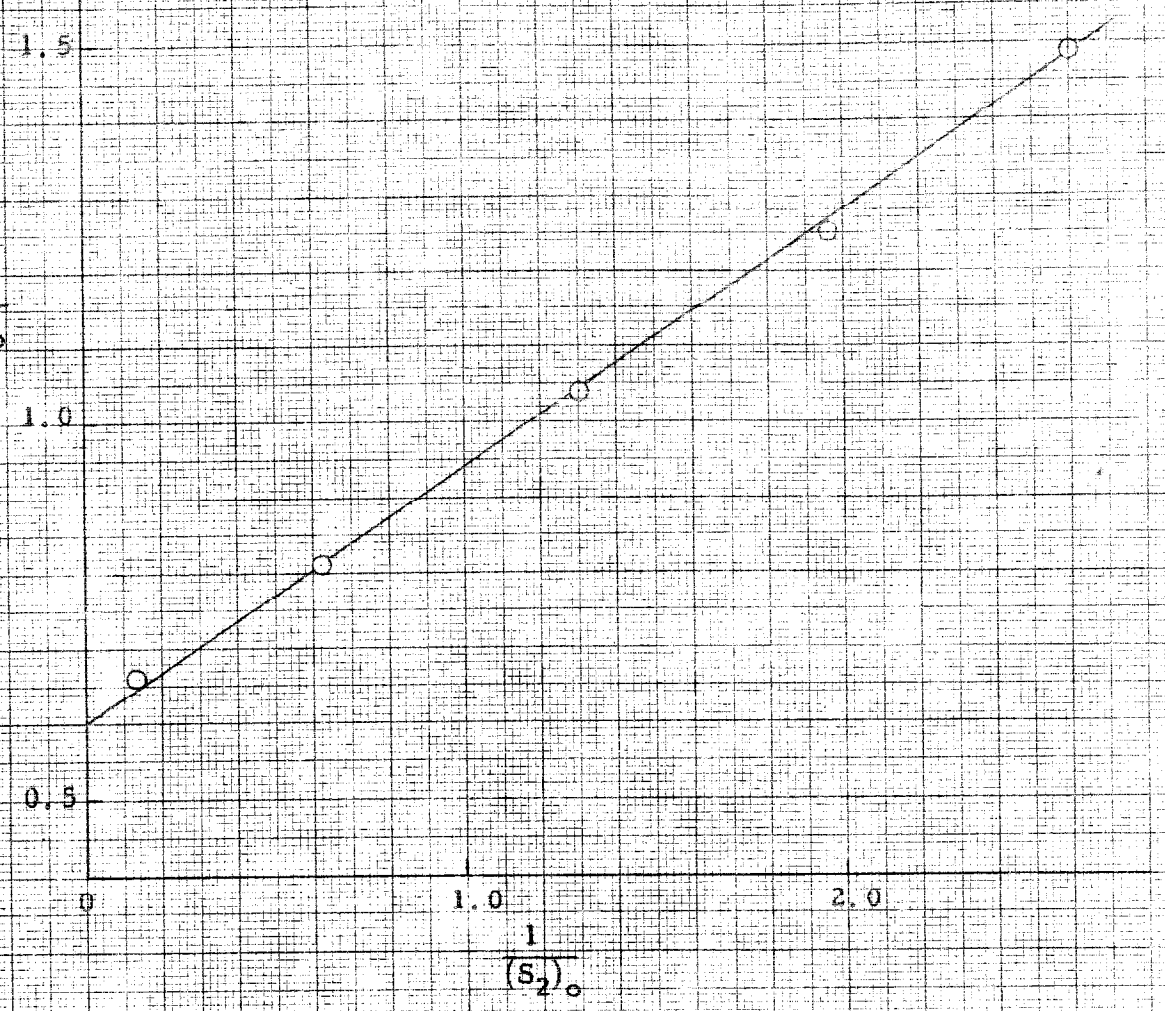


Fig. 10. $\frac{1}{v_o}$ versus $\frac{1}{(S_2)_o}$ plot for L-serine and tryptophane desmolase from C83re-C102-15300. v_o in units of $M \cdot min.^{-1} \times 10^{-6}$ and initial L-serine concentrations $(S_2)_o$ in units of $M \times 10^{-2}$. Other conditions are the same as for Fig. 4.

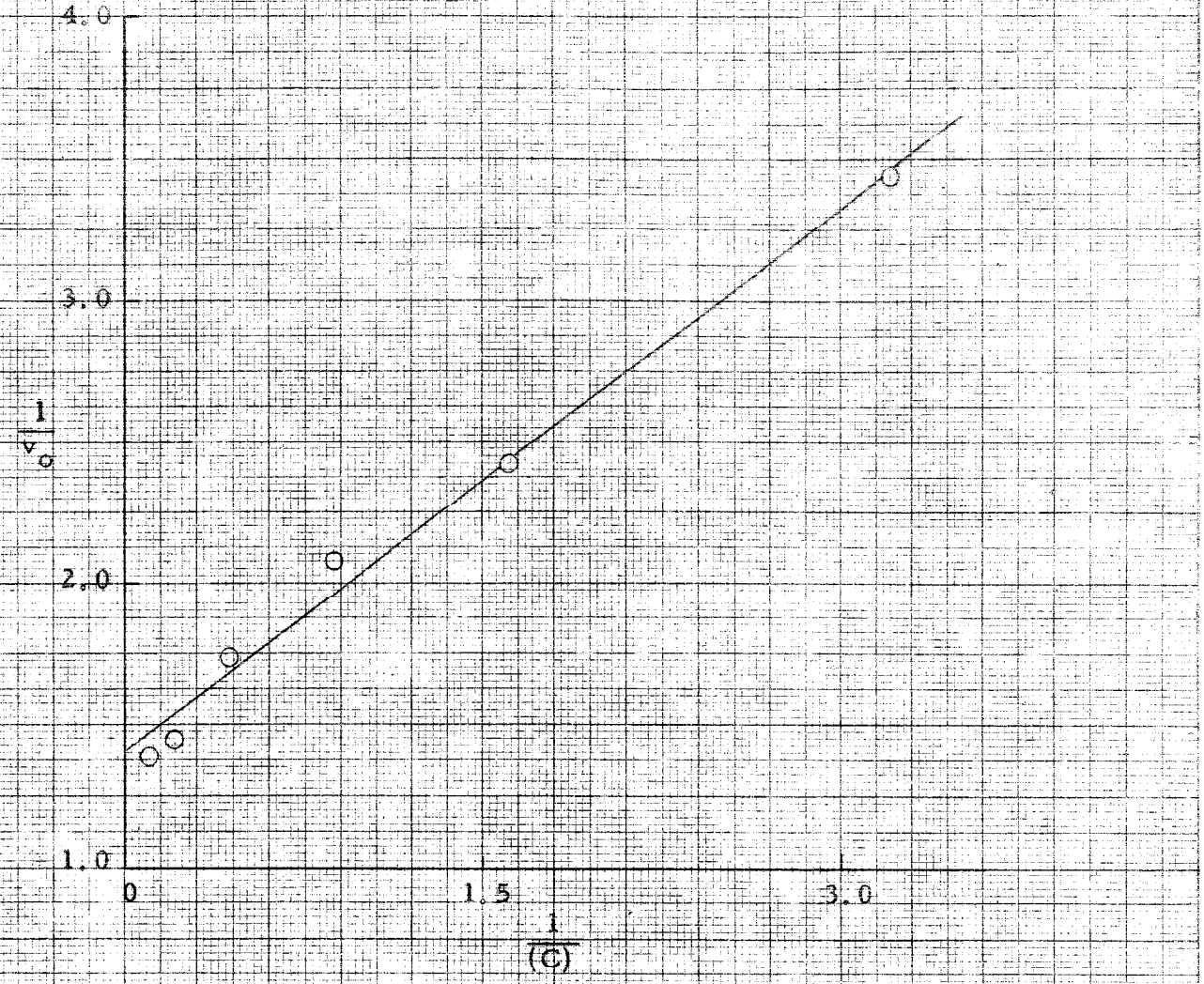


Fig. 11. $\frac{1}{v_o}$ versus $\frac{1}{(C)}$ plot for pyridoxal phosphate and tryptophane desmolase from C83re-C102-15300. v_o in units of $M \cdot min.^{-1} \times 10^{-6}$ and pyridoxal phosphate concentrations (C) in $M \times 10^{-5}$. Other conditions are the same as for Fig. 5.

Table IX

Comparison of the K_m Values and the Inhibitory Effect of L-Tryptophane on Tryptophane Desmolase from Strain C83re-C102-15300 and Strain C102-15300.

Source of Tryptophane Desmolase	K_m Values*, M			% Inhibition by 2×10^{-3} M L-Tryptophane**
	Indole	L-Serine	Pyridoxal Phosphate	
C102-15300	2.3×10^{-5}	6.3×10^{-3}	3.4×10^{-6}	29
C83re-C102-15300	2.8×10^{-5}	5.7×10^{-3}	4.4×10^{-6}	26

* Conditions are those given in Table IV.

** Determined at 37.0° C in 0.2 M phosphate buffer, pH 7.6. Initial concentrations are indole, 4.74×10^{-4} M; DL-serine, 0.160 M; and pyridoxal phosphate, 4.6×10^{-5} M.

Thus the tryptophane desmolase preparations from both the reference and the backmutated strains are alike in all of their properties that have been tested.

It is appropriate at this time to consider what conclusions can reasonably be drawn from the data presented here. The C83 mutant originated from a cross of ultraviolet-irradiated conidia of wild type 3A with protoperithecia of wild type 6a (4, 27). These wild type strains were reisolates from a cross of wild type Em 5256A and Em 5297a. Yanofsky (7) has used the wild type strain Em5256 in his studies of the properties of tryptophane desmolase. Although wild type strains 3A and 6a have not been tested for tryptophane desmolase activity, they can reasonably be assumed to have this activity, since

a) one of their parents has this activity, and b) all wild type strains tested so far have tryptophane desmolase activity. No tryptophane desmolase has been found in the mutant C83 or in extracts from it. The data presented in Table VI indicate that the characteristics of a tryptophane requirement and of the lack of tryptophane desmolase activity are due to a single gene.

Ultraviolet irradiation has thus caused a single gene change which is associated with a drastic limitation of the amount of active tryptophane desmolase which the organism can produce.

The apparent reversal of this process has also been shown. The C83 mutant will backmutate to a strain which contains active tryptophane desmolase. Again ultraviolet irradiation has caused what is apparently a single gene change that is associated with a drastic change in the amount of active tryptophane desmolase which the organism can produce. Clearly then it is reasonable to conclude that a single gene exerts a control on either the production or destruction of active tryptophane desmolase.

Unfortunately no conclusions concerning the nature of this control can be drawn from the data. The data are consistent with the conclusion that the gene determines the specificity of the tryptophane desmolase, but they are equally consistent with the conclusion that the gene has no influence on the determination of specificity. Since there is no phenotypic difference between the backmutated strain

C83re-C102-15300 and the reference strain C102-15300 that is indicative of a difference in genetic constitution at the C83 locus, there is no reason to conclude that the specificity of the tryptophane desmolase from the two strains should be different, whether it is assumed that this genétic material determines specificity or has no influence on its determination. The fact that no differences could be found in the specificity of the two tryptophane desmolase preparations is in accordance with either assumption. Similarly the data do not indicate whether the change in the gene influences the production or the destruction of the active tryptophane desmolase, since all that we are reasonably sure of is that there is a much smaller amount of active tryptophane desmolase in the C83 mutant strain than in the backmutant and the reference strains.

It is apparent that experiments in which one modifies the nuclear genetic material in some unknown manner and then tries to observe the end effect of such a modification in protein molecules are of limited value in determining the actual action of the genetic material as it is concerned with protein synthesis. This is not to say that such experiments are not of any value, but rather that they cannot of themselves give the answer. In such a complicated situation of chemical balance that must exist in the organism, the relationship between an initial action of a gene and the end effect which is observed as the results of this action is certainly apt to be a complex one. It will

probably be necessary that our knowledge of protein synthesis (i.e., of the nature of the precursors of protein molecules) and of the chemical nature of the nuclear genetic material will have to be greatly increased before a conclusive answer to the question of the nature of genetic control of protein formation can be given. However, an increase in our knowledge of the possible end effects of a gene change on protein molecules will undoubtedly improve the chances of successfully attacking the problems of protein synthesis and the chemical nature of nuclear genetic material.

The fact that a backmutation of the C83 gene has occurred indicates that the C83 mutant is not a result of a deficiency. If the C83 mutant characteristics resulted from an actual deficiency of chromosomal material, it is hard to imagine how a backmutation could have taken place. It is of course possible that some inert chromosomal material in the immediate neighborhood of a deletion could, as a result of ultraviolet irradiation, be changed to duplicate the material that was deleted. However, it is certainly simpler and more satisfying to consider the mutation as a result of a structural change in the chromosome material that does not involve any large loss of that material. At any rate, considering the present state of cytological techniques for observing Neurospora chromosomes, the fact that backmutation can occur is about the best evidence that can be offered to indicate that a mutation has not resulted from a deficiency. This then

effectively invalidates the idea that the difference between the C83 and the S1952 alleles could be due to the fact that the C83 mutant represents a deficiency but that the S1952 mutant does not.

Tryptophane Desmolase Activity in the Histidineless Mutant, C84.

In the preceding paragraphs it has been shown that a genetic change (wild type to mutant C83 or mutant C83 to wild type) has caused a drastic alteration in the amount of tryptophane desmolase activity that can be extracted from the mold. Can other genetic changes alter the amount of tryptophane desmolase activity in a similar or more moderate manner? The suppressor effect of Yanofsky (8) on the S 1952 mutant indicates that they can. Similarly Straus (28) has found that a single gene mutant with low carboxylase activity has associated with it a low tryptophane desmolase activity.

It was mentioned earlier that Mitchell (11) has found that several nutritional mutants of Neurospora have associated with their particular growth requirement a relatively high tryptophane desmolase activity (i. e., two to three times that found in wild type). One of these mutants, C84, has a histidine requirement that cannot be satisfied by any other compound so far tested (12). Crude extracts from cultures of C84A grown at 25° C in minimal medium supplemented with 50 μg. L-histidine per ml. for four days have a specific tryptophane desmolase activity that is 2.8 times that of wild type 8a extracts grown under the same conditions (these figures are the average of five independent specific activity determinations with each strain).

It seemed possible that this higher activity might be associated with the histidineless C84 mutant gene and thus represent a pleiotrophic

effect of this gene. In order to test this possibility a cross of histidine-less C84A with wild type 8a was made. Asci from this cross were dissected and the ascospores isolated and transferred to minimal agar supplemented with 50 μg. L-histidine per ml. The progeny were then tested for growth on minimal medium with and without the addition of L-histidine and classified as C84 or wild. These strains were then grown for four days at 25° C in minimal medium supplemented with 50 μg. L-histidine per ml. and crude extracts of the resulting cultures then tested for tryptophane desmolase activity. The results from nine asci are presented in Table X.

All of the wild type are seen to possess an activity comparable to that of the parent wild type. The distribution of the relative specific activity values for each wild type spore pair (the average value being used where both members of the pair were examined) of the progeny is presented in Fig. 12. Also presented in Fig. 12 are the distributions for the relative specific activity values for each wild type spore pair of the progeny of C83A x 8a (see Table VI) and for the relative specific activity values of eleven independent determinations of the specific activity of the parent wild type 8a. Since there is no difference in the average specific activity of the crude extracts of the 8a strain when grown for four days at 25° C in minimal medium supplemented with 100 μg. L-tryptophane per ml. or when grown for four days at 25° C in minimal medium supplemented with 50 μg. L-histidine per ml.,

Table X

Tryptophane Desmolase Activity in Crude Extracts from the Progeny of C84A x 8a.

Ascus Number	Relative Specific Activity of Tryptophane Desmolase*			
	C84	C84	Wild type	Wild type
1-8	0.00, 0.00 (0.00)	0.13	0.95, 1.13 (1.04)	1.15
1-9	0.00	0.11	0.94, 0.94 (0.94)	1.07
15-1	0.00, 0.44 (0.22)	0.00	1.09, 1.18 (1.14)	0.90, 0.91 (0.90)
23-1	0.00, 0.00 (0.00)	0.00, 0.00 (0.00)	1.00	0.97
26-1	0.00	0.00, 0.00 (0.00)	1.03, 1.03 (1.03)	1.03, 0.87 (0.95)
2-11	0.00, 0.00 (0.00)	2.52	1.32, 1.35 (1.34)	1.35
21-1	0.00	2.36, 2.66 (2.51)	0.92, 1.16 (1.04)	0.92
19-1	1.91	2.84, 2.49 (2.66)	1.01, 1.05 (1.03)	1.24, 1.44 (1.34)
24-1	2.04	3.54	0.94, 0.89 (0.92)	1.06, 0.99 (1.02)

*The specific activity (initial rate of indole disappearance in $M \cdot (min.)^{-1} \cdot (mg.\ protein\text{-}nitrogen\ per\ ml.)^{-1}$) relative to the average specific activity of the parent 8a when grown for the same time (4 days) under the same conditions (25° C and in minimal medium supplemented with 50 μg. L-histidine per ml.). The average of five specific activity values for 8a extracts is 1.69×10^{-5} $M \cdot (min.)^{-1} \cdot (mg.\ protein\text{-}nitrogen\ per\ ml.)^{-1}$ and the average of five specific activity values for

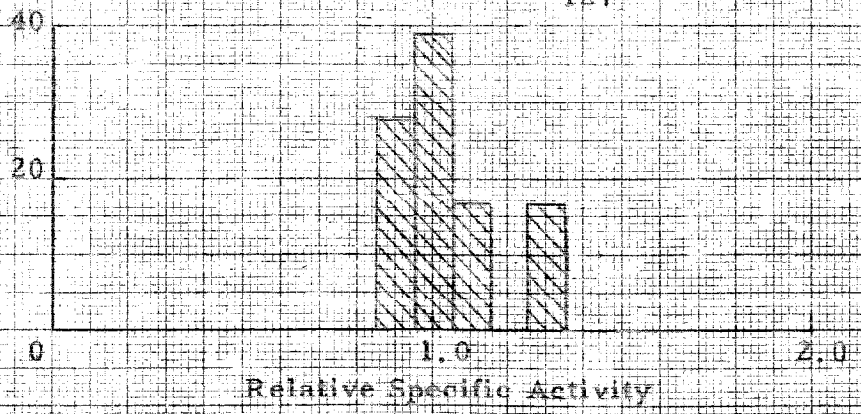
C84A extracts is 4.72×10^{-5}M·(min.)$^{-1}$·(mg. protein-nitrogen per ml.)$^{-1}$, or relative specific activity values of 1.00 and 2.80 respectively.

When two values are given for a single spore pair, they represent the values obtained for each member of the pair. The average of the two values is given in parentheses. The single values are for cultures from only one member of the pair.

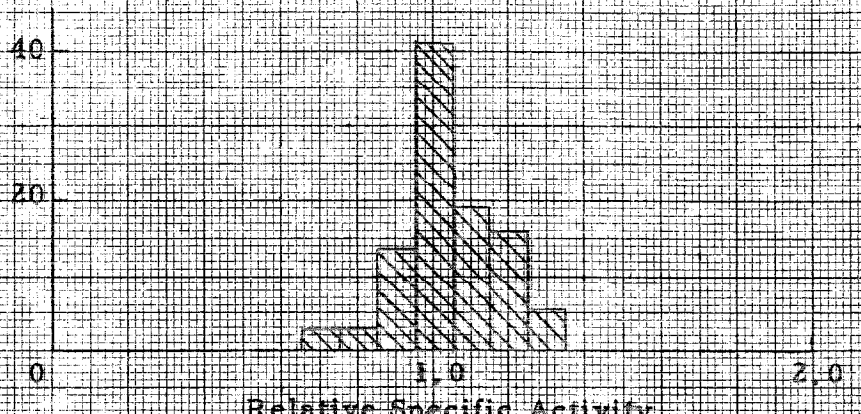
specific activity values determined under both conditions are included in Fig. 12-c.

Considering the number of determinations in each case, the distributions do not differ significantly, and thus it is reasonable to conclude that the wild type progeny of C84A x 8a are like the parent wild type. However, the relative specific activity values of the C84 progeny certainly differ by a significant amount from those of the wild type. The values for the C84 mutants can be divided into two groups: 1) those which are significantly higher than the values for wild type and approximate the average value of 2.8 for the histidineless parent C84A; and 2) those which are zero or very low. Using this classification, the asci dissected fall into three groups: 1) five asci with a 2:2 ratio between low and wild type relative specific activities; 2) two asci with a 1:1:2 ratio between low, high and wild type relative specific activities; and 3) two asci with a 1:1 ratio between high and wild type relative specific activities.

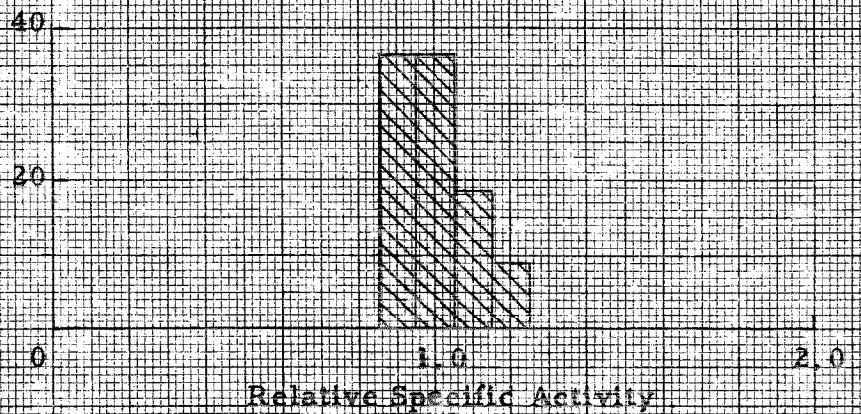
The ascus patterns that are observed can be explained if it is assumed that two genes (one of which is the C84 gene) are effective in changing the tryptophane desmolase activity from that of wild type. Let the other gene be represented by x. Then the cross of C84A to 8a can



A. Distribution of relative specific activity values for wild type spore pairs from C84A x 8a (18 spore pairs).



B. Distribution of relative specific activity values for wild type spore pairs from C83A x 8a (37 spore pairs).



C. Distribution of relative specific activity values for wild type 8a (11 determinations).

Fig. 12. Distribution of relative activity values.

be represented as follows:

	C84A x 8a			— phenotypes
	$\underline{C84}\ \underline{x}$ x $\underline{C84^+}\ \underline{x^+}$			— genotypes
Ascus type 1	$\underline{C84}\ \underline{x^+}$	$\underline{C84}\ \underline{x^+}$	$\underline{C84^+}\ \underline{x}$	$\underline{C84^+}\ \underline{x}$
Ascus type 2	$\underline{C84}\ \underline{x^+}$	$\underline{C84}\ \underline{x}$	$\underline{C84^+}\ \underline{x}$	$\underline{C84^+}\ \underline{x^+}$
Ascus type 3	$\underline{C84}\ \underline{x}$	$\underline{C84}\ \underline{x}$	$\underline{C84^+}\ \underline{x^+}$	$\underline{C84^+}\ \underline{x^+}$

Thus the genotypes $\underline{C84^+}\ \underline{x^+}$ and $\underline{C84^+}\ \underline{x}$ would be phenotypically the same in that they do not require L-histidine and that they have the same specific tryptophane desmolase activity. The $\underline{C84}\ \underline{x^+}$ genotype then represents the phenotype which requires L-histidine and has a low relative specific activity, whereas $\underline{C84}\ \underline{x}$ represents strains which require L-histidine and have a high relative specific activity. In this interpretation, x is effectively a modifier of C84, since it has no observed effect in the presence of $C84^+$.

As crude extracts from cultures of 12 of the 18 spore pairs that require histidine have a low relative specific activity, the C84 and x genes cannot be considered linked; hence we would expect that the probability of obtaining a $\underline{C84}\ \underline{x^+}$ spore pair would be equal to that of obtaining a $\underline{C84}\ \underline{x}$ spore pair. The probability of getting the observed result of twelve histidine-requiring, low relative specific activity spore pairs to six histidine-requiring, high relative activity spore pairs or a result which deviated from the most probable ratio of nine of one type to nine of the other by more than that observed would be 0.234. Thus the observed result is not significantly different from that which would be expected from the theory. Furthermore, the observed ratio of 5:2:2 for the

ascus types 1, 2 and 3 is not significantly different from what the theory would predict (the most probable ratio under the theory being 1:2:1). It is then possible to say that the results are in agreement with the genetic interpretation outlined above.

Though this simplest genetic interpretation is in agreement with the data, other equally valid interpretations involving additional genes can also be formed. However, all of these interpretations have in common the assumption that the C84 gene or one closely linked to it must exert an influence on the tryptophane desmolase activity. Therefore, while these data are too meager to give any reliable indication as to the total number of genes involved in changing the tryptophane desmolase activity from that of wild type, they do indicate that the C84 gene is involved.

It should be noted at this point that the C84 and C83 genes are not allelic. C84 has been found to be in the linkage group E (29) by Hass et al. (12). Thus they found that in an examination of 4124 germinating ascospores from a cross of C84 to inositolless 37401 of linkage group E, only 3.2% were wild type. The linkage group of C83 is not known, but Pittenger (30) has found that an examination of 1236 germinating ascospores from a cross of C83 to 37401 revealed that 24.9% were wild. These data provide good evidence that the C84 and C83 genes are not allelic.

The fact that the C84 progeny of the C84A to 8a cross which

show no tryptophane desmolase activity in crude extracts of four-day-old cultures will grow in the absence of L-tryptophane suggests that they do not actually lack the tryptophane desmolase, or at least do not lack the enzyme during some stage of their growth period. Four of the C84 progeny of this type from different spore pairs were therefore grown for varying lengths of time at 25° C in minimal medium supplemented with 50 μg. L-histidine. At the end of the growth period crude extracts were made and the specific tryptophane desmolase activity measured. Under the same conditions, the specific tryptophane desmolase activities of crude extracts of the parents, 8a and C84A; of one of the wild type progeny; and of one of the C84 progeny which showed a high relative specific activity were measured as a function of time of growth. The results of these experiments are summarized in Fig. 13. For the sake of clarity the results from experiments on only one of the four tested C84 progeny with low specific activity are shown in Fig. 13. However, all four strains exhibited the same type of curve except that the rise in specific activity (at ca. 90 hours for the strain given in Fig. 13) either occurred at a later time or did not occur at all during the 166 hours over which the experiments were conducted.

These curves emphasize the differences between the two types of C84 progeny and their difference from the wild type progeny. It is also apparent from these curves that there is no significant difference

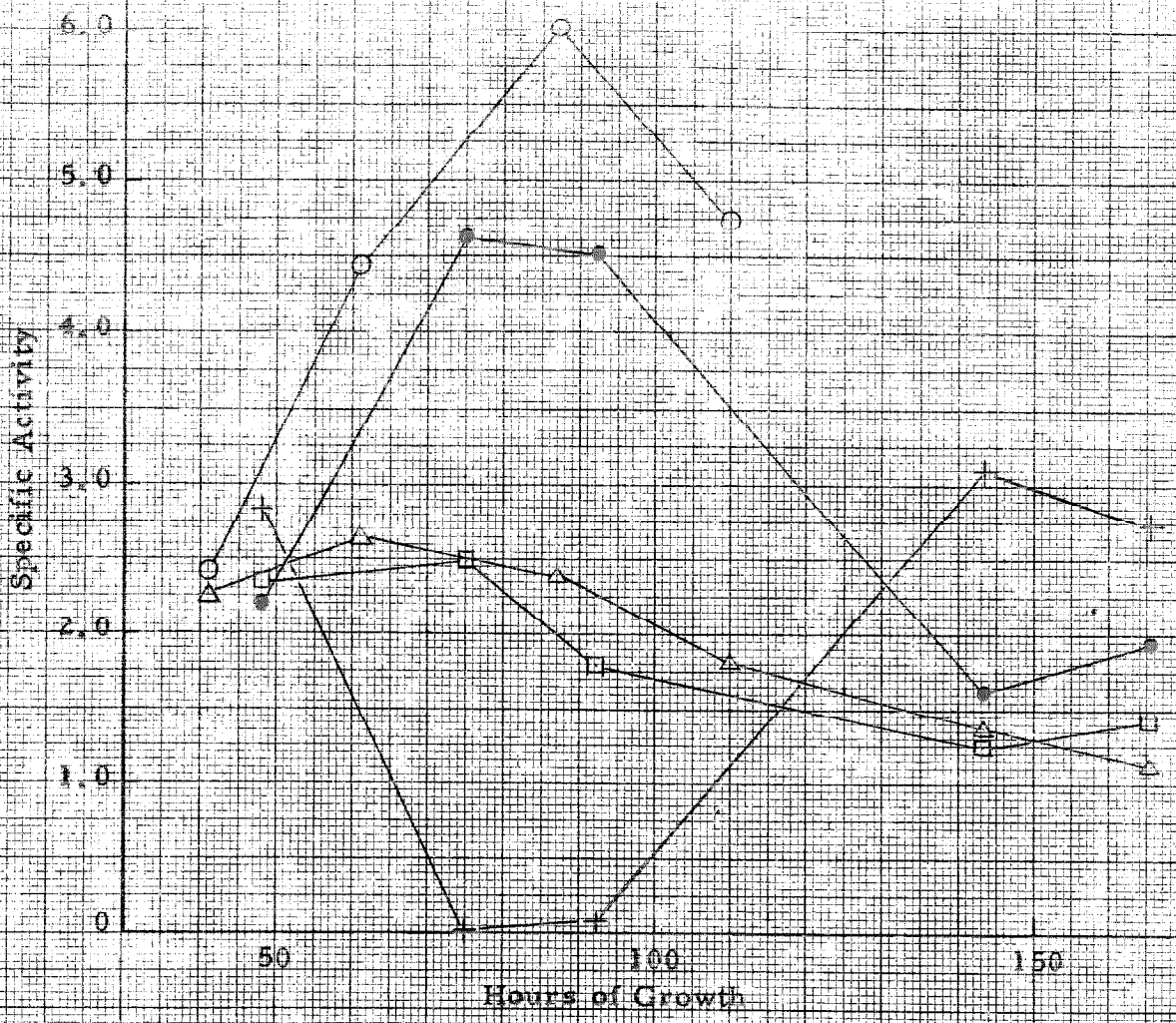


Fig. 13. Specific tryptophane desmolase activity of crude extracts from C84A, 8a and progeny of C84A x 8a as a function of time of growth. Specific activity is in units of $M(\text{min.})^{-1}$ (mg. protein-nitrogen per ml.)$^{-1} \times 10^{-5}$.

between the wild type progeny tested and its wild type parent 8a, nor between the histidine-dependent, high specific tryptophane desmolase activity progeny and its comparable parent, C84A. The nature of the curve for the histidine-dependent, low specific tryptophane desmolase activity progeny shown in Fig. 13 is surprising, particularly in the rise of specific activity after a period of zero or very small activity. This rise in specific activity suggests that the strains do not actually lack tryptophane desmolase at the time when crude extracts of them show no activity, but rather that no activity can be found in these extracts because the enzyme is more difficult to extract from the mold or because of the presence of some strong inhibitor in the extract.

Neither of these possibilities has been critically investigated. However, experiments utilizing mixtures of active and inactive crude extracts do indicate that an inhibitor is present in the inactive extracts. Table XI shows the results of such a mixing experiment.

From these data it is apparent that something in the inactive crude extracts of C84 (1-8-6) strain is inhibiting the activity of the 8a extracts. The nature of this inhibiting material is not known. It was thought that it might be 4-(trihydroxypropyl)-imidazole, which is known to be accumulated by the C84 mutant when grown on limiting concentrations of L-histidine (i.e., 25 μg. L-histidine per ml.) (12, 31). However, no inhibition was observed when this compound was present at a concen-

Table XI

Inhibition by Inactive Extracts from the C84 Progeny of C84A x 8a

Vol. active* extract, ml.	Vol. inactive* extract, ml.	Observed v_o** $M \cdot (min.)^{-1}$	Calculated v_o*** $M \cdot (min.)^{-1}$	$\frac{(v_o\text{-observed})}{(v_o\text{-calculated})}$
0.50	0.00	3.3×10^{-6}	3.3×10^{-6}	1.00
0.38	0.12	2.2×10^{-6}	2.5×10^{-6}	0.88
0.25	0.25	1.0×10^{-6}	1.6×10^{-6}	0.62
0.12	0.38	0.1×10^{-6}	0.8×10^{-6}	0.12
0.00	0.50	0.0×10^{-6}	0.0×10^{-6}	----

* Active extract is the crude extract of 8a. Inactive extract is the crude extract of C84 (1-8-6), one of the histidine-requiring, low specific tryptophane desmolase activity isolates from ascus 1-8 (Table X). Both strains were grown for 92 hours at 25° C in minimal medium supplemented with 50 μg. L-histidine per ml.

** v_o values determined at 37.0° C and pH 7.6. Initial concentrations are indole, 4.22×10^{-4} M; DL-serine, 0.171 M; and pyridoxal phosphate, 4.8×10^{-5} M.

*** Calculated on the assumption that the v_o values are proportional to the amount of active extract (see Fig. 3) and that the inactive extract does not contain any tryptophane desmolase, inhibitor or activitor.

tration of 1×10^{-3} M, and thus it seems unlikely that it could be the inhibiting material present in the inactive crude extracts of the histidineless strains.

Although the results of the mixing experiments do show that an inhibiting material is present in the inactive extracts, they do not indicate whether or not tryptophane desmolase is present in these extracts. The answer to that question will have to await the results of further experiments such as those designed to test for the presence of tryptophane desmolase in the extracts of the C83 mutant (see Table V).

It is apparent then that the data presented in this section cannot be used to evaluate the question of the effect of the C84 gene on the actual amount of tryptophane desmolase present in the mold. The only reasonable conclusion that can be drawn from the data is that the C84 gene influences the tryptophane desmolase activity in the mold. Even here it is assumed that the activity of the crude extracts represents a measure of the activity present in the mold. This is certainly a reasonable assumption but nevertheless an assumption, and hence the conclusion must remain a tentative one.

If this conclusion is utilized, it would seem that a single gene change (from $C84^+$ to C84) has caused two observable effects: 1) an L-histidine growth requirement and 2) a change in tryptophane desmolase activity. The relationship between these two effects is not apparent as

it is between the two effects caused by the C83 gene (i.e., an L-tryptophane requirement and a low tryptophane desmolase activity). It is probable that the L-histidine requirement is indicative of an alteration in the activity of some enzyme system concerned with the formation of L-histidine which is not tryptophane desmolase. Following this line of reasoning, one is forced to the conclusion that the C84 gene influences the activity of two enzyme systems. This tentative conclusion would then seem to be incompatible with the one gene-one enzyme hypothesis since this hypothesis requires "that a given gene be concerned in a primary way with only a single enzyme"(1). The word "primary" eliminates the contradiction but at the same time weakens the hypothesis and, at present, makes it impossible to prove or disprove. Thus the question of which effect of the C84 gene — that of causing a requirement for L-histidine or that of altering the tryptophane desmolase activity — is the primary one and which the secondary, is obviously meaningless at this stage in our knowledge of the development of enzyme activity by the organism.

Experimental: Methods and Materials

Crude Extracts: Crude extracts were prepared by two methods. One method (Method A) resulted in more concentrated crude extracts than did the other (Method B). Method A was utilized for the preparation of crude extracts that were to be purified (Table I) or that were used in attempts to find tryptophane desmolase activity in strain C83-C102-15300 (Table V). Method B was used when assaying for activity in crude extracts of the parents and progeny of the two crosses, C83A x 8a and C84A x 8a.

Method A: The strains C102-15300, C83-C102-15300, and C83re-C102-15300 were grown on minimal medium (32) or minimal medium supplemented with 100 μg. L-tryptophane per ml., depending on the growth requirement of the strain. Either 8 or 16 liters of medium were used for each preparation of mycelium. The carboys containing the medium were incubated at 25° C and the medium subjected to forced aeration. After the desired time (4 days for the C102-15300 and C83re-C102-15300 strains; 2, 3, 4 or 5 days for strain C83-C102-15300) the mycelium was removed by filtering through cotton cloth and washed three times with distilled water. The mycelium was then used immediately for the preparation of the crude extract or frozen at -16° C and stored at this temperature until used.

The mycelium was ground in a mortar in the presence of 0.5 g. of sand and 1.0 ml. of 0.2 M dibasic potassium phosphate containing

2 mg. DL-serine per ml. for every gram (wet weight) of the mycelium. Mitchell and Gordon (15) have found that the presence of DL-serine helps to stabilize the tryptophane desmolase in crude extracts — hence it was used in the grinding operation. Generally ca. 15 g. of mycelium were ground for 10 minutes in a mortar that was 16 cm. in diameter and 10 cm. high. The ground material was then centrifuged for 20 minutes in a Servall centrifuge at ca. 5,000 r.p.m. The supernatant was used as the crude extract. All operations were carried out in the cold room maintained between 2-4° C.

Method B: The parents or progeny of the two crosses C83A x 8a or C84A x 8a were grown on 20 ml. of minimal medium supplemented with the appropriate growth factor contained in 125-ml. Erlenmeyer flasks. The flasks were incubated at 25° C and, after the appropriate growth period, the mycelial pads were removed and washed once with distilled water.

The mycelium was immediately ground in a mortar in the presence of 2.0 ml. of 0.2 M dibasic potassium phosphate containing 2 mg. DL-serine per ml., 2.0 ml. of 0.2 M phosphate buffer at pH 7.7, and 0.5 g. of sand for every gram (wet weight) of mycelium. The conditions of grinding were standardized as follows: approximately 1 g. of mycelium was ground for 5 minutes in mortars having a diameter of 13 cm. and a height of 7 cm. The ground material was then centrifuged in a Servall centrifuge for 20 minutes at ca. 5,000 r.p.m.

The supernatant was used as the crude extract and its tryptophane desmolase activity determined three hours after the grinding operation was initiated. All operations were carried out at room temperature.

Tryptophane Desmolase Activity: Solutions of the substrates, pyridoxal phosphate and inhibitor (if any) in 0.2 M phosphate buffer of the desired pH value were added to 6-inch test tubes. These tubes were then equilibrated at 37.0 $\pm$ 0.1° C in a water bath. At time t = o the appropriate amount of tryptophane desmolase extract (also equilibrated at 37.0 $\pm$ 0.1° C) was added to these 6-inch test tubes and the mixture then stirred for ca. 20 seconds. The test tubes were allowed to remain in the water bath and 1.00 ml. aliquots were removed at convenient intervals and analysed for indole by the method of Fearon (33). Thus the 1.00 ml. aliquots were immediately placed in 6-inch test tubes containing 5.00 ml. of a solution of 1.00 g. of xanthydrol in 1 liter of glacial acetic acid and the contents shaken. These tubes were then heated at 100° C for thirty minutes, cooled, and the intensity of the color determined with a Klett-Summerson photoelectric colorimeter equipped with a green filter (filter no. 54 — transmission range ca. 500-570 millimicrons). The colorimeter readings were always found to be directly proportional to the indole concentrations up to 8×10^{-4} M, although the proportionality constants were found to be dependent on the age of the xanthydrol solution and on the pH and serine content of the calibrating solutions. Therefore, the proportionality constant was

always determined from indole solutions of the same pH value and serine concentrations as those utilized in the enzymatic reaction mixture. Since a calibration curve was prepared for each enzyme run, the age of the xanthydrol solution that was used for the calibration solutions and the reaction solutions was the same. The change of the proportionality constant with age of the xanthydrol solution is small — a change of only 10% was observed over a period of three months. The proportionality constant is not dependent upon the presence or absence of pyridoxal phosphate in the concentrations used in these experiments.

The initial rate of disappearance of indole (v_o) was determined from a plot of colorimeter readings versus time. The initial part of the curve was always found to be linear and hence zero order. Typical curves are given in Fig. 14.

The initial rates of indole disappearance utilized in the calculation of specific activities for the crude extracts of the parents and progeny of the two crosses — C83A x 8a and C84A x 8a — and for following the purification of crude extracts were determined under the following standard conditions: temperature = 37.0° C; pH = 7.6; initial concentration of indole = 4.22×10^{-4} M; initial concentration of DL-serine = 0.171 M; and initial concentration of pyridoxal phosphate = 4.8×10^{-5} M.

The protein-nitrogen content of the extracts was determined by the Kjeldahl method after precipitation with trichloroacetic acid.

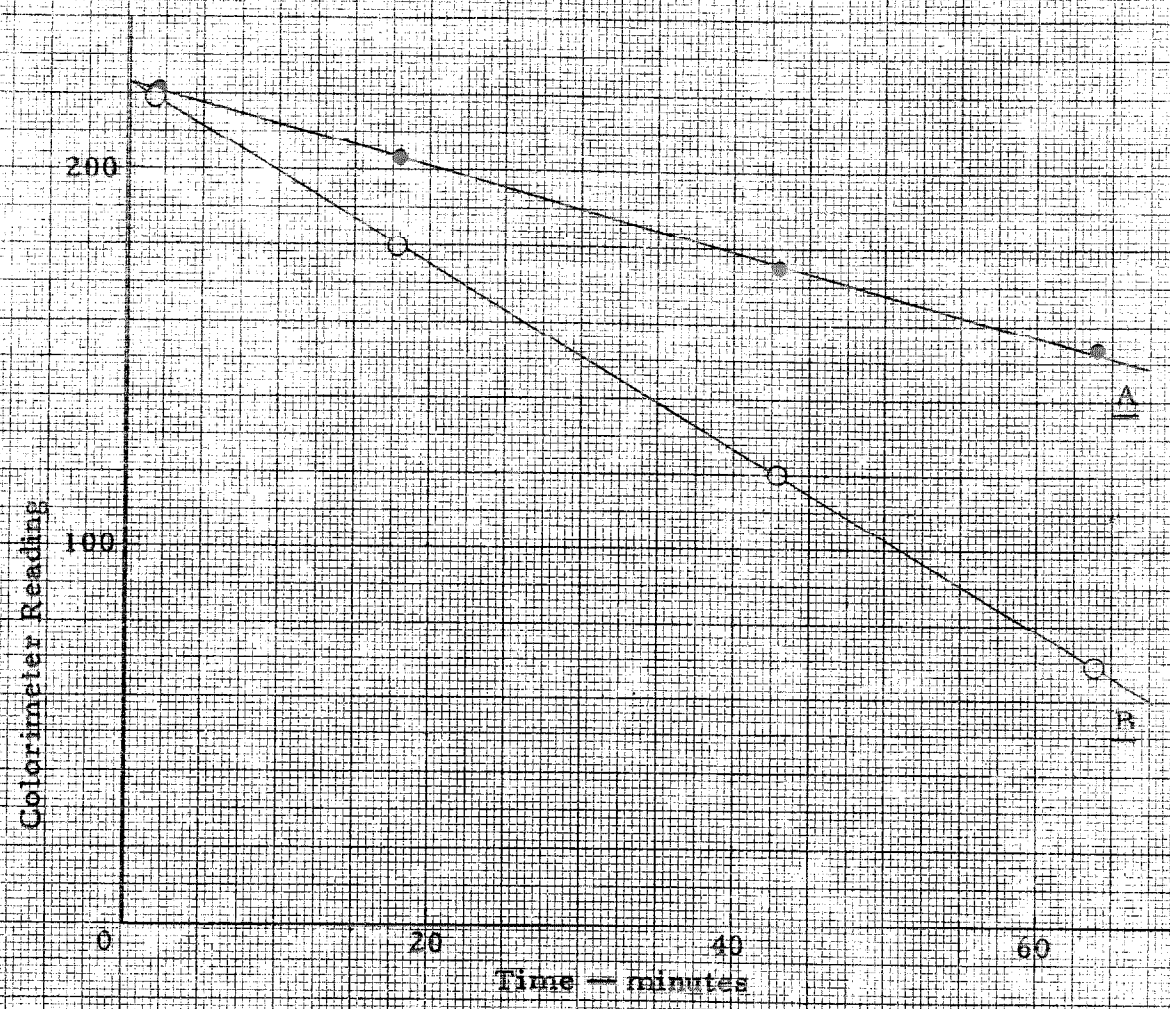


Fig. 14. Determination of initial velocities at 37.0° C and pH 7.6 for two different crude extracts, A and B. Initial concentrations: indole, 4.22×10^{-4} M; DL-serine, 0.171 M; pyridoxal phosphate, 4.8×10^{-5} M; protein-nitrogen, 0.12 and 0.24 mg. per ml. for A and B respectively.

Tryptophane and serine were determined from separate 1.00 ml. aliquots of the reaction solution. Immediately after withdrawal from the reaction solution, the 1.00 ml. aliquot was mixed with 1.00 ml. of 2% perchloric acid. The protein that precipitated was centrifuged down and the clear supernatant used for tryptophane and serine determinations. The tryptophane was determined by the method of Gordon and Mitchell (34). The serine was determined by a modification of the method of Boyd and Logan (35). A 0.20 ml. aliquot of the supernatant solution was mixed with 0.20 ml. of 0.01 M metaperiodate. After standing at room temperature for two hours and then at 3° C for 6.5 hours, 3.00 ml. of 0.039 M sodium arsenite and 2.50 ml. of a chromotropic acid solution (720 mg. chromotropic acid in 20 ml. of water mixed gradually in an ice bath with 30 ml. concentrated sulfuric acid) were added. The mixture was heated at 100° C for 25 minutes, cooled, and the intensity of color measured with a Klett-Summerson photoelectric colorimeter equipped with a green filter (filter number 54). One ml. aliquots of solutions containing known amounts of DL-serine were treated in the same fashion as the aliquots of the reaction mixture. The standard curve is linear for DL-serine concentrations from 0 to 3×10^{-3} M.

Materials

Eastman Kodak Co. (white label) indole was recrystallized from hot petroleum ether (86-100° C) to yield colorless lamelar crystals, m.p. 52-52.5° C.

D-Serine and L-serine were obtained from the California Foundation for Biochemical Research and, according to the manufacturer, have the following properties:

D-serine — $[\alpha]_D$ = -14.4° (c. 10% in 1 N HCl); lit. (36),
$[\alpha]_D^{25}$ = -14.3 ± 0.2° (c. 10% in 1 N HCl).

Anal. Calcd. for $C_3H_7O_3N$ (105): N, 13.33.

Found: N, 13.25.

L-serine — $[\alpha]_D$ = +14.3° (c. 10% in 1 N HCl); lit. (36),
$[\alpha]_D^{25}$ = +14.4° ± 0.2° (c. 10% in 1 N HCl).

Anal. Calcd. for $C_3H_7O_3N$ (105): N, 13.33.

Found: N, 13.41.

DL-Serine was obtained from Winthrop-Stearns, Inc. Pyridoxal phosphate was obtained from Merck and Co. in the form of the calcium salt (Lot No. 9R1068) and is stated to have a purity of 66%. The xanthydrol and chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid) were obtained from the Eastman Kodak Co. The 4-(trihydroxypropyl)-imidazole was kindly supplied by B. N. Ames (see 31). The L-tryptophane was obtained from the Van Camp Laboratories and the L-histidine from Merck and Co. All other chemicals used in these investigations were of reagent grade.

References

1. Beadle, G. W., in "Genetics in the 20th Century" by L. C. Dunn, p. 221 ff., MacMillan Co., New York, 1951.

2. Tatum, E. L. and Bonner, D., Proc. Nat. Acad. Sci. 30, 30 (1944).

3. Umbreit, W. W., Wood, W. A. and Gunsalus, I. C., J. Biol. Chem. 165, 731 (1946).

4. Mitchell, H. K. and Lein, J., J. Biol. Chem. 175, 481 (1948).

5. Gordon, M. and Mitchell, H. K., Genetics 35, 110 (1950).

6. Nason, A., Kaplan, N. D. and Colowick, S. P., J. Biol. Chem. 188, 397 (1951).

7. Yanofsky, C., J. Biol. Chem. 194, 279 (1952).

8. Yanofsky, C., Proc. Nat. Acad. Sci. 38, 215 (1952).

9. Horowitz, N. H. and Mitchell, H. K., Ann. Rev. Biochem. 20, 477 (1951).

10. Giles, N. H., Cold Spring Harbor Symposia Quant. Biol. 16, 283 (1951).

11. Mitchell, H. K., unpublished results.

12. Haas, F., Mitchell, M. B., Ames, B. N., and Mitchell, H. K., Genetics 37, 217 (1952).

13. Mitchell, M. B. and Mitchell, H. K., Proc. Nat. Acad. Sci. 38, 442 (1952).

14. Reissig, J. L., Thesis, Calif. Inst. of Tech., 1952.

15. Mitchell, H. K. and Gordon, M., unpublished results.

16. Stadie, W. C. and Zapp, J. A., J. Biol. Chem. 150, 165 (1943).

17. Laidler, K. J. and Socquet, I. M., J. Phys. and Colloid Chem. 54, 530 (1950).

18. Schwert, G. W. and Hakala, M. T., Arch. Biochem. Biophys. 38, 55 (1952).

19. Lineweaver, H. and Burk, D., J. Am. Chem. Soc. 56, 658 (1934).

20. Straus, O. H. and Goldstein, A., J. Gen. Physiol. 26, 559 (1943).

21. Goldstein, A., J. Gen. Physiol. 27, 529 (1944).

22. Partridge, C. W. H., Bonner, D. M. and Yanofsky, C., J. Biol. Chem. 194, 269 (1952).

23. Kolmark, G. and Westergaard, M., Hereditas 35, 490 (1949).

24. Jensen, K. A., Kolmark, G. and Westergaard, M., Hereditas 35, 521 (1949).

25. Dickey, F. H., Cleland, G. H. and Lotz, C., Proc. Nat. Acad. Sci. 35, 581 (1949).

26. Giles, N. H. and Lederberg, E. Z., Am. J. Bot. 35, 150 (1948).

27. Lein, J., Mitchell, H. K. and Houlahan, M. B., Proc. Nat. Acad. Sci. 34, 435 (1948).

28. Straus, B. S., to be published.

29. Houlahan, M. B., Beadle, G. W., and Calhoun, H. C., Genetics 34, 493 (1949).

30. Pittenger, T. H., unpublished results.

31. Ames, B. N., Mitchell, H. K. and Mitchell, M. B., to be published.

32. Beadle, G. W. and Tatum, E. L., Am. J. Bot. 32, 678 (1945).

33. Fearon, W. R., Analyst 69, 122 (1944).

34. Gordon, M. and Mitchell, H. K., J. Biol. Chem. 180, 1065 (1949).

35. Boyd, M. J. and Logan, M. A., J. Biol. Chem. 146, 279 (1942).

36. Fischer, E. and Jacobs, W. A., Ber. 39, 2942 (1906).

PROPOSITIONS

1. The structure of the triphosphate grouping of adenosine-5' triphosphate rests primarily on titrimetric studies, since its synthesis is not unambiguous (1, 2). I propose that the utilization of C^{14} labelled dibenzyl chlorophosphonate at one stage in the synthesis would provide a method by which the ambiguity could be eliminated.

1. Baddiley, J., Michelson, A. M., and Todd, A. R., J. Chem. Soc. 1949, 582.

2. Michelson, A. M. and Todd, A. R., J. Chem. Soc. 1949, 2487.

2. Since it has been shown that mixtures of trifluoroacetic anhydride and carboxylic, sulfonic, nitrous or nitric acids react with alcohols to yield esters (3), consideration should be given to the use of mixtures of trifluoroacetic anhydride and phosphates as phosphorylating agents of alcohols. In particular the use of the mixed anhydrides of trifluoroacetic acid and a nucleotide in the formation of di- and polynucleotides should be considered.

3. Bourne, E. J., Stacey, M., Tatlow, J. C., and Tedder, J. M., J. Chem. Soc. 1952, 1695.

3. I propose that biochemical pressure mutants of microorganisms should exist.

4. From the anomalous sedimentation behavior of alpha-chymotrypsin it has been inferred that the protein undergoes a reversible dimerization (4). This could be tested by a kinetic analysis at high enzyme concentrations.

4. Schwert, G. W. and Kaufman, S., J. Biol. Chem. 190, 807 (1951).

5. I propose an interpretation of the differences in suppressor action on the two tryptophaneless mutants, C83 and S1952 (5, and Part II of this thesis).

5. Yanofsky, C., Proc. Nat. Acad. Sci. 38, 215 (1952).

6. Since it has been shown that organic peroxides can function as mutagenic agents (6), consideration should be given to the influence of peroxide-producing enzymes such as lipoxidase (7) on the spontaneous mutation rate.

6. Dickey, F. H., Cleland, G. H., and Lotz, C., Proc. Nat. Acad. Sci. 35, 581 (1949).

7. Bergstrom, S. and Holman, R. T., Adv. Enzym. 8, 425 (1948).

7. Since indole has a high affinity for tryptophane desmolase, consideration should be given to the possible coprecipitation of indole and the enzyme as a means of purifying the enzyme. Alternatively, the

polymer of indole that is formed in acid solutions might be used as a specific absorbent for tryptophane desmolase. These techniques might also be applied to the purification of alpha-chymotrypsin.

8. Two methods are suggested for testing the possibility that the P_z protein of Cohn and Torriani (8) functions in the induced formation of β-galactosidase in E. coli (9).

   a. Equilibrium dialysis studies of the P_z protein and the inductors of β-galactosidase would determine whether or not the inductors are specifically bound to the P_z protein.

   b. Since β-galactosidase activity cannot be induced in amino acid requiring mutants of E. coli unless the amino acid is present, addition of the amino acid labelled with C^{14} to E. coli starved for the amino acid and in a medium containing a β-galactosidase inductor might produce radioactive β-galactosidase containing more C^{14} than the bulk of the protein. Hence an examination of the radioactivity present in the P_z protein would give an indication of any specific role it played in the formation of β-galactosidase.

8. Cohn, M., and Torriani, A. M., C. R. Acad. Sci. 232, 115 (1951).

9. Monod, J. and Cohn, M., Adv. Enzymol. 13, 67 (1952).

9. It has been shown that the egg of Arbacia punctulata can be centrifugally dissected into quarters, one of which contains the nucleus but no mitochondria (10). Since this quarter can develop into a normal pluteus which contains mitochondria, this system could be utilized to study the development of mitochondria. Therefore, it is proposed that the enzyme systems normally associated with animal mitochondria be studied in the microsomal and mitochondrial fractions of ground up cells which are at various stages in their development. In this manner a developmental relationship between the microsomes and mitochondria might be detected.

    10. Harvey, E. B., J. Exp. Zool. 102, 253 (1946).

10. I propose that sonic vibrations might be utilized to increase the efficiency of fractionation columns.

11. The argument that if two substrates, S_1 and S_2, of the same enzyme have K_S and k_3 values such that $(K_S)_1 < (K_S)_2$ and $(k_3)_1 > (k_3)_2$, then the corresponding enzyme-substrate dissociation constants will be in the same order as are the K_S values $\left(\left(\frac{k_2}{k_1}\right)_1 < \left(\frac{k_2}{k_1}\right)_2\right)$ is not valid (11).

    11. Hogness, D. S. and Niemann, C., J. Am. Chem. Soc. 74, 3183 (1952).