I. SOME STUDIES IN ENZYME KINETICS.

II. THE OXIDATION OF 3-INDOLEACETIC ACID BY PLANT ENZYMES.

III. THE SYNTHESIS OF SOME α-ALKYL α-AMINO ACIDS AND THEIR DERIVATIVES.

Thesis by

David Treadway Manning

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ABSTRACT

The kinetics of the alpha-chymotrypsin catalyzed hydrolysis of N-carboethoxy-L-tyrosinamide have been determined in aqueous solution at 25°C and at both pH 7.9 and pH 8.2. The enzyme-inhibitor dissociation constants of N-carboethoxy-D-tyrosinamide, N-carboethoxy-D-tyrosinmethylamide, and N-carboethoxy-L-tyrosinmethylamide have been evaluated in aqueous solution at 25°C, the former at pH 7.9 and the latter two at pH 7.6. The L isomers of each enantiomorphic pair appear to have a greater affinity for the enzyme than do their respective D-isomers.

An investigation into the nature of the enzymatic oxidation products of 3-indoleacetic acid (IAA) has been made. 3-Indolealdehyde, o-formamidoacetophenone, o-aminoacetophenone, and 4-hydroxyquinoline have been eliminated as possible major products of the reaction.

A study of synthetic routes to the alpha-alkyl alpha-amino acids has been made. DL-alpha-Methylphenylalanine and DL-alpha-methyltyrosine have been synthesized. All attempts to resolve these compounds by enzymatic means have failed.

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SECTION I

PART I

THE KINETICS OF INTERACTION OF THE ENANTIOMORPHIC N-CARBOETHOXY-TYROSINAMIDES WITH ALPHA-CHYMOTRYPSIN IN AQUEOUS SOLUTION AT 25°C.

Introduction

Investigations concerning the structural requirements of alpha-amino acid derivatives of the general formula $R_1CHR_2R_3$ capable of interacting with the proteolytic enzyme alpha-chymotrypsin have led to the view[1-4] that such derivatives combine with the enzyme at its catalytically active site by virtue of specific interactions between the three principal structural features, R_1, R_2, and R_3 of the amino acid derivative and three complementary centers, ρ_1, ρ_2, and ρ_3 assumed to exist at the active site of the enzyme. In customary terminology the structural characters R_1, R_2, and R_3 represent the alpha-acylamino group, the alpha-amino acid side chain, and the functional derivative of the carboxyl group of the amino acid derivative, respectively[2]. To gain insight into the nature of the forces involved in enzyme-substrate and enzyme-inhibitor interactions it has been of interest to investigate the effect upon the magnitudes of these forces, as revealed by the effect upon the various kinetic constants involved, of varying these structural features.

According to the classical formulation[1,5,6] the interaction of a substrate with alpha-chymotrypsin and its subsequent hydrolysis in aqueous solution may be represented by

$$(1) \qquad [E] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_3} [E] + \text{products}$$

where the Michaelis constant K_s is given by

$$(2) \qquad K_s = \frac{k_2 + k_3}{k_1}$$

and k_3 represents the specific rate constant of the hydrolytic step. If, in addition to the substrate, an added inhibitor is present in the system then the following equilibrium must also be considered;

$$(3) \qquad [E] + [I] \underset{k_5}{\overset{k_4}{\rightleftharpoons}} [EI]$$

for which the equilibrium constant is termed K_I.

While certain requirements[1-4] must be met by all of the structural characteristics of an amino acid derivative to render it a specific substrate for alpha-chymotrypsin, considerable variation may be made in the nature of the alpha-acylamino group R_1 without serious reduction of this property[3]. A number of alpha-amino acid derivatives differing only in the nature of the acyl group upon the alpha-amino nitrogen have therefore been investigated in the hope of correlating known electrostatic, steric, or other property of such a group with either the affinity of the enzyme for the substrate or with the susceptibility of the R_3 carboxyl function toward hydrolysis. Since previous determinations of the kinetic constants of the acetyl,[7,8,10] trifluoroacetyl,[3] chloroacetyl,[3] and nicotinyl[8-11] derivatives of various alpha-amino acid amides have indicated a possible relation between their K_s values and corresponding molecular volumes of these groups[3] the question arose as to the effect of the bulky carboethoxy group upon the K_s and k_3 values. Accordingly, the enantiomorphic carboethoxytyrosinamides were prepared with the purpose of testing the L isomer as a specific substrate and the D isomer as a competitive inhibitor of alpha-chymotrypsin. In the case of the L isomer it was

felt that any bulk-attraction or electrostatic influence of the N-carboethoxy group might be manifested in the value of the breakdown constant k_3 or in the steady state constant K_s which may be considered, in the case of the acylated amino acid amide substrates, as a measure of affinity of the substrate for the enzyme.[2,8,9,10] If, as was expected, the D isomer proved to be a competitive inhibitor of alpha-chymotrypsin then its affinity for the enzyme would be expressed by K_I, the true equilibrium constant for the enzyme-inhibitor complex (equation (3)).

Treatment of Kinetic Data

Returning to the previously stated formulation for a system of enzyme, substrate, and added competitive inhibitor,

$$(1) \qquad [E] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_3} [E] + \text{products}$$

$$(3) \qquad [E] + [I] \underset{k_5}{\overset{k_4}{\rightleftharpoons}} [EI],$$

and making the following assumptions,[1,5,6,10] that [S] and [I] are much greater than [E] and that a steady state is rapidly attained in which

$$(4) \qquad \frac{d[S]}{dt} \gg \frac{d[ES]}{dt} \qquad \text{and} \qquad \frac{d[S]}{dt} \gg \frac{d[EI]}{dt}$$

it follows that the rate of enzymatic hydrolysis of a substrate in the

presence of an added inhibitor is given by

$$(5) \qquad v = -\frac{d[S]}{dt} = k_3[ES] = \frac{k_3 [E] [S]}{[S] + K_s}$$

When $[S] \gg K_s$, v approaches the value $k_3[E]$ and at the maximum concentration of enzyme-substrate complex ($[ES] = [E]$), when the enzyme is saturated with substrate, the velocity becomes

$$(6) \qquad V_m = k_3[ES]_m = k_3[E]$$

Substituting V_m for $k_3[E]$ into equation (4) and inverting leads to the useful form[a]

$$(7) \qquad \frac{1}{v} = \frac{(1 + \frac{I}{K_I})K_s}{V_m} \cdot \frac{1}{[S]} + \frac{1}{V_m}$$

This equation suggests that at a constant concentration of added inhibitor [I], the reciprocal of the velocity of a substrate obeying the above kinetic formulation bears a linear relationship to the reciprocal of the substrate concentration at any time t. In the widely used Lineweaver-Burk treatment,[6] initial velocities are estimated from constructed tangents at the $t = 0$ points of plots of [S] versus t or of ln[S] versus t. At time zero equation (7) becomes

$$(8) \qquad \frac{1}{v_o} = \frac{(1 + \frac{[I]}{K_I})K_s}{V_m} \cdot \frac{1}{[S]_o} + \frac{1}{V_m}$$

and with no added inhibitor present ($[I] = 0$) a plot of the reciprocals of these estimated initial velocities against the corresponding

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[a] This is the inverted form of the Michaelis-Menten[12] equation.

reciprocal initial substrate concentrations results in a straight line having a slope of $\frac{K_s}{V_m}$ and an intercept of $\frac{1}{V_m}$ from which these constants may be obtained. Since the intercept is independent of added competitive inhibitors, the plotting of $\frac{1}{[S]_o}$ vs. $\frac{1}{v_o}$ data for a series of such experiments conducted with increasing amounts of inhibitor present will result in a family of straight lines having an intercept common with that of the line representing uninhibited hydrolysis but having slopes greater than the $[I] = 0$ slope by factors of $(1 + \frac{[I]}{K_I})$. The advantage in estimation of instantaneous velocities at time zero arises from the fact that during the hydrolysis of an acylated amino acid amide substrate the hydrolysis product, i.e., the acylated amino acid, accumulates. Since such compounds are, in general, competitive inhibitors of alpha-chymotrypsin the velocity at any time t greater than zero will be less than it would be in its absence and this velocity retarding effect increases throughout the reaction.[7,10] Hence, the estimation of initial velocities from reactions limited to about 40 per cent total hydrolysis has been the general practice. Otherwise it would be feasible to determine an entire series of $\frac{1}{[S]}$ and $\frac{1}{v}$ values from one kinetic experiment beginning at an appropriately high initial substrate concentration.

While equations (7) and (8) are in theory entirely adequate for determining the desired kinetic constants, the practical difficulty arises of estimating initial velocities from the kinetic data, i.e., from the substrate concentration measured at time t. This situation is best illustrated by integration of equation (7) to give

$$(9) \qquad k_3[E]t = (1 + \frac{[I]}{K_I})K_s \ln \frac{[S]_o}{[S]} + [S]_o - [S]$$

which reduces to

$$(10) \qquad k_3[E]t = K_s \ln \frac{[S]_o}{[S]} + [S]_o - [S]$$

in the absence of added competitive inhibitors. Equation (10) relates, implicitly, substrate concentration to any explicit value of t for a given set of reaction constants and conditions. The two right hand members of this equation may be interpreted as first and zero order terms respectively.[7] Since in the general case the reaction kinetics are intermediate between zero and first order with respect to the substrate concentration a plot of either $[S]$ or of $\ln[S]$ versus the corresponding time values will give a curving line whose rate of departure from linearity depends upon the relative magnitudes of K_s and the substrate concentrations $[S]$. It may be seen from equation (6) that in the range of large $[S]_o$ values, reactions conducted over intervals of low total fractional hydrolysis will receive little contribution from the logarithmic term of equation (10) and approximate zero order kinetics. Under these conditions equation (10) becomes

$$(11) \qquad k_3[E]t = [S]_o - [S] \quad \text{and} \quad \ln \frac{[S]_o}{[S]} = \ln 1 = 0$$

At low substrate concentrations where the fraction of hydrolysis becomes appreciable the logarithmic term predominates and the kinetics approach the first order extremity of

$$(12) \qquad k_3[E]t = K_s \ln \frac{[S]_o}{[S]}$$

A high value of K_s may also increase the first order character of the kinetics as is indicated by this constant multiplying the first order term. A clearer exposition of the interplay between [S] and K_s in determining the position of the reaction order between the two extremes is given by the equation

$$(13) \qquad v = \frac{V_m \cdot [S]}{K_s + [S]}$$

which derives from equation (5) when $[I] = 0$. As seen from equation (13) when $K_s > [S]$ the velocity of the reaction approaches $\frac{V_m}{K_s} \cdot [S]$, or first order in [S], while in the region $[S] > K_s$ the expression reduces to $v = V_m$ which is to say that when the enzyme is saturated with substrate the velocity is independent of the substrate concentration and is equal to the maximum velocity, i.e., the true zero order velocity.

Thus, while a tangent constructed at the $t = 0$ point of either the [S] vs. t (zero order) or ln[S] vs. t (first order) plot will yield, theoretically, the true initial velocity (v_o) value associated with a particular value of $[S]_o$, the practical difficulty of drawing such a tangent line increases with the tendency of the plot to curve away from a straight line. This difficulty has, in the past, been overcome to some extent by the estimation of initial velocities from [S] vs. t plots when dealing with initial substrate concentrations higher than the value of K_s and from ln[S] vs. t plots when operating in the region $K_s > [S]_o$. For a value of $[S]_o = K_s$ both types of plots depart from linearity with equal rapidity and at such a concentration neither plot is preferable to the other in velocity estimation.

The alpha-chymotrypsin catalyzed hydrolysis of N-carboethoxy-L-tyrosinamide was followed by the titrimetric method developed by Huang and Niemann[10] in which aliquots of reaction mixture are removed at successive time intervals and analyzed for content of hydrolysis product by a formol titration. Fifty-six such experiments were performed at pH 7.9 with the aqueous system buffered by a tris-(hydroxymethyl)-amino-methane-hydrochloric acid buffer at a concentration of 0.02 F with respect to the amine component. In all experiments the enzyme concentration was 0.208 mg. protein-nitrogen per ml. and the initial substrate concentrations varied from 5.0×10^{-3} M to 20.0×10^{-3} M. One experiment each was performed at $[S]_o = 25.0 \times 10^{-3}$ M and 30.0×10^{-3} M and as there was some indication that the solubility limit of the substrate had been reached further runs at concentrations in excess of 20×10^{-3} M were not attempted.

In the case of the present hydrolysis studies, plots of ln[S] vs. t appeared virtually linear over a sixty minute experimental interval while the zero order plots invariably curved abruptly below a straight line. The linearity of the first order and extreme downward curvature of the zero order plots (Figures 1 and 2) are explained by the generation, throughout the course of the reaction, of the inhibiting split product N-carboethoxy-L-tyrosine. This enhancement of the importance of the first order term (of equation (10)) by split product inhibition had been noted previously by Huang and Niemann[10] in their study of the hydrolysis of nicotinyl-L-tryptophanamide. While the effect upon a plot of $([S]_o - [S])$ vs. t is to increase further the inherent negative curvature from a straight line, the effect upon the log plot is to compensate for the downward deviation.

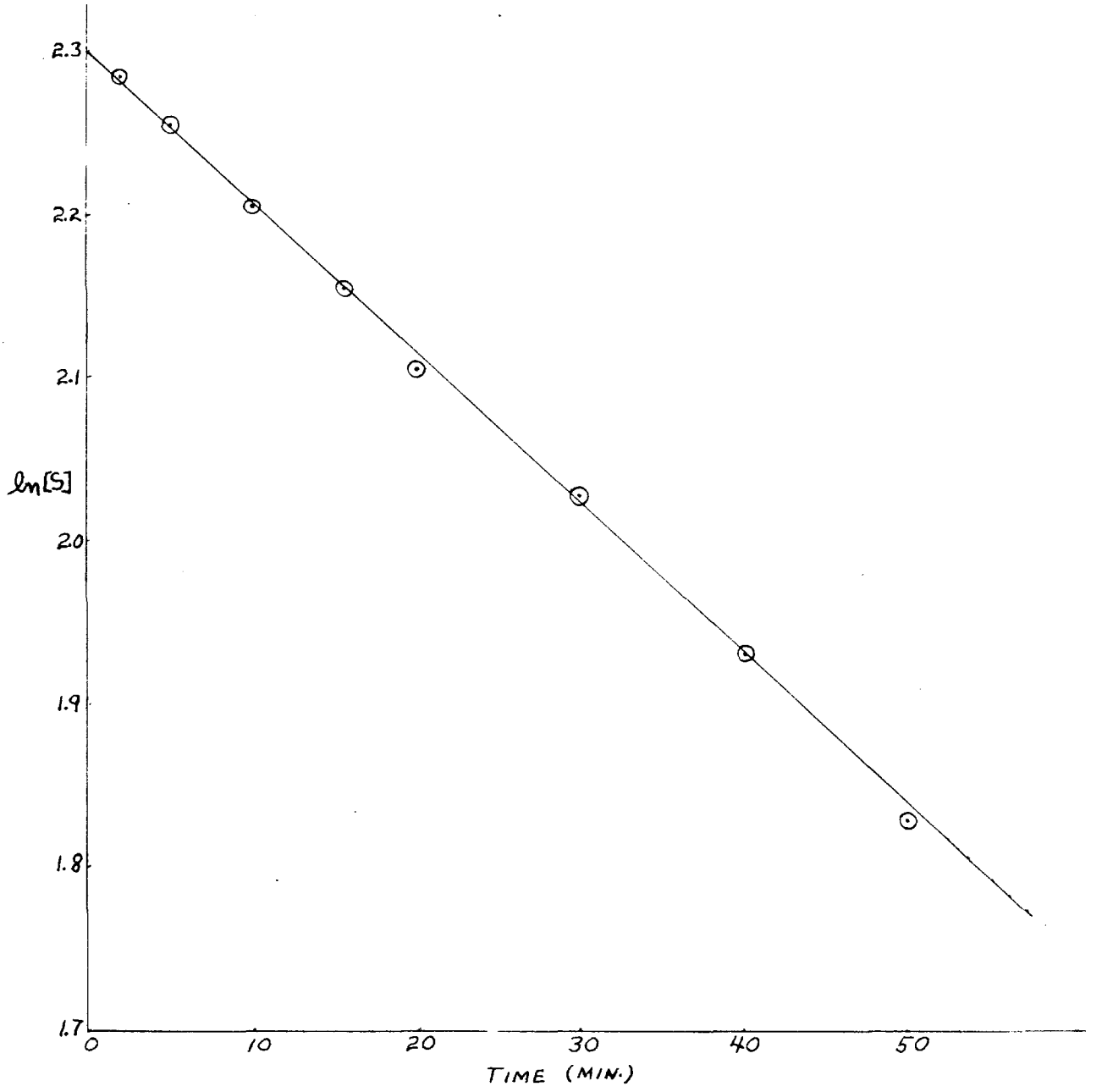

Fig. 1. First Order Plot of Run No. 42.
$[S]_o = 10 \times 10^{-3}$ M, [E] = 0.208 mg. protein-nitrogen/ml.

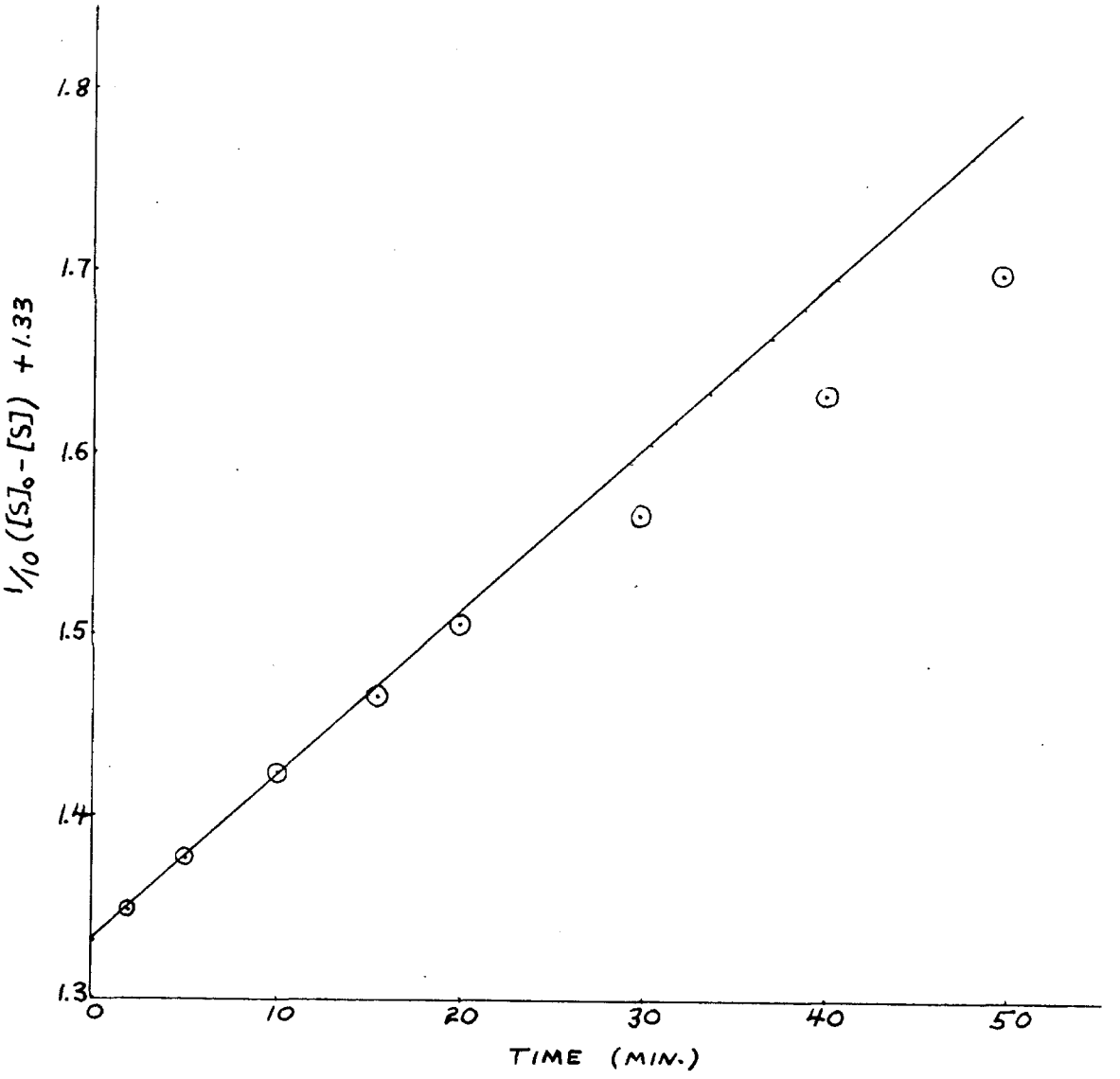

Fig. 2. Zero Order Plot of Run No. 42.
$[S]_o = 10 \times 10^{-3}$ M, [E] = 0.208 mg. protein-nitrogen/ml.

To remedy the difficulty of estimating the true initial velocity of an enzymatic hydrolysis it was decided to employ the velocity correction method developed by Jennings and Niemann.[13] In this method, the individual time values (t), corresponding to measured $([S]_o - [S])$ or $\ln[S]$ values of an enzymatic hydrolysis are reduced to new parameters (t') which lie upon the initial tangent line. In the case of the first order logarithmic plots the procedure is as follows: for a plot of $\ln[S]$ vs. t, the equation

$$\ln[S] = \frac{V_m \cdot t'}{K_s + [S]_o} + \ln[S]_o \qquad (14)$$

may be written, representing the line tangent to the $\ln[S] = f(t)$ curve at $t = 0$. (See Fig. 3.) When multiplied by $\frac{1}{[S]_o}$, the slope of this tangent line gives the true initial velocity v_o. It should be mentioned that the experimental curve depicted in the above diagram is meant to represent the ideal case in which split product inhibition does not tend to reduce the downward curvature.

By combining equations (14) and (10), a first order correction factor may be derived which, when divided into the time intervals (t) corresponding to the experimentally determined values of $\ln[S]$ on the lower curved line (Fig. 3), gives time parameters (t') lying on the tangent line.

$$\frac{t}{t'} = \frac{K_s}{K_s + [S]_o} + \frac{[S]_o - [S]}{(K_s + [S]_o) \ln \frac{[S]_o}{[S]}} \qquad (15)$$

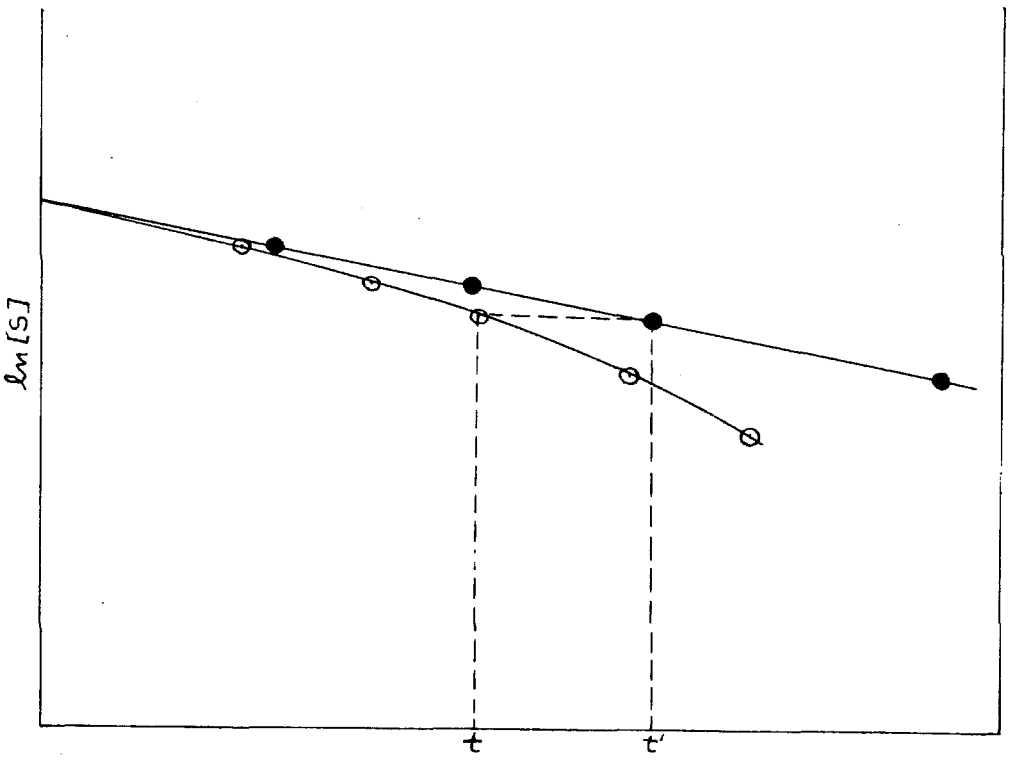

Fig. 3. Hypothetical first-order plot illustrating velocity correction method: open circles are experimental points; solid circles represent these values shifted to initial tangent (Eq. 14) by correction factors.

In a similar manner t/t' correction factors may be derived to convert values on the zero order, $[S] = f(t)$ plots to new values defining initial tangent lines.[13] The usual procedure in determining initial velocities by this method is as follows. Initial velocities are first estimated from zero and first order plots and from these an approximate K_s is calculated according to the Lineweaver-Burk method (equation (8)). This value of K_s is then inserted into equation (15) (or in the corresponding equation for zero order correction factors) from which correction factors may be obtained. The corrected values of the initial velocities may then be used to determine an improved value of K_s. Since the true initial velocity of an enzymatic hydrolysis obeying kinetics intermediate between zero and first order will, in general, fall between the velocities estimated from the two non-linear plots it is evident that one of the groups of correction factors must be somewhat less than and the other somewhat greater than unity with the result that, in a given determination, one of the estimated velocities will be decreased and the other increased by their respective correction factors to give the same intermediate corrected velocity.

In the enzymatic experiments with N-carboethoxy-L-tyrosinamide at pH 7.9 initial velocities were estimated from both zero order and first order plots and their reciprocals plotted on separate Lineweaver-Burk plots.[6] From these separate plots two approximate values of K_s were obtained by a least squares treatment of the data (see Table I). From these values of K_s, separately applied, zero order and first order correction factors were calculated and applied to the correction of the first order and zero order initial velocities. As may be seen from

Table II the agreement between corrected zero and first order velocities is good for a majority of the fifty-six experimental determinations. In cases where the discrepancy between corrected velocities was about 10 per cent or greater the $([S]_o - [S])$ vs. t' and ln[S] vs. t' plots for the run in question were checked for possible errors in velocity estimation. In runs no. 11, 13, 21, 22, 25, 30, 32, 35, 39, 46, and 48 it was found possible to reestimate either the corrected zero or first order velocity in a manner bringing them closer to the same value. Finally the corrected zero order and first order initial velocities were averaged (last column of Table I) and their reciprocals plotted versus their corresponding reciprocal initial substrate concentrations (see Fig. 4). From these values K_s and V_m were obtained by a least squares determination (Table III).

The enzyme-inhibitor dissociation constant K_I of N-carboethoxy-D-tyrosinamide was next determined. Sixteen hydrolysis experiments with N-carboethoxy-L-tyrosinamide as the substrate were performed with the D isomer present as added inhibitor in concentrations of either $[I] = 5.0 \times 10^{-3}$ M or $[I] = 10.0 \times 10^{-3}$ M. When the reciprocal initial velocities, estimated from first order plots, were plotted in the Lineweaver-Burk manner two straight lines corresponding to the two concentrations of added inhibitor resulted. The behavior of these lines suggested competitive inhibition by extrapolating to near the ordinate $(\frac{1}{v_o})$ intercept for uninhibited hydrolysis and the slopes of the lines increased in the direction of increasing inhibitor concentration.

TABLE I

Kinetic Constants for N-Carboethoxy-L-tyrosinamide from Uncorrected Initial Velocity Data[a]

Kinetic Order	K_s[b]	k_3[d]
0	7.23	0.66
1[c]	5.35	0.69

[a] All determinations at pH 7.9 and [E] = 0.208 mg. protein-nitrogen per ml.

[b] In units of 10^{-3} M.

[c] The first order kinetic constants were actually determined from a total of only 23 runs.

[d] In units of 10^{-3} mole/min/mg. protein-nitrogen/ml.

TABLE II

Initial Velocity Data for N-Carboethoxy-L-tyrosinamide[a]

Run	$[S]_o$	Uncorr. $v_o's$[b]		Corr. $v_o's$[b]		Aver. Corr. $v_o's$[b]
		0 ord.	1st ord.	0 ord.	1st ord.	
1	5.0	$.056_2$	.063	$.059_2$	$.060_5$	$.059_8$
2	5.0	$.060_1$	0.69	$.064_7$	.065	$.064_8$
3	5.0	$.056_3$	.062	$.058_6$	$.058_5$	$.058_5$
4	5.0	$.064_1$	.069	$.064_1$	.065	$.064_5$
5	5.0	$.061_3$	.068	$.062_4$	.065	$.063_7$
6	5.0	$.050_5$	$.045_5$	$.050_5$	-	$.050_5$
7	5.0	.050	.059	.053	.056	$.054_5$
8	5.0	$.060_3$	$.060_5$	$.064_6$	$.056_5$	$.060_5$
9	5.0	$.060_8$	.068	.063	$.065_5$	$.064_2$
10	5.70	$.064_5$	$.072_4$	$.064_5$	.068	$.066_2$
11	5.70	.061	$.071_6$	$.063_7$	$.065_2$[c]	$.064_5$
12	5.70	$.073_3$	.082	$.074_5$	.077	$.075_7$
13	5.70	$.060_3$	.077	$.065_5$	$.070_9$[c]	$.068_2$
14	5.70	$.063_1$	.071	$.066_2$	$.067_5$	$.066_8$
15	5.70	$.070_6$	.076	$.073_7$	$.076_5$	$.075_1$
16	5.70	$.072_1$	.084	$.077_3$	.078	$.077_6$
17	5.70	$.070_9$	.081	$.076_2$	$.076_5$	$.076_4$
18	5.70	$.056_6$	.068	$.057_2$	.065	$.061_1$
19	5.70	$.055_1$	.072	$.060_6$	$.064_2$[c]	$.062_4$
20	5.70	$.062_7$	$.067_5$	$.064_1$	.063	$.063_5$

TABLE II (cont.)

Run	$[S]_o$	Uncorr. $v_o's$[b]		Corr. $v_o's$[b]		Aver. Corr. $v_o's$[b]
		0 ord.	1st ord.	0 ord.	1st ord.	
21	5.70	$.063_4$	.077	$.066_2$	$.071_6$[c]	$.068_9$
22	5.70	$.060_9$	$.071_7$	$.062_9$	.062	$.062_4$
23	5.70	.057	.068	$.058_3$	$.062_5$	$.060_4$
24	5.70	$.060_1$	$.071_1$	$.065_7$	$.068_5$	$.067_1$
25	6.66	$.060_9$	.071	$.066_7$[c]	$.069_5$	$.068_1$
26	6.66	$.081_8$	.081	$.081_8$	$.074_5$	$.078_1$
27	6.66	$.073_3$	.081	$.075_1$	.076	$.075_5$
28	6.66	$.075_7$	.079	$.077_6$	.075	$.076_3$
29	6.66	$.072_6$	.076	$.072_6$	$.072_5$	$.072_6$
30	6.66	$.061_5$	$.073_8$	$.063_2$	$.067_2$[c]	$.065_2$
31	6.66	$.065_4$	.075	$.068_5$	$.072_2$	$.070_3$
32	8.00	$.075_3$	.087	$.074_3$	$.082_2$[c]	$.078_2$
33	8.00	$.074_3$	.078	$.075_7$	.075	$.075_4$
34	8.00	$.081_7$	.085	$.080_6$	.082	$.081_3$
35	8.00	$.067_1$	.080	$.070_5$	$.073_3$[c]	$.071_9$
36	8.01	$.078_5$	$.086_5$	$.081_8$	.083	$.082_4$
37	8.02	$.090_2$	.096	$.090_2$	.092	$.091_1$
38	8.33	$.082_6$	.089	.088	.083	.085
39	8.33	$.072_6$	.089	$.074_8$	.080[c]	$.077_4$

TABLE II (cont.)

Run	$[S]_o$	Uncorr. $v_o's$[b]		Corr. $v_o's$[b]		Aver. Corr. $v_o's$[b]
		0 ord.	1st ord.	0 ord.	1st ord.	
40	10.0	$.083_4$	.093	.087	.083	.085
41	10.0	$.087_8$	.093	$.087_8$	.088	$.087_9$
42	10.0	$.092_1$	.092	$.092_1$	.090	.091
43	12.50	$.087_4$	$.095_3$	$.089_4$	.091	$.090_4$
44	12.65	$.085_1$	.096	$.087_9$	$.091_5$	$.089_7$
45	13.15	$.096_4$	.099	$.093_3$	$.097_5$	$.095_4$
46	13.15	.101	.103	$.097_2$	$.097_7$	$.097_4$
47	15.15	.089	.100	$.091_6$	.096	$.093_8$
48	17.63	$.097_2$	$.113_5$	$.097_2$	$.109_4$	$.103_3$
49	20.0	.103	.108	.103	.102	$.102_5$
50	20.0	.110	.106	.112	$.101_2$	$.106_6$
51	20.0	$.107_3$	.111	$.106_4$	.107	$.106_7$
52	20.0	$.108_9$	.112	$.106_6$	.106	$.106_3$
53	20.0	$.100_4$	.104	$.102_3$	-	$.102_3$
54	20.0	$.105_8$	.108	$.107_3$	.104	$.105_6$
55	20.0	$.104_5$	.106	.102	.101	$.101_5$
56	20.0	$.105_2$	.106	$.105_2$	.101	$.103_1$

[a] All determinations made at pH 7.9 and [E] = 0.208 mg. protein-nitrogen per ml.

[b] Velocity values are in units of 10^{-3} M·min.$^{-1}$

[c] Corrected initial velocity reestimated.

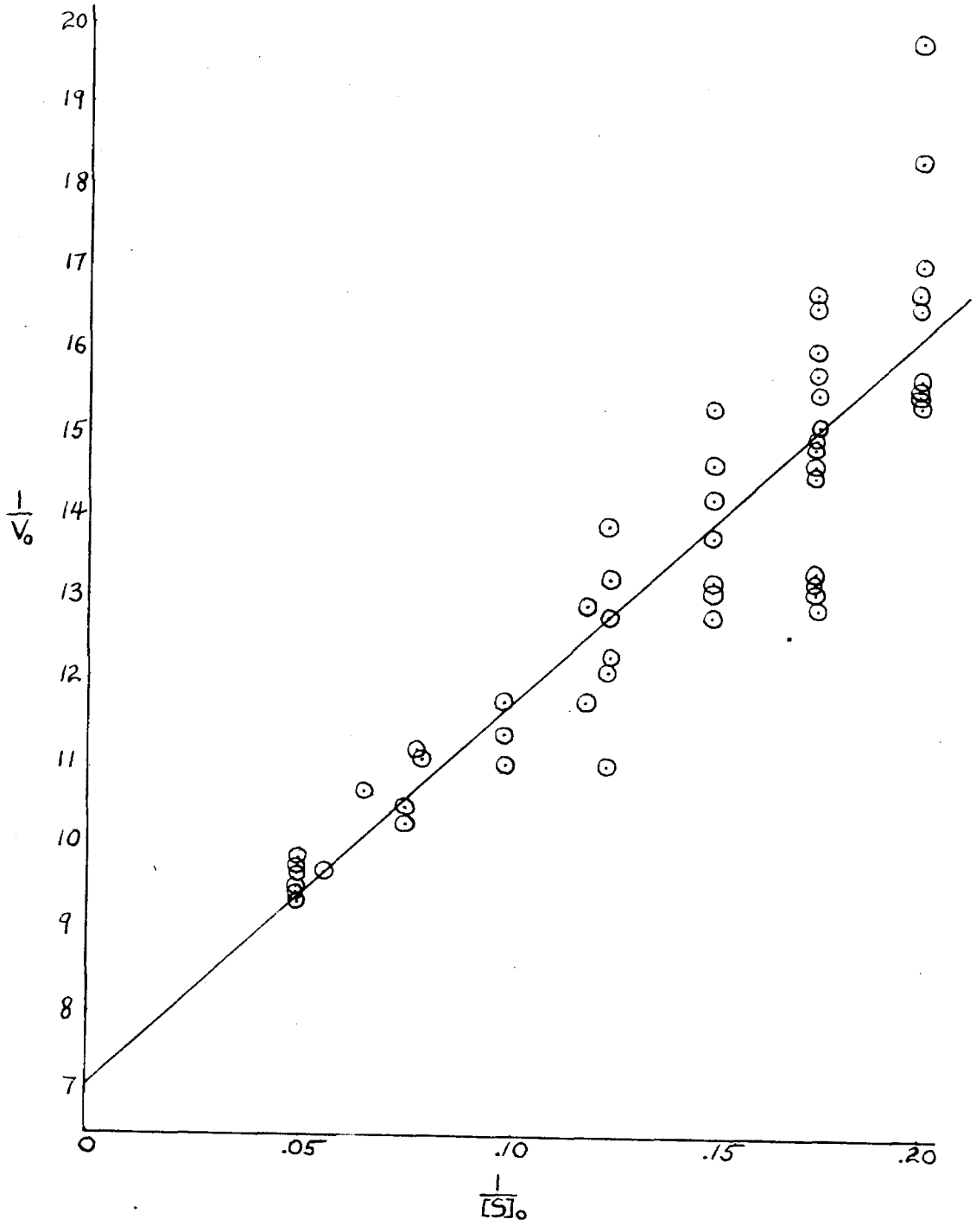

Fig. 4. Lineweaver-Burk treatment of the average corrected velocity data for N-Carboethoxy-L-tyrosinamide at pH 7.9 and [E] = 0.208 mg. protein-nitrogen/ml.

TABLE III

Summary of The Kinetic Constants of The Enantiomorphic N-Carboethoxy-tyrosinamides at pH 7.9

Isomer	K_s^a	k_3^b	K_I^a	
			[I] = 5.0[a]	[I] = 10.0
L	6.43 ± .50[c]	0.68 ± .04[c]		
D			21.07	20.34
			(Mean = 20.7 ± 2.5)	

a In units of 10^{-3} M.

b In units of 10^{-3} mole/min/mg. protein-nitrogen/ml.

c Standard deviations.

As indicated by equation (8) a plot of $\frac{1}{v_o}$ vs. $\frac{1}{[S]_o}$ for a system containing an added competitive inhibitor will be a straight line having an "apparent K_s" equal to

$$(16) \qquad \text{"apparent } K_s\text{"} = K_s\left(1 + \frac{[I]}{K_I}\right)$$

Knowing K_s and using a tentative value of K_I, a value for the "apparent K_s" may be calculated and then applied to the correction of the estimated initial velocities of runs made in the presence of added inhibitor in exactly the same manner as K_s is used to determine correction factors for uninhibited hydrolysis. Employing a K_s of approximately 6×10^{-3} M and an approximate value of 15×10^{-3} M for K_I (suggested by uncorrected first order data), a series of first order correction factors were calculated for the runs performed at each of the two inhibitor concentrations and the reciprocals of these corrected first order velocities plotted as before. Employing the final value of 6.43×10^{-3} M for K_s (Table III) and estimating by eye the best straight lines intercepting the $\frac{1}{v_o}$ axis at the predetermined value of 7.10 and passing through the points corresponding to $[I] = 5.0 \times 10^{-3}$ M and $[I] = 10.0 \times 10^{-3}$ M, an average value of 23.1×10^{-3} M for K_I (for the two inhibitor concentrations) was obtained. Using this improved value of K_I and the final value of K_s, a recalculation of the first order correction factors was then effected. The correction factors estimated from this improved value of K_I differed from the original correction factors by amounts of only 0-0.75 per cent and it was not possible therefore to refine further the originally corrected velocities

TABLE IV

Initial Velocity Data for N-Carboethoxy-L-tyrosinamide Inhibited by N-Carboethoxy-D-tyrosinamide[a]

$[I] = 5.0 \times 10^{-3}$ M

Run	$[S]_o \times 10^3$ M^{-1}	1st order v_o[b]	
		Uncorr.	Corr.
1	5.00	.057	$.055_2$
2	5.70	$.062_3$	$.059_6$
3	6.66	$.067_2$	$.065_1$
4	6.66	.064	$.061_6$
5	7.82	.072	$.068_3$[c]
6	10.00	.081	$.078_7$
7	14.28	$.094_5$	$.090_4$

$[I] = 10.0 \times 10^{-3}$ M

Run	$[S]_o \times 10^3$ M^{-1}	1st order v_o[b]	
		Uncorr.	Corr.
1	4.00	$.042_1$	$.041_2$
2	5.00	$.048_5$	.048
3[d]	5.70		
4	5.70	$.055_3$	$.053_7$[c]
5	5.70	$.055_3$	$.054_6$[c]
6	6.66	$.057_7$	$.056_6$
7	7.82	$.063_9$	$.061_8$
8	10.00	$.070_8$	.068
9	10.00	.076	$.073_6$

[a] All determinations made at pH 7.9 and [E] = 0.208 mg. protein-nitrogen per ml.

[b] Velocity values are in units of 10^{-3} M·min.$^{-1}$

[c] Corrected initial velocity reestimated.

[d] Erratic run rejected.

by the recalculated correction factors. A summary of this velocity data is given in Table IV. As indicated in Table IV, however, the estimations of corrected velocities were, in a few cases, revised before being entered into the final Lineweaver-Burk plot (Fig. 5). It should be noted that these reestimations were merely a matter of rejudgement upon the originally calculated values of t' vs. $\ln[S]$ and did not result from any recalculation of correction factors. The corrected reciprocal first order initial velocities and reciprocal initial substrate concentrations for each inhibitor concentration were plotted as before and values of K_I determined for each line by a least squares treatment. The lines corresponding to the two inhibitor concentrations were first made to pass through the predetermined ordinate intercept by giving this value of $\frac{1}{V_m}$ the high arbitrary statistical weight of n = 100. The values of K_I thus determined for the two values of inhibitor concentration were found to be in excellent agreement (Table III). The exclusive use of corrected first order initial velocities in determining K_I was considered sufficiently accurate especially in view of the 25 per cent - 50 per cent enhancement[a] of the first order term (see equation (9)) by the multiplier $(1 + \frac{[I]}{K_I})$. It should be noted that in this determination of K_I competitive inhibition has been assumed to occur by deliberately causing the lines for inhibited hydrolysis to pass through the predetermined intercept. In Table V are shown the intercepts for the two inhibitor concentrations which have been calculated on the basis of inhibited hydrolysis data alone. The intercept for the substrate alone and its standard deviation are shown for comparison. The overlap of error limits indicates that, within the

[a] This was also noted by Huang and Niemann[10].

TABLE V

Data for N-Carboethoxy-D-tyrosinamide as an Inhibitor Disregarding The Intercept Predetermined from Uninhibited Hydrolysis.

(L isomer included for comparison)

Isomer	$1/V^a$ (ordinate intercept)	Stan Dev. in 1/V
L	7.10	± 0.38
D (at 5×10^{-3} M)	7.40	- [b]
D (at 10×10^{-3} M)	7.53	± 0.66

[a] Units of 10^3 sec. M^{-1}.

[b] Not calculated since its difference in value from that of the L is less than the error of the latter.

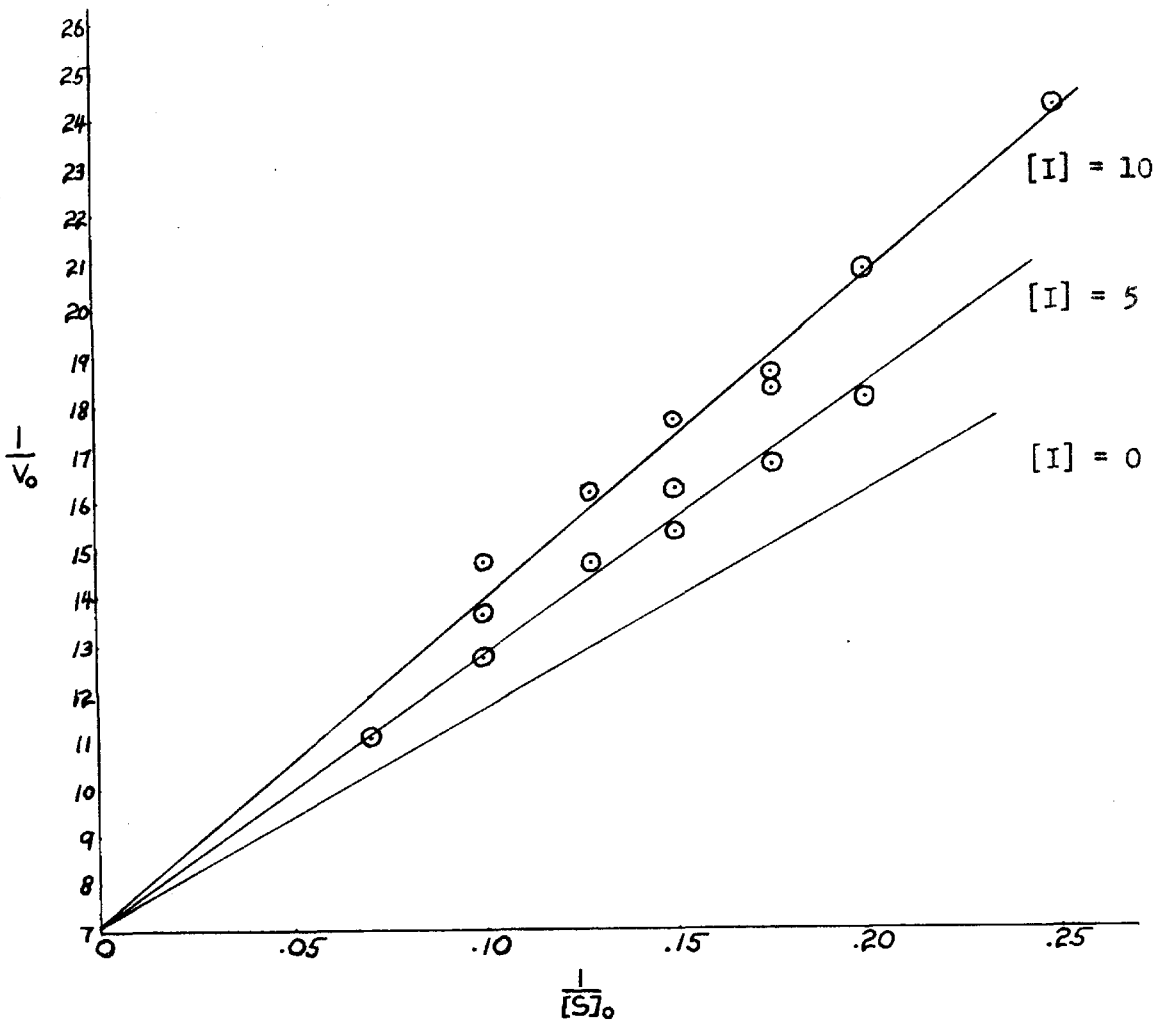

Fig. 5. Lineweaver-Burk plot of N-Carboethoxy-D-tyrosinamide as a Competitive Inhibitor of its L Antipode.

limits of experimental error, the three lines extrapolate to the same intercept. Hence, within these experimental limits, competitive inhibition is indicated. Assuming competitive inhibition, the use of the more precise intercept determined from the substrate alone is desirable considering the great sensitivity of K_I to the intercept and the good agreement between K_I values noted in Table III. The justification of this assumption awaits the independent evaluation of K_I for N-carboethoxy-D-tyrosinamide against a second substrate.

N-Carboethoxy-D-tyrosinamide as an Inhibitor Against Acetyl-L-tyrsoin-hydroxamide

Six experiments[a] were performed in which N-carboethoxy-D-tyrosinamide, at a concentration of 10×10^{-3} M, inhibited the chymotryptic hydrolysis of acetyl-L-tyrosinhydroxamide. The kinetics were followed by the spectrophotometric technique described in a later section (page 52). From an approximate apparent K_s of 65×10^{-3} M, first order correction factors were calculated and from these reciprocal corrected velocities obtained.

In Table VI is a summary of the uncorrected and corrected initial velocity data. An ordinate intercept was first calculated from the six points using a least squares treatment. Its value of 0.68 is somewhat below the expected value of 1.11 calculated from the data of Foster and Niemann[17]. A second least squares treatment was then performed causing the line to pass through the intercept determined for acetyl-L-tyrosinhydroxamide alone (Fig. 6). While the resultant K_I of

[a] In these experiments, [E] = 0.0266 mg. protein-nitrogen/ml.

TABLE VI

N-Carboethoxy-D-tyrosinamide as Inhibitor Against

Acetyl-L-tyrosinhydroxamide

$[I] = 10 \times 10^{-3}$ M

Run	$[S]_o^a$	Uncorr. 1st ord. v_o^b	Corr. 1st ord. v_o^b
1	5.00	.064	.064
2	5.00	.062	.062
3	6.02	.076	.075
4	7.50	.094	.092
5	10.00	.125	.121
6	10.00	.123	.120

[a] In units of 10^{-3} M.

[b] In units of 10^{-3} M/min.

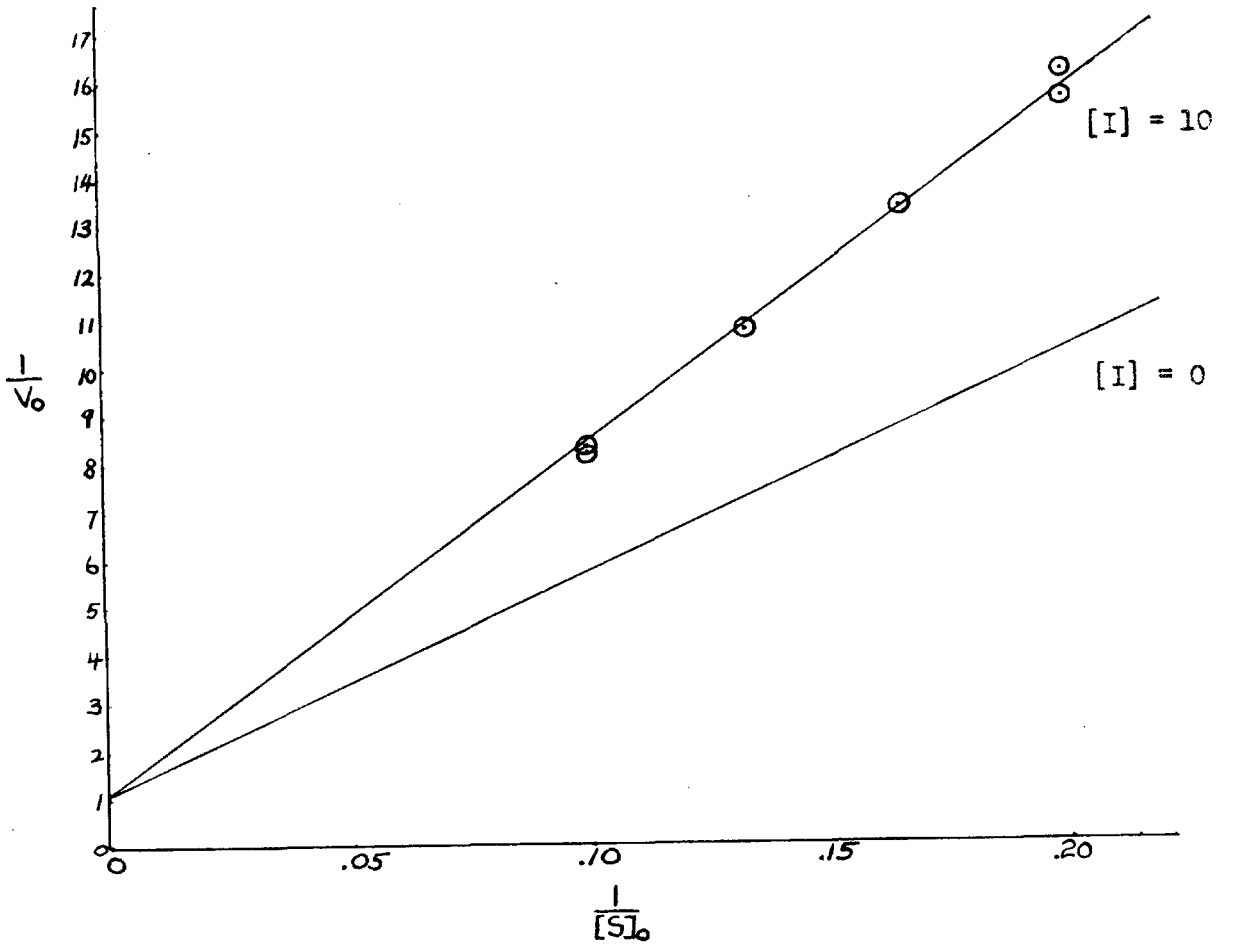

Fig. 6. N-Carboethoxy-D-tyrosinamide as an Inhibitor against Acetyl-L-tyrosinhydroxamide.

17.3×10^{-3} M is below the mean value of 20.7×10^{-3} M found against N-carboethoxy-L-tyrosinamide, it lies within the limit of error of this latter value. This error derives from the uncertainty in K_s (6.43 ± 0.5) of the carboethoxy substrate which figures into the calculation of K_I by equation (16). Table VII summarizes the calculations based upon the data of Table VI.

TABLE VII

Least Squares Data for N-Carboethoxy-D-tyrosinamide as Inhibitor Against Acetyl-L-tyrosinhydroxamide

Ordinate Intercept	$0.68^{a} \pm 0.31^{b}$
K_I(assuming competitive inhibition)	17.3×10^{-3} M $\pm 1.2 \times 10^{-3}$ M

[a] In units of 10^{3} M^{-1}·min.

[b] Standard deviation. For calculation, see Youden, "Statistical Methods for Chemists", 1951, John Wiley and Sons, Inc.

Discussion of Results

The discovery that, in the case of the enantiomorphic N-carboethoxytyrosinamides, the K_I of the D isomer (19.0×10^{-3} M)[a] has a larger value than K_s of its L antipode (6.4×10^{-3} M) was unexpected in view of the fact that in the case of every other D and L pair of acylated alpha-amino acid amides studied K_I has always been found to be less than K_s. Since it is generally assumed[2,8,9,10] that in the case of the acylated-L-amino acid amide substrates K_s is approximately equal to $\frac{k_2}{k_1}$, i.e., is a measure of the affinity of enzyme for substrate in the ES complex, the usually observed order for D and L pairs of $K_s > K_I$ has been interpreted as signifying that, in such pairs, the D inhibitors have greater affinities for the enzyme than do their corresponding L isomers.[7] This apparently lower stability of the ES complex has been considered to reflect a strained configuration of the substrate molecule in its act of meeting the geometrical requirements of the ES union. The EI complex has been considered to exist without this strain.[2,14] According to one view, the specificity of alpha-chymotrypsin (and of proteolytic enzymes in general) for L amino acid (naturally occurring) derivatives results from the residence of this strain, in part, in the carboxylic acid function R_3 thereby lowering its activation energy toward nucleophilic attack.[14] This strain would result from the interference offered by the alpha-hydrogen atom toward $R_1\rho_1$, $R_2\rho_2$, $R_3\rho_3$ interaction during the act of duplicating the

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[a] Averaging the value of K_I obtained with its L antipode as the substrate with the value of K_I determined against acetyl-L-tyrosinhydroxamide as the substrate.

strain free EI type union in the inverted fashion. Another possible explanation for the specific hydrolysis of L derivatives is, of course, that the R_3 group in EI may be unfavorably oriented with respect to subsequent attack.(1) In agreement with the concept of similar mode of combination in ES and EI is the observation[a] that for three pairs of acylated alpha-amino acid amides, differing (within each pair) only in the nature of the acyl group (R_1), the ratios of the K_s's of the L members of each pair are roughly equal to the corresponding ratios of the K_I's of the D members. This ratio is approximately independent of the nature of R_2 (the amino acid side chain) and is close to two in the cases investigated.(8) For these observations to suggest the same mode of combination for substrate and inhibitor requires that they also suggest

$$(17) \qquad K_s \simeq \frac{k_2}{k_1}, \qquad \text{i.e.,} \qquad k_2 >> k_3$$

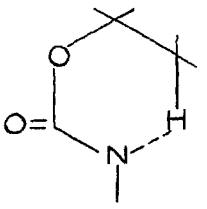
for the acylated alpha-amino acid amides. In Table VIII, the K_s and k_3 values of N-carboethoxy-L-tyrosinamide are compared with those of several other acyl-L-tyrosinamides. It will be noted that the K_s values fall into two general groups; the high values of the acetyl, chloroacetyl, and trifluoroacetyl derivatives and the lower values of the bulkier benzoyl, nicotinyl, and isonicotinyl substrates. With a K_s of

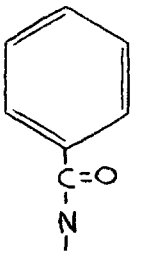
---

[a] Specifically, the acetyl and nicotinyl derivatives of the enantiomorphic tyrosinamides, phenylalaninamides, and tryptophanamides. These ratios are not equal in the cases of some chloroacetyl and trifluoroacetyl derivatives, suggesting the possibility of alternate modes of combination in these cases.

6.4×10^{-3} M the carboethoxy substrate lies in this latter category. The significance of the relation of the nature of the acyl group to K_s has been discussed by Shine and Niemann[3] for the first four members of Table VIII. Evidently, the electronic character of the acyl group has little influence on the magnitudes of the kinetic constants. On the other hand, the bulk of the acyl group, expressable as its molecular refractivity, seems to be related to the size of K_s, the lower K_s's (higher affinities) being associated with the more voluminous acyl groups. The position of the formyl derivative in this apparent category is not understood. The possibility has been suggested that the high affinity (low K_s) of the carboethoxy derivative may result from a cyclic configuration (equation (18)) of the carboethoxy group, assumed upon the occasion of ES interaction, in which it may approximate the molecular volume of a larger group, i.e., benzoyl.[15]

(18)





This relation of size of the acyl group to affinity for enzyme is considered in agreement with the present concept that the forces responsible for the formation of the ES complex are primarily van der Waals forces.[3,16]

TABLE VIII

Kinetic Constants for a Number of Acylated L-tyrosinamides at pH 7.75-8.0.

Substrate	Molec. Refr. of Acyl	K_s[a]	k_3[b]
Acetyl-L-tyrosinamide	10.3	32	2.4
Chloroacetyl-L-tyrosinamide	15.1	27	4.0
Trifluoroacetyl-L-tyrosinamide	10.2	26	2.6
Nicotinyl-L-tyrosinamide	25.8	12	5
Isonicotinyl-L-tyrosinamide		9	6.4
Benzoyl-L-tyrosinamide		2.5	4.0
Formyl-L-tyrosinamide		12	.45
N-Carboethoxy-L-tyrosinamide		6.4	.68

[a] In units of 10^{-3} M.

[b] In units of 10^{-3} mole/min/mg. protein-nitrogen/ml.

The right hand column of Table VIII indicates that k_3 may also be related to the size of the acyl group, the magnitude of this constant generally increasing with the bulk of the acyl group. With this in mind, the low value of 0.45 for the formyl derivative is understandable while the relatively low value for N-carboethoxy-L-tyrosinamide is surprising. It seems not unreasonable to assume that this low value of k_3 for the carboethoxy substrate is a manifestation of an unusually unstrained (and therefore stable) ES complex originally suggested by the "inverse" order of affinities of the D and L isomers.

The pH-Activity Relationship For The Substrate

Since the pH optima for the chymotryptic hydrolysis of all previously investigated acyl derivatives of L-tyrosinamide have been found to be within the region pH 7.75-8.0, the kinetic constants of N-carboethoxy-L-tyrosinamide were determined at the usual pH of 7.9 before making a careful investigation of the pH-activity curve.

The pH-activity relationship for the system N-carboethoxy-L-tyrosinamide-alpha-chymotrypsin in aqueous solution at 25°C was determined by performing a large number of short-term hydrolysis experiments buffered at the desired pH with a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer at a concentration of 0.02 M with respect to the amine component. With this buffer it was possible to work over a range of pH 7.25 to pH 8.7, limiting the reaction time to the first 20 minutes for an enzyme concentration of 0.266 mg. protein-nitrogen per ml. The usual procedure was to determine, titrimetrically, the increase in liberated acid over the 20 minute reaction interval, taking the total change in end point for this period as a measure of activity. The uncorrected zero order initial velocities (essentially, these were determined) were, in general, estimated from six total experimental points, three in the 1-$2\frac{1}{2}$ minute range and three at 18-$19\frac{1}{2}$ minutes. It was felt that limiting the estimation of end points change to this brief hydrolysis period adequately eliminated the complication of a significant pH change during reactions performed near the extremes of the pH range studied and also minimized the problem of split-product inhibition.

In Figure 7, the twenty minute hydrolysis increments, expressed as fractions of the maximum hydrolysis increment observed, are plotted versus the corresponding pH's of the determinations. In the usual case where the pH varied over an interval of 0.02-0.10 units from beginning to end of the run the initial and final pH values were averaged and these values plotted vs. the relative hydrolysis increments.

Figure 7 indicates that the pH-activity curve of N-carboethoxy-L-tyrosinamide possesses a maximum near pH 8.25, differing in this respect from all previously investigated derivatives of L-tyrosinamide, the majority of which pass through a maximum at or near pH 7.9. At this pH and at the experimental initial substrate concentration of 10×10^{-3} M, N-carboethoxy-L-tyrosinamide is hydrolyzed at a rate of approximately 95 per cent of that at its maximum. In Figure 6, the pH-activity curve of acetyl-L-tyrosinamide[7] is shown for comparison. While the curves for the two derivatives are seen to be of the same general shape, that for the carboethoxy derivative is shifted toward the higher pH's by a value of approx. 0.35-0.40 pH unit. The possession of a rather high pH maximum seems to be a further manner in which N-carboethoxy-L-tyrosinamide departs from the typical behavior of the acyl-L-tyrosinamide substrates.

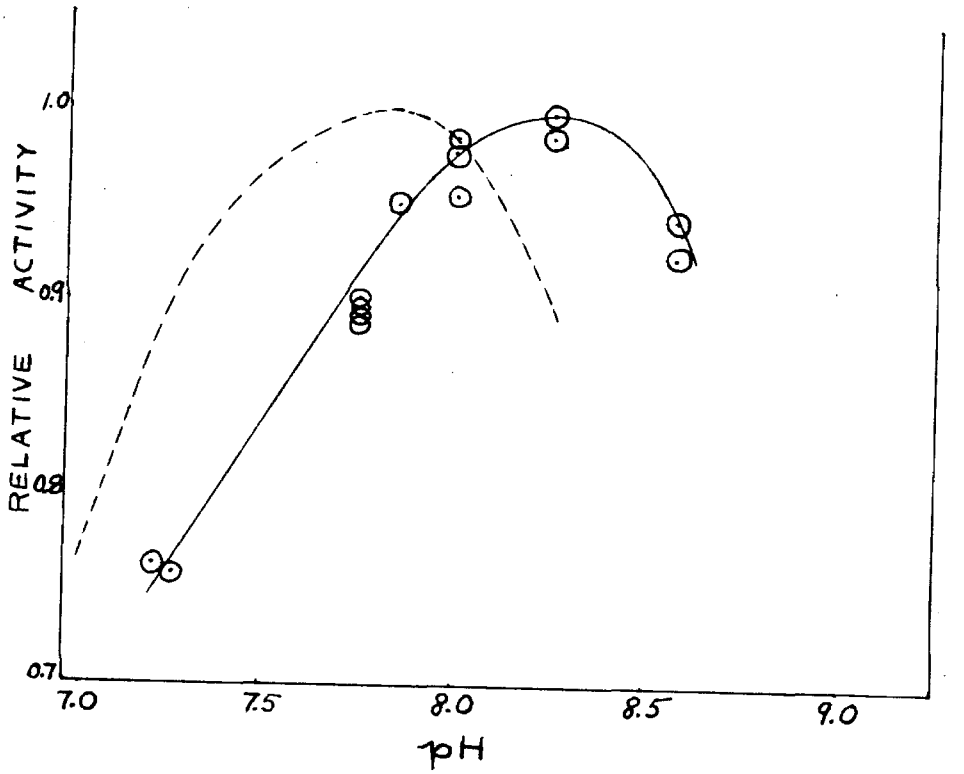


Fig. 7. N-Carboethoxy-L-tyrosinamide $[S]_o = 10 \times 10^{-3}$ M; [E] = 0.266 mg. protein-ntirogen per ml.; 0.02 M-tris-(hydroxymethyl)-aminomethane-hydrochloric Acid Buffer.

– – – – – For Comparison, Acetyl-L-tyrosinamide, $[S]_o = 20 \times 10^{-3}$ M; [E] = 0.125 mg. protein-nitrogen/ml. After Thomas, MacAllister, and Niemann[7].

The Redetermination of The Kinetic Constants of N-Carboethoxy-L-tyrosinamide at pH 8.10-8.25

While the knowledge of K_s and k_3 for N-carboethoxy-L-tyrosinamide at pH 7.9 may be considered of interest for the sake of comparison with the kinetic constants of other acyl-L-tyrosinamides at the same pH, it was nevertheless desirable to have information regarding the carboethoxy substrate at its optimum pH of hydrolysis. Accordingly it was decided to redetermine the constants at a pH near the optimum value of 8.25.

To obviate the tedious process of estimating initial velocities from two types of plots and correcting these estimates to values for use in the Lineweaver-Burk treatment, it was decided to employ the recently developed method of Foster and Niemann[17] which does not require the separate evaluation of initial velocities.

For a system consisting of enzyme and substrate with no added inhibitor but in which competitive inhibition by one of the hydrolysis products occurs throughout the reaction, the following representation may be given[1,5,6]

$$(19a) \qquad [E] + [S] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [ES] \xrightarrow{k_3} [E] + [P]$$

$$(19b) \qquad [E] + [P] \underset{k_7}{\overset{k_6}{\rightleftharpoons}} [EP]$$

The course of such an enzymatic hydrolysis, inhibited by liberated reaction product, is given by the integrated rate expression

$$(20)\quad k_3[E]t = \left(1 + \frac{[S]_o}{K_p}\right) K_s \ln \frac{[S]_o}{[S]} + \left(1 - \frac{K_s}{K_p}\right) ([S]_o - [S])$$

where K_p is given by

$$(21)\qquad K_p = \frac{k_7}{k_6}$$

From equation (20) it is evident that for an experiment conducted at a particular value of $[S]_o$, a plot of $\frac{1}{t}([S]_o - [S])$ versus $\frac{1}{t} \ln \frac{[S]_o}{[S]}$ will be a straight line of slope $-K_s \frac{(K_p + [S]_o)}{(K_s - K_p)}$. Since a straight line drawn through the origin of such a plot will have a slope equal to $[S]_o$, that particular straight line drawn through the origin having a slope equal to the initial substrate concentration of a given experiment will intersect the line defined by the data of that experiment at an ordinate value equal to the corresponding initial velocity v_o. If then a straight line is drawn through a series of these intersections derived from a series of experiments conducted at the same enzyme concentration but differing in initial substrate concentration it will have a slope of $-K_s$, an ordinate intercept of $k_3[E]$ and an abcissa intercept of $k_3[E]/K_s$. Besides eliminating the necessity of a preliminary evaluation of initial velocities, this treatment has the further advantage of offering a method for determining the value of K_p. Of course, since the intersections of the experimentally determined lines with the constructed lines of slope equal to $[S]_o$ yield values of the true initial velocity v_o, K_s and k_3 may be alternatively determined by a Lineweaver-Burk treatment of these velocities. A disadvantage in this latter method lies in the fact that

K_s thus determined is a function not only of the slope of the $\frac{1}{v_o}$ vs. $\frac{1}{[S]_o}$ line but also of its intercept and therefore inherits the uncertainty of determination of this latter quantity.

A total of eighteen experiments, buffered by 0.02 M THAM at pH 8.10-8.25, were performed, following the kinetics by the titrimetric method as before. In these runs the enzyme was an Armour preparation of alpha-chymotrypsin at a concentration of 0.532 mg. protein-nitrogen per ml. Since the Foster-Niemann method of analysis enables the direct interpretation of experimental data taken at high total hydrolysis, the reactionswere allowed to proceed to 63 per cent - 94 per cent hydrolysis over time intervals of approximately 50 minutes. By studying the reaction system at intermediate and later stages of the reaction, the experimental error is decreased and it becomes possible to obtain an estimate of K_p from the experimental lines having slopes equal to $-K_s(K_p + [S]_o)/(K_p - K_s)$.[17] In general the calculated values of $\frac{1}{t} \ln \frac{[S]_o}{[S]}$ behaved erratically over the first 10-20 minutes of each reaction. At these initial stages of the reaction the values of $\ln[S]_o - \ln[S]$ correspond to small differences between rather large values and the error is therefore quite large.

The experimental data was plotted both according to the Foster-Niemann method (Fig. 7) and in the Lineweaver-Burk manner (Fig. 8) using a least squares treatment in each case to determine the best straight line. In the case of the latter plot, the $\frac{1}{v_o}$ values are the reciprocals of the $\frac{1}{t}([S]_o - [S])$ values obtained from the Foster-Niemann plot intersections. In Table IX these velocity values are

summarized. By omitting the data from runs 1, 2, 3, 6, 10, 12, which fell rather far from the lines defined by the majorities of the points of both plots, values of K_s and k_3, in excellent agreement between plots, were obtained (Table X).

From the expression

$$(22) \qquad K_p = K_s \frac{Sl - [S]_o}{Sl + K_s}$$

where Sl is the slope of a line defined by the experimental points, values of K_p were calculated and tabulated in Table XI. From equation (22) it is evident that at low values of Sl, i.e., close to K_s, the denominator becomes a small difference between two relatively large numbers (since Sl is negative in sign) with a large resultant uncertainty in the calculated K_p. This is further complicated by the fact that at low values of $[S]_o$, the slope was often very hard to determine as the $\frac{1}{t} \ln \frac{[S]_o}{[S]}$ vs. $\frac{1}{t} ([S]_o - [S])$ points had a tendency to fluctuate rather badly. In theory, the slopes of these lines must be greater in absolute magnitude than K_s for K_p to have a finite positive value. Table XI indicates that, at the lower $[S]_o$ values, the estimation of a reasonable value for K_p was not possible. In most previous cases studied, K_p is roughly three times the value of K_s of the parent substrate. As runs 4, 5, 7, 8, 9, 13, and 14 gave experimental points the majority of which suggested straight lines of slope greater than $-K_s$, the value of K_p estimated from these runs were averaged giving a mean value of 32.3 ± 13.4. Setting K_p equal to this value and K_s equal to 7.27, the expression for the slope becomes

TABLE IX

Initial Velocity Values of N-Carboethoxy-L-tyrosinamide at pH 8.10-8.25 and 25°C

[E] = 0.532 mg. protein-nitrogen/ml.

Run	$[S]_o$[a]	v_o[b]	$\frac{1}{t} \ln \frac{[S]_o}{[S]}$[c]	$1/[S]_o$	$1/v_o$	Comment
1	13.15	.246	.0187	.0761	4.06	reject
2	5.0	.161	.0320	.200	6.21	reject
3	6.66	.189	.0283	.150	5.29	reject
4	10.0	.272	.0272	.100	3.68	
5	20.0	.337	.0169	.050	2.97	
6	2.0	-	-	-	-	reject
7	10.0	.253	.0254	.100	3.95	
8	5.70	.187	.0327	.1754	5.35	
9	20.0	.327	.0163	.050	3.06	
10	2.0	.084	-	.500	11.83	reject
11	3.33	.145	.0437	.300	6.90	
12	8.00	-	-	-	-	reject
13	8.00	.242	.0303	.125	4.12	
14	6.66	.216	.0323	.1501	4.63	
15	5.70	.202	.0352	.1754	4.95	
16	5.70	.200	.0352	.1754	5.00	
17	5.70	.187	.0329	.1754	5.33	
18	5.70	.192	.0337	.1754	5.19	

[a] In units of 10^{-3} M.

[b] In units of 10^{-3} M/min.

[c] In units of $min.^{-1}$

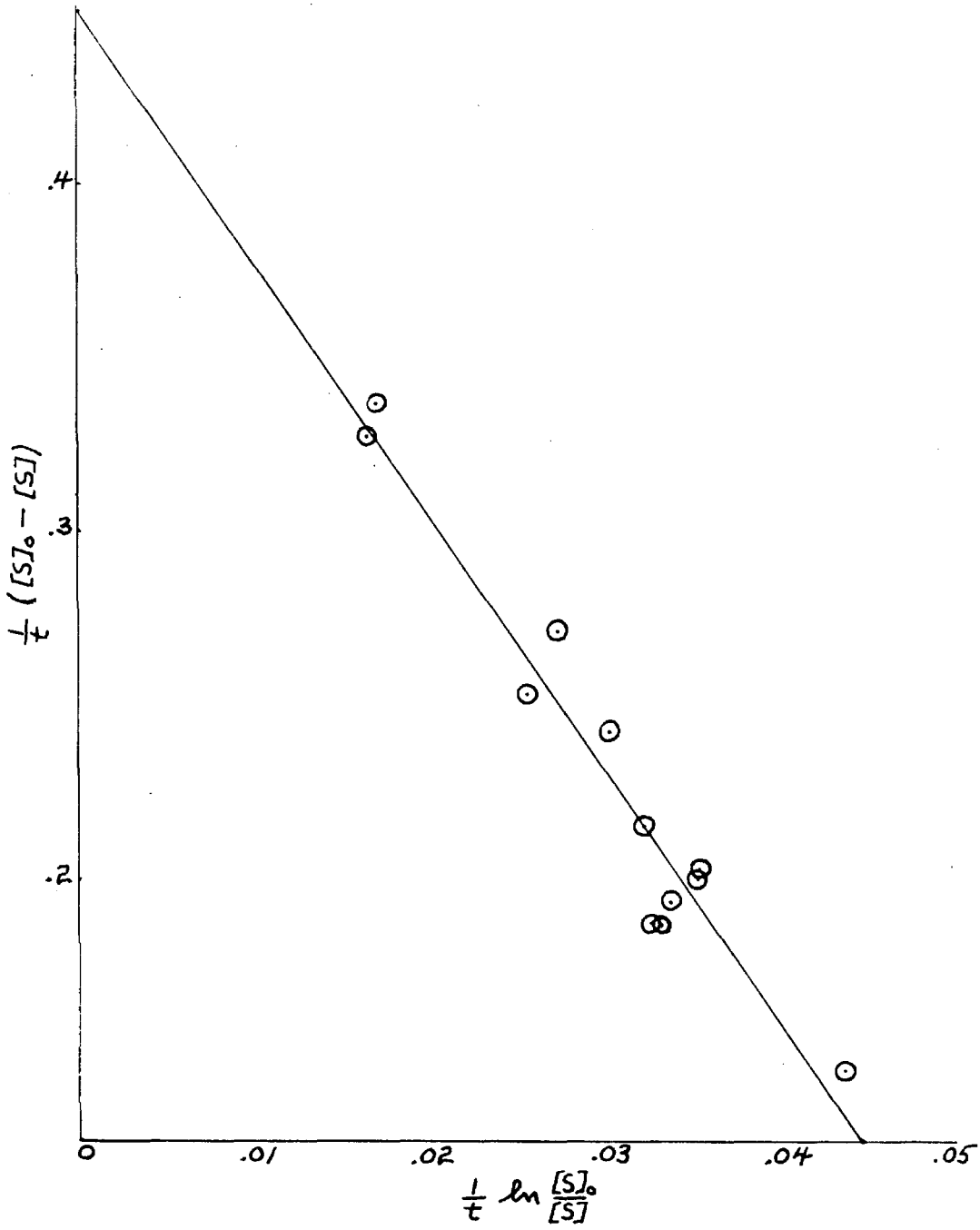


Fig. 8. Foster-Niemann Plot for N-Carboethoxy-L-tyrosinamide at pH = 8.10-8.25.

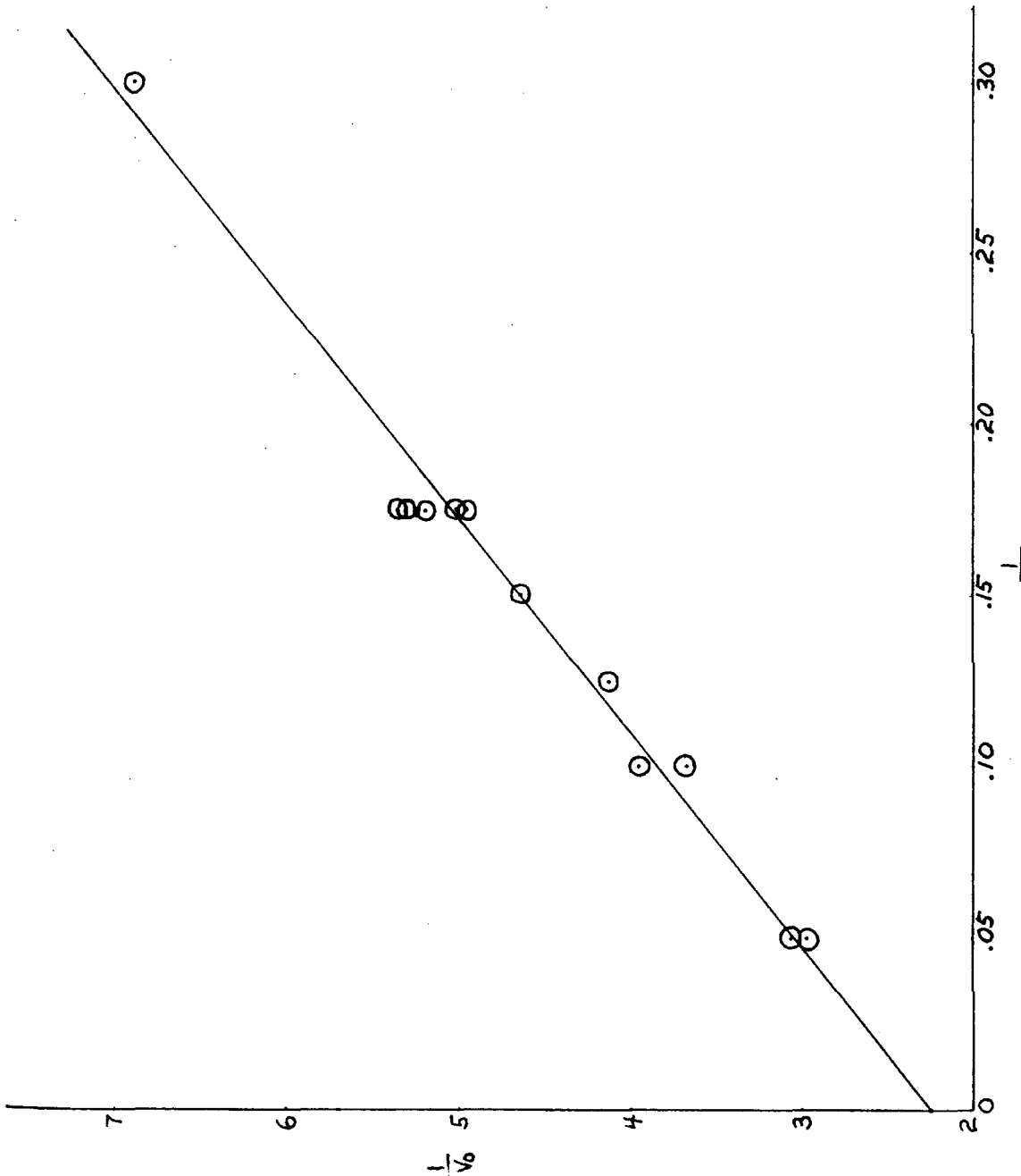


Fig. 9. Lineweaver-Burk Plot for N-Carboethoxy-L-tyrosinamide at pH = 8.10-8.25. V_o's from Foster-Niemann Plot.

TABLE X

N-Carboethoxy-L-tyrosinamide

Dependence of Kinetic Constants upon pH

pH	Type of Data Analysis	Constant	
		K_s[a]	k_3[b]
7.85-7.9	Lineweaver-Burk	6.43 ±.50[c]	0.68 ±.04[c]
8.10-8.25	Lineweaver-Burk	7.17	0.84
8.10-8.25	Foster-Niemann	7.27 ±.60[c]	0.84 ±.03[c]
8.10-8.25	(mean)	7.22	0.84

[a] In units of 10^{-3} M.

[b] In units of 10^{-3} mole/min/mg. protein-nitrogen/ml.

[c] All error limits are standard deviations.

TABLE XI

Estimates of K_p for N-Carboethoxy-L-tyrosine at pH 8.10-8.25 and 25°C

Run	$[S]_o$[a]	Slope[a]	Calc. K_p[a]	Comment
1	13.15	K_s	0	Rej. in K_s det.
2	5.0	K_s	0	"
3	6.66	-8.14	124	"
4	10.0	-22.45	16.5	"
5	20.0	-47.4	12.2	
6	2.0	-	-	
7	10.0	-12.77	30.1	"
8	5.70	-8.66	75.2	
9	20.0	-42.7	12.8	
10	2.0	K_s	0	"
11	3.33	K_s	0	"
12	8.00	-	-	"
13	8.00	-18.69	23.3	
14	6.66	-9.34	56.2	
15	5.70	K_s	0	
16	5.70	K_s	0	
17	5.70	-7.48	456	
18	5.70	K_s	0	

[a] In units of 10^{-3} M.

(23) $\quad Sl = -9.38 - 0.29[S]_o$

Since (23) would predict a slope of only -15.2 for an $[S]_o$ value of 20×10^{-3} M (where virtually all of the experimental points fell on lines of slopes -42 to -47) it seems fairly certain that K_p for N-carboethoxy-L-tyrosine is less than this average value, probably in the vicinity of $12\text{-}18 \times 10^{-3}$ M.

Discussion of Results

The value of K_p for N-carboethoxy-L-tyrosine appears to lie approximately in the range expected from the K_s of its parent substrate.

The behavior of the carboethoxy substrate observed upon changing the pH of the hydrolytic reaction by approximately 0.35 pH unit is somewhat unexpected. Such changes in K_s with pH are more characteristic of a charged, rather than of an uncharged, substrate.[18] Somewhat surprising also is the decrease in affinity (higher K_s) at the pH optimum over that observed at the lower pH. While this difference in K_s is doubtless of significance it should be emphasized that the two values differ by an amount only slightly greater than the standard deviations in these values. This decrease in affinity at the maximum is more than offset by the increase in k_3 which is related to K_s and to the initial velocity by

(24) $$v_o = \frac{k_3[E]\,[S]_o}{K_s + [S]_o} \quad .$$

SECTION I

PART II

THE DETERMINATION OF THE ENZYME-INHIBITOR DISSOCIATION CONSTANTS FOR THE ENANTIOMORPHIC N-CARBOETHOXYTYROSINMETHYLAMIDES AT pH 7.6 AND 25°C.

Introduction

The observation of the so-called "inverse" order of K_s and K_I for the enantiomorphic N-carboethoxytyrosinamides prompted speculation concerning its significance. While it would seem to indicate the existence of a case in which a substrate is more tightly bound to the enzyme than is its D antipode, nothing certain is known about the magnitudes of the constants k_1 and k_2 and hence no definite conclusions may be drawn. Only if it could be taken for granted that in the case of N-carboethoxy-L-tyrosinamide $k_2 >> k_3$ so that K_s would represent, for all practical purposes, a true equilibrium constant would the suggested order of affinities be a certainty. An alternate approach is to study compounds sufficiently similar to N-carboethoxytyrosinamide in structure to permit comparison, but differing from that compound in some minimum structural detail rendering both D and L isomers competitive inhibitors of chymotrypsin.[a] A similar "inverse" order of K_I's in such a case would give force to the idea that the K_s to K_I relationship of the enantiomorphic N-carboethoxytyrosinamides represents an order of affinities. Since N-carboethoxy-L-tyrosinmethylamide is hydrolyzed only very slowly[b] by alpha-chymotrypsin and therefore approximates a true inhibitor ($k_3 \tilde{=} 0$) in behavior it was decided to determine the K_I of this compound and of its D antipode and compare the two.

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[a] Suggested by Dr. Niemann, this approach originated in a proposition by D.W. Thomas, Ph.D. Thesis, Calif. Inst. of Technology, 1951.

[b] Actually inferred from an unpublished experiment with acetyl-L-tyrosinbutylamide in these laboratories.

Procedure and Treatment of Data

The enzyme-inhibitor dissociation constants of D and L N-carboethoxytyrosinmethylamide were evaluated against acetyl-L-tyrosinhydroxamide as the specific substrate at 25°C in aqueous solution buffered at pH 7.6 by tris-(hydroxymethyl)-aminomethane-hydrochloric acid (THAM) with the amine component at a concentration of 0.27 F. The hydrolyses were followed by an extension of the method of Hogness and Niemann[19] which depends upon the colorimetric estimation of the colored complex formed between the hydroxamic acid and ferric ion. In the present experiments the absorbance of residual acetyl-L-tyrosinhydroxamide, as its ferric complex, was measured at a wavelength of 515mμ with a Beckman Model B Spectrophotometer.

Since the Beer's Law expression

$$[S]_t = K \times A \qquad (25)$$

where $[S]_t$ represents the concentration of the hydroxamide-ferric complex (and hence of the hydroxamide itself) and A is the absorbance, is known to apply to this system at λ = 515mμ, a plot of ln A versus time has an initial slope which, upon multiplication by $[S]_o$, yields the first order initial velocity.

In the experiments with the methylamides values of ln A were plotted versus time and the reciprocal first order initial velocities estimated from these plots entered into Lineweaver-Burk plots. In these preliminary plots all lines for inhibited hydrolysis were drawn through the intercept for acetyl-L-tyrosinhydroxamide determined by Foster and Niemann[17]. From these plots provisional values of apparent

K_s were estimated and these used to calculate first order velocity correction factors. Tables XII and XIII present a summary of the first order velocity data for the methyl amides each of which was tested at concentrations of $[I] = 10 \times 10^{-3}$ M and $[I] = 20 \times 10^{-3}$ M. Due to the high K_s of acetyl-L-tyrosinhydroxamide (42×10^{-3} M) and the enhancement of the first order terms by the added inhibitors the hydrolyses were close to first order kinetically and the first order correction factors close to unity.

Plotting the reciprocal corrected velocities in the Lineweaver-Burk manner, ordinate intercepts were calculated by a least squares treatment. These intercept values and their standard deviations are shown in Table XIV compared with the intercept for uninhibited hydrolysis. In a second series of least squares determinations, competitive inhibition was assumed to occur by causing the lines to pass through the ordinate (1/V) intercept for plain hydrolysis of acetyl-L-tyrosinhydroxamide. The values of K_I assuming competitive inhibition are presented in Table XV. Table XIV indicates that while the ordinate intercept values for the two concentrations of N-carboethoxy-D-tyrosinmethylamide differ from the substrate intercept by amounts somewhat greater than the combined calculated standard deviations of both intercepts there is no progressive increase of intercept with increasing inhibitor concentration that would suggest the occurrence of inhibition of a type other than competitive. Furthermore, the values of K_I calculated upon the assumption of competitive inhibition at the two inhibitor concentrations are in good agreement with each other.

TABLE XII

Initial Velocity Data for Acetyl-L-tyrosinhydroxamide Inhibited By N-Carboethoxy-D-tyrosinmethylamide[a]

$[I] = 10 \times 10^{-3}$ M

Run	$[S]_o^c$	First order v_o^b	
		Uncorr.	Corr.
1	5.00	.071	$.069_4$
2	6.02	$.082_8$	$.081_1$
3	7.50	.100	$.098_4$
4	7.50	.100	$.098_4$
5	10.00	.133	$.130_2$
6	15.00	$.173_5$	$.170_3$

$[I] = 20 \times 10^{-3}$M

Run	$[S]_o^c$	First order v_o^b	
		Uncorr.	Corr.
1	5.00	$.052_1$	$.052_1$
2	8.00	$.082_4$	$.081_6$
3	15.00	$.148_1$	$.145_4$

[a] All determinations made at pH 7.6 and [E] = 0.0266 mg. protein-nitrogen per ml.

[b] Velocity values are in units of 10^{-3} M·min.$^{-1}$

[c] In units of 10^{-3} M.

TABLE XIII

Initial Velocity Data for Acetyl-L-tyrosinhydroxamide Inhibited By N-Carboethoxy-L-tyrosinmethylamide[a]

$[I] = 10 \times 10^{-3}$ M

Run	$[S]_o$[c]	First order v_o[b]	
		Uncorr.	Corr.
1	5.00	$.053_5$	.053
2	5.00	$.051_7$	$.051_3$
3	6.02	$.062_2$	$.061_8$
4	7.50	$.076_5$	$.075_7$
5	10.00	$.100_4$	$.098_3$
6	15.00	$.140_8$	$.137_3$

$[I] = 20 \times 10^{-3}$ M

Run	$[S]_o$[c]	First order v_o[b]	
		Uncorr.	Corr.
1	10.00	$.064_2$	$.064_2$
2	15.00	$.089_6$	$.088_9$
3	20.00	.120	$.118_3$
4	30.00	$.155_3$	$.153_9$
5	40.00	.205	.201

[a] All determinations made at pH 7.6 and [E] = 0.0266 mg. protein-nitrogen per ml.

[b] Velocity values are in units of 10^{-3} M·min.$^{-1}$

[c] In units of 10^{-3} M.

TABLE XIV

Data for The Enantiomorphic N-Carboethoxytyrosinmethylamides as Inhibitors Disregarding the Intercept Predetermined[b] from Uninhibited Hydrolysis. ($1/V^a$ for Acetyl-L-tyrosinhydroxamide = 1.106 ± .065)[b]

Isomer	Conc.	$1/V^a$ (Ordin. int.)	Stan. Dev. in 1/V
D	10×10^{-3} M	1.40	± 0.23
D	20×10^{-3} M	.70	± 0.27
L	10×10^{-3} M	1.27	± 0.32
L	20×10^{-3} M	1.61[c]	± 0.24
L	20×10^{-3} M	1.39[d]	± 0.37

a In units of 10^3 M^{-1} sec.

b Determined by Foster and Niemann[17].

c Including the experimental point as $[S]_o = 10 \times 10^{-3}$ M.

d Omitting the point at $[S]_o = 10 \times 10^{-3}$ M.

TABLE XV

Summary of the Enzyme-Inhibitor Complex Dissociation Constants of The Enantiomorphic N-Carboethoxytyrosinmethylamides at pH 7.6 and 25°C

Isomer	K_I^a (assuming competitive inhibition)	
	[I] = 10 x 10^{-3} M	[I] = 20 x 10^{-3} M
D	22.2 ± 1.6[d]	21.4 ± 0.9[d]
	Mean = 21.8 ± 1.6[d]	
L	10.5 ± 0.5[d]	8.8[c] ± 0.5[d] 9.1[b]
	Mean = 9.5 ± 1.2[d]	

[a] In units of 10^{-3} M.

[b] Including the experimental point at $[S]_o$ = 10 x 10^{-3} M.

[c] Omitting the point at $[S]_o$ = 10 x 10^{-3} M.

[d] Estimated by eye from Fig. 10 and Fig. 11.

Hence the assumption of competitive inhibition seems justified. The case of the L-methylamide is less certain. Here the $[I] = 10 \times 10^{-3}$ M line has an intercept within standard deviation range of the substrate intercept while the $[S]_o = 10 \times 10^{-3}$ M point must be omitted to cause the $[I] = 20 \times 10^{-3}$ M line to intersect the ordinate axis within this range. There appears to be a slight increase in intercept with inhibitor concentration. Moreover the assumption of competitive inhibition yields values of K_I at the two inhibitor concentrations which differ by an amount greater than the combined estimated errors with the lower value of K_I at the higher inhibitor concentration (Table XV). Such an apparent decrease in K_I with increasing [I] could result from overestimating the slope of the line by drawing the intercept too low, i.e., through the substrate intercept. It is clear that the few experimental points of Figure 11 and the rather arbitrary significance of standard deviation do not allow any final judgement as to the behavior of the intercept with changing inhibitor concentration. This apparent behavior does, however, indicate the desirability of further experiments at high substrate concentrations using some method of plotting (such as a plot of v_o versus $v_o/[S]_o$)[20] better suited to revealing possible differences in intercept.

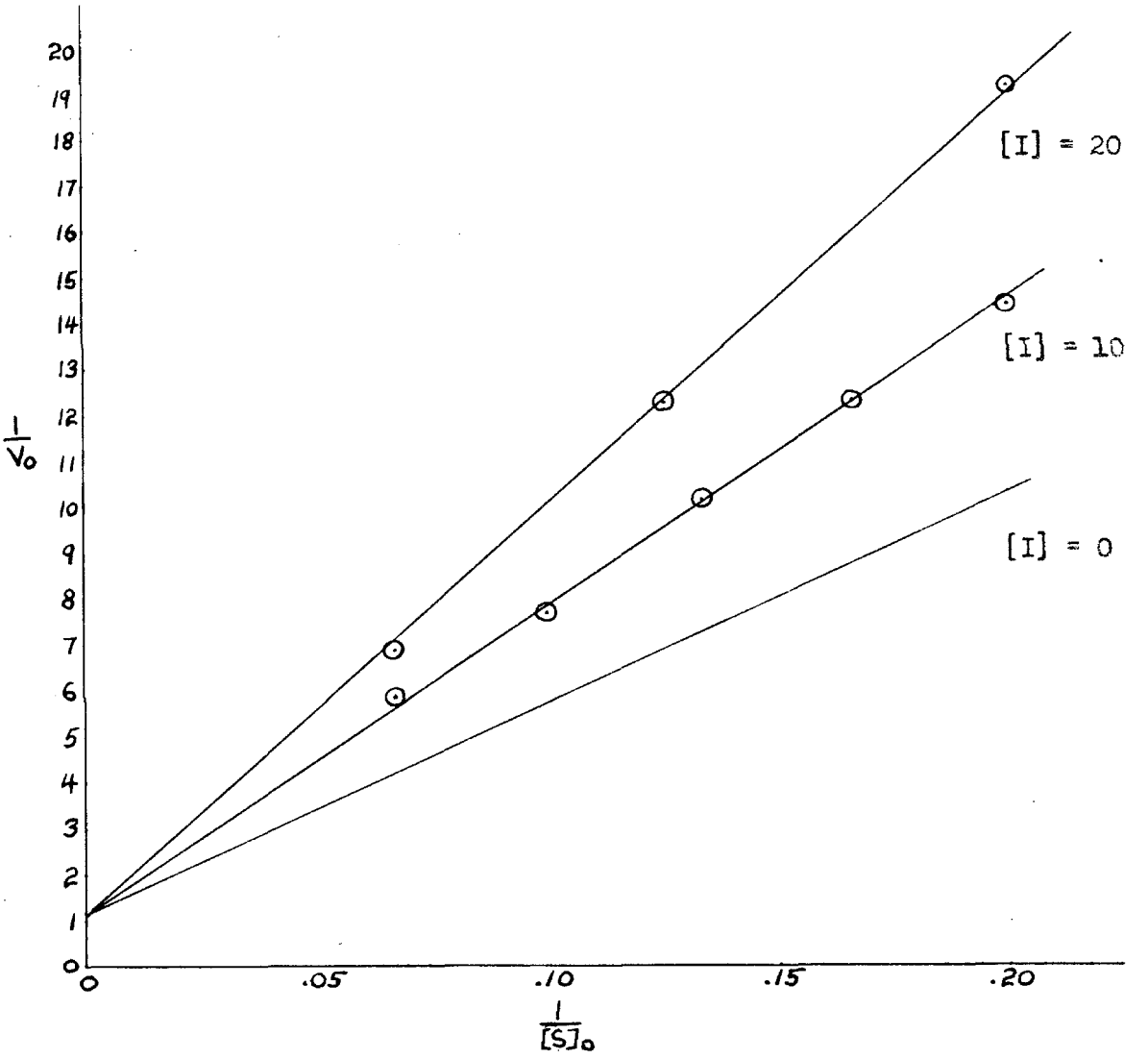

Fig. 10. N-Carboethoxy-D-tyrosinmethylamide as an Inhibitor against Acetyl-L-tyrosinhydroxamide.

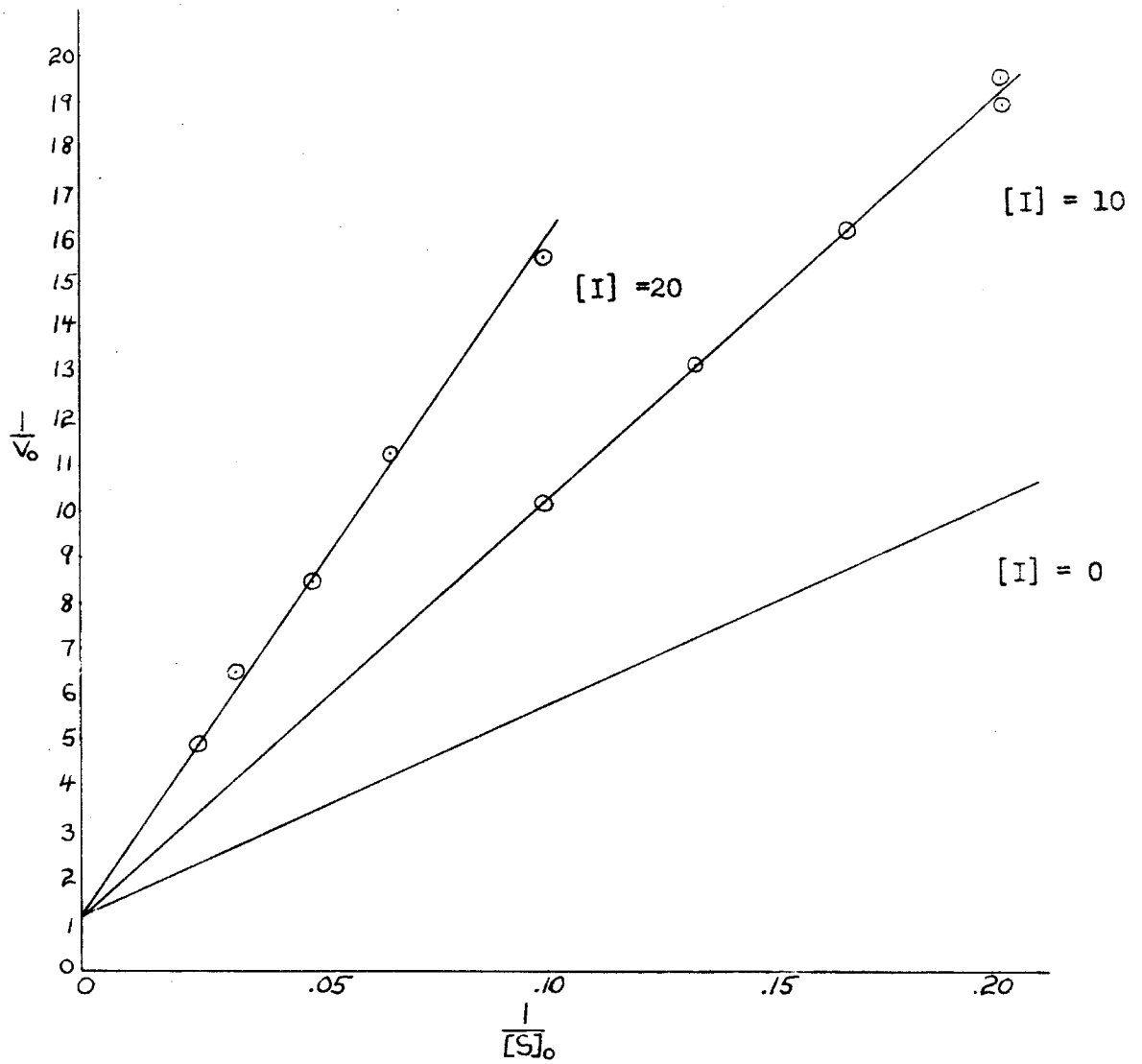

Fig. 11. N-Carboethoxy-L-tyrosinmethylamide as an Inhibitor against Acetyl-L-tyrosinhydroxamide.

Discussion of Results

While the occurrence of purely competitive inhibition in the sense of competitive three point attachment of the inhibitor cannot be considered established by the above data it is reasonable to assume that the K_I values calculated for the methylamides on the assumption of competitive behavior indicate magnitudes of enzyme-inhibitor affinity in the EI complexes. The occurrence of an actual increase in intercept with increasing [I] in the case of the L isomer would result if a certain fraction of trimolecular IES interactions took place in the system. Such interactions could be of the noncompetitive or uncompetitive IES type or, alternatively, a simultaneous attachment of substrate and inhibitor at the same active site. This type of interaction was found to occur in the case of the inhibition of the hydrolysis of the bifunctional substrate, methyl hippurate, by indole[4]. It was noted in the previous part of this chapter that N-carboethoxy-L-tyrosinamide has a surprisingly high apparent affinity for the enzyme and at the same time a low k_3. The same is true of the affinity of the L-methylamide. A possible explanation of this behavior is that in the cases of the L isomers other modes of combination with the enzyme may be possible than that in which the L molecule duplicates the combining mode of its D isomer in the inverted manner. Such might be the case if the R_1 carboethoxy group, in molecules of the L configuration, were able to interchange roles with the R_2 para-hydroxybenzyl side chain by assuming the cyclic hydrogen bridged configuration mentioned earlier in this chapter. The occurrence of a certain percentage of such

alternate type interactions could be responsible for the low value of k_3 of the L amide provided that such alternately oriented ES complexes were unproductive from the standpoint of hydrolysis. Another possible explanation for the low k_3 is the one stated previously, i.e., that it is a manifestation of the relatively unstrained ES complex suggested by the low K_s. It is possible that a predisposition toward such alternative modes of combination in the case of the L-methylamide might render it a potential monofunctional inhibitor in a certain fraction of its interactions. The L-methylamide would then be capable of participating in the trimolecular type $\overline{\text{IES}}$ complex noted in the methyl hippurate-indole case. Such behavior would result in an increase in intercept with increasing inhibitor concentration on the Lineweaver-Burk plot and this possibility emphasizes the necessity of a more thorough investigation of the possible influence of the L-methylamide on the substrate intercept.

The results summarized in Table XVI indicate that the "inverse" order of K_s and K_I observed with the D and L amides also obtains in the case of the K_I values of the methylamides. Since the K_I values of the methylamides may be translated into affinities of enzyme for inhibitor the indication is that K_s of N-carboethoxy-L-tyrosinamide is indeed a measure of its affinity for chymotrypsin and, that in this enantiomorphic substrate-inhibitor pair, the substrate is bound more tightly in ES than is the inhibitor in EI. In Table XVI is a summary of the affinity data for the four compounds including the free energies of dissociation of their respective complexes with chymotrypsin.

TABLE XVI

Summary of the ES and EI Dissociation Constants of The Enantiomorphic N-Carboethoxytyrosinamides[a] and N-Carboethoxytyrosinmethylamides[b] at 25°C

Compound	K_s^e	K_I^e	$\Delta F = -RT \ln K$
L-amide	6.43		+ 2987
D-amide		19	+ 2345
L-methylamide		9.5	+ 2756
D-methylamide		21.8	+ 2264

a Determined at pH 7.9.

b Determined at pH 7.6.

c Assuming $k_2 >> k_3$.

d Averaging the value of K_I obtained against its L antipode as the substrate with the value of K_I determined with acetyl-L-tyrosinhydroxamide as the substrate.

e In units of 10^{-3} M.

A certain degree of independence of relative binding strengths of the members of the DL pairs from R_3 is indicated by comparing the differences in ΔF's of the amides ($\Delta(\Delta F) = 642$) to $\Delta(\Delta F) = 492$ for the methylamides. The ratio of the K_I value of the L-methylamide to the K_s value of the L-amide is ca. 1.48 while the ratio of the K_I values of the corresponding D isomers is ca. 1.15. This small difference in relative binding strengths is in the wrong direction to suggest any influence of k_3 on the size of K_s of the L-amide (known to be small anyway) and is possibly a reflection of alternate modes of combination, which as suggested earlier, may be open to the L isomers alone.

Experimental

L-Tyrosine Ethyl Ester - Prepared according to the method of MacAllister.[a]

A suspension of 20.0 g. of L-tyrosine (Lemke) in 100 ml. of absolute ethanol was saturated with anhydrous hydrogen chloride (Matheson) at 0-5°. The mixture was heated under refluxing conditions for 26 hours and the excess solvent removed by evaporation in vacuo to yield a solid cake of the ester hydrochloride. Neutralization by aqueous potassium carbonate gave the crude ester which was recrystallized once from ethyl acetate to give 11.6 g. of small stout prisms, m.p. 101-102.3°. Found by MacAllister, m.p. 105-106° (after several recrystallizations).

L-Tyrosinamide - Prepared according to the method of MacAllister.[a]

A solution of 9.8 g. of L-tyrosine ethyl ester in 60 ml. of absolute methanol was saturated with anhydrous ammonia (Dow) at 0-5°. After warming slowly to room temperature the mixture was allowed to stand for 46 hours and the volatile material was removed by evaporation in vacuo. The resulting crude material was recrystallized from methanol-water to yield 3.34 g. of chunky prisms, m.p. 154.3-154.9°. Found by MacAllister, m.p. 155-156°.

N-Carboethoxy-L-tyrosinamide - A solution of 3.34 g. of L-tyrosinamide in 23 ml. dioxane-water was stirred with a 10 per cent excess of ethyl chlorocarbonate (Eastman) at 0-5°, with concurrent addition of aqueous potassium carbonate to react with the liberated hydrogen chloride.

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[a] R.V. MacAllister, Ph.D. Thesis, Calif. Inst. of Technology, 1950.

The crude N-carboethoxy-L-tyrosinamide was recrystallized from water to give fine white needles, m.p. 156.6-158.1°, Lit[21], m.p. 155-157°. $[\alpha]^{25}$ = + 1.90 (C = 5 per cent in methanol).

Anal. calcd. for $C_{12}H_{16}O_4N_2$ (252.3) : C, 57.1; H, 6.4; N, 11.1; Found: C, 57.0; H, 6.4; N, 11.2.

DL-Tyrosine Ethyl Ester - Prepared in the same manner as the L ester. Treatment of 100 g. of DL-tyrosine (Dow) with ethanolic-hydrogen chloride yielded a crude crop of ester weighing 65.7 g., m.p. 104.9-107.9°. The material was used without further purification.

N-Carboethoxy-DL-tyrosine Ethyl Ester - To a solution of 100 g. of DL-tyrosine ethyl ester in 500 ml. of pyridine, maintained at 5-10°, 58 g. of ethyl chlorocarbonate (Eastman) was added dropwise with mechanical stirring over a period of 50 min. The pyridine was removed by evaporation in vacuo and the resulting syrup, dissolved in 300 ml. of chloroform, extracted in the cold with dilute hydrochloic acid to remove unreacted ester and pyridine. Evaporation of the chloroform yielded 91.0 g. of small clear prisms, m.p. 87.2-90.3°.

Enzymatic Resolution of N-Carboethoxy-DL-tyrosine Ethyl Ester - 50 mg. alpha-chymotrypsin (Armour lot no. 10705) was added to a mechanically stirred solution[a] of 75 g. of N-carboethoxy-DL-tyrosine ethyl ester in a mixture of 422 ml. methanol and 1445 ml. water, maintained at 30-35°, and containing sufficient phenol red indicator (0.2 per cent Braun)

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[a] Some ester actually remained undissolved and had to be separated from the main reaction mixture and treated with additional enzyme.

to follow visibly the pH of the reaction. As the asymmetric hydrolysis proceeded, 5.46 g. of sodium hydroxide in 336 ml. of aqueous solution was added at a rate which maintained the pH of the reaction mixture within the range, pH 7-8. From time to time it was necessary to replace methanol lost by evaporation. After 30 min. an additional 25 mg. of chymotrypsin was added, followed by an occasional small portion of a water suspension of "Viobin". After $3\frac{1}{2}$ hours the reaction was essentially complete and the solution was reduced to ca. 400 ml. by evaporation *in vacuo* at 30-40°. The reaction mixture was brought to pH 8.5 and extracted with 4-200 ml. portions of chloroform. Evaporation of the chloroform yielded the syrupy D-ester which could not be crystallized.

N-Carboethoxy-D-tyrosinamide - A syrupy preparation of N-carboethoxy-D-tyrosine ethyl ester obtained from an enzymatic resolution of 55.0 g. of N-carboethoxy-DL-tyrosine ethyl ester was dissolved in 150 ml. of absolute methanol and the solution saturated with anhydrous ammonia (Dow) at 0-5°. After standing at room temperature for 2 days the volatile material was removed by evaporation *in vacuo* and the resulting syrup slowly crystallized. Repeated recrystallization from water yielded 7.6 g. of fine white needles, m.p. 156.6-158.1°, $[\alpha]_D^{25} = -19.4$. (C = 5 per cent in methanol).

Anal. calcd. for $C_{12}H_{16}O_4N_2$ (252.3) : C, 57.1; H, 6.4; N, 11.1. Found: C, 57.4; H, 6.2; N, 11.1.

N-Carboethoxy-D-Tyrosinmethylamide - A syrupy preparation of N-carboethoxy-D-tyrosine ethyl ester obtained from an enzymatic resolution of 75.0 g. of N-carboethoxy-DL-tyrosine ethyl ester was dissolved in 250 ml. of absolute methanol and cooled to 0-5°. Methylamine (Matheson) was passed into the solution until it had increased to about 400 ml. in total volume and the mixture was allowed to warm slowly to room temperature and stand undisturbed for 70 hours. Upon removal of volatile substances by evaporation, a syrup was obtained which rapidly crystallized in bunches of silky white needles. These were recrystallized twice from water to give 10.1 g. of fine white needles, m.p. 152.4-154.4° $[\alpha]_D^{22}$ = -20.9 (C = 5 per cent in methanol).

Anal. calcd. for $C_{13}H_{18}O_4N_2$ (266.3) : C, 58.6; H, 6.8; N, 10.5. Found: C, 58.6; H, 6.8; N, 10.4.

L-Tyrosinemethylamide - Methylamine (Matheson) was passed into a solution of 40.5 g. of L-tyrosine ethyl ester in 300 ml. of absolute methanol at 0-5° until the total volume had increased to ca. 500 ml., and the resulting solution was allowed to warm slowly to room temperature and stand undisturbed for 4 days. Removal of volatile substances left an uncrystallizable syrup which was, however, suitable for conversion to the carboethoxy derivative.

N-Carboethoxy-L-Tyrosinmethylamide - The syrupy L-tyrosine methylamide from above was dissolved in 300 ml. of ethyl acetate-acetone and placed in a 1000-ml. Florence flask with a second phase consisting of 300 ml. of saturated aqueous potassium carbonate. The biphasic system was

cooled to 6° and 40.6 ml. of ethyl chlorocarbonate (Eastman) was added in small portions over a 20 minute period with continuous shaking and cooling. The precipitate which formed was recrystallized repeatedly from water to give 7.0 g. of white needles, m.p. 151.5-153.5°, $[\alpha]_D^{22}$ = +19.2 (C = 5 per cent in methanol).

Anal. calcd. for $C_{13}H_{18}O_4N_2$ (266.3) : C, 58.6; H, 6.8; N, 10.5. Found: C,58.6; H, 6.9; N 10.5.

Acetyl-L-Tyrosinhydroxamide - This material was prepared by R.R. Jennings. It was recrystallized once from isopropanol and the resulting fine white solid washed repeatedly with cold isopropanol and tried *in vacuo*. $[\alpha]_D^{20}$ = +39.0° (C = 5 per cent in water). Lit[19], $[\alpha]_D^{25}$ = +38.3° (C = 5 per cent in water).

Enzyme Solutions - Two crystalline preparations of alpha-chymotrypsin were used in these studies. In all experiments utilizing N-carboethoxy-L-tyrosinamide as the substrate at pH 7.9, including those in the presence of its D antipode, the enzyme was an Armour preparation, lot no. 90402, containing 10.4 per cent protein-nitrogen. In the pH optimum study, in the hydrolyses of N-carboethoxy-L-tyrosinamide at pH 8.10-8.25, and in the evaluation of all inhibitors against acetyl-L-tyrosinhydroxamide, the preparation was likewise an Armour one, lot no. 10705, containing 13.3 per cent protein-nitrogen. Both preparations were of bovine origin and contained added magnesium sulfate. Aqueous stock solutions of enzyme were prepared daily and were kept in the refrigerator between the intervals in which they were brought to 25° prior to introduction into the substrate reaction mixtures.

Buffer Solutions - All hydrolysis experiments were buffered by the tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer described by Iselin and Niemann.[22] The amine component of the buffer was recrystallized three times from water and made up to buffer solutions of the desired pH and concentration by titration with solutions of hydrochloric acid of the appropriate concentration. These adjustments of pH were followed by means of a Beckman pH meter.

Formaldehyde - Merck reagent grade formaldehyde (36-38 per cent in aqueous solution) adjusted to pH 7-8 was used in the titrimetrically analyzed experiments.

Ferric Chloride Solution - The ferric chloride solution used for the spectrophotometric determination of acetyl-L-tyrosinhydroxamide was prepared as follows. 54.0 g. of reagent ferric chloride hexahydrate was dissolved in 500 ml. of water and 16.3 ml. of concentrated hydrochloric acid (assay 37.5 per cent HCl) added. This mixture was then made up to 1000 ml. with absolute methanol. The resulting solution was turbid when first prepared but became clear after filtering through a Whatman No. 1 paper and standing about 2 days.

Enzyme Experiments - All enzymatic hydrolyses were conducted at 25° in aqueous solutions containing a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer in the manner described by Huang and Niemann.[10] The titrimetric method of analysis used with the runs involving N-carboethoxy-L-tyrosinamide was identical with that described by Huang and Niemann[10] and earlier Iselin and Niemann.[22]

In the experiments involving acetyl-L-tyrosinhydroxamide as the specific substrate the hydrolyses were followed by an extension of the method of Hogness and Niemann.[19] In the present experiments, the absorbance, at any time t, of unhydrolyzed acetyl-L-tyrosinhydroxamide, as its ferric complex, was measured at a wavelength of 515mμ with a Beckman Model B Spectrophotometer. From the reaction mixtures buffered at pH 7.6 by tris-(hydroxymethyl)-aminomethane-hydrochloric acid, 0.27 F with respect to the amine component, 1-ml. aliquots were pipetted out and introduced at the chosen time intervals into 10-ml. G.S. volumetric flasks containing 7.5 ml. of absolute methanol and 1 ml. of the ferric chloride solution, stoppered and mixed. The absorbance of the colored complex was then measured with the spectrophotometer set at 100 per cent transmission against a blank, prepared in the same manner as the test samples, but containing, instead of 1 ml. of reaction mixture, 1 ml. of a blank solution consisting of enzyme, buffer and water. In the case of determinations made in the range $[S]_o = 15 \times 10^{-3}$ M to $[S]_o = 40 \times 10^{-3}$ M it was necessary to increase the dilution of the colored ferric complex to enable its spectrophotometric measurement. This was achieved by increasing the 10-ml. sample scale to 25 ml., introducing a reaction aliquot into a mixture of 1 ml. of the ferric chloride solution and 22.5 ml. methanol, contained in a 25-ml. G.S. volumetric flask, and adding methanol to 25 ml.

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SECTION II

EXPERIMENTS ON THE OXIDATION OF 3-INDOLEACETIC ACID BY PLANT ENZYMES

Introduction

That 3-indoleacetic acid occurs widely in plants where it functions as the chief growth regulating hormone (auxin) has been established by a long series of investigations[1-5]. This substance is biosynthesized in the apical meristems and young leaves of plants, from which it is translocated downward to control a wide variety of plant physiological responses, the most studied of which is that of cell elongation. 3-indoleacetic acid is formed in the plant from tryptophane, a fact indicated by the existence in plants of an enzyme system capable of this conversion and apparently distributed in such a way that its highest concentrations are found in those tissues known to synthesize and export auxin. In addition to this synthetic potentiality another enzyme system, which functions in the oxidative destruction of IAA, has been shown to be present in a number of plant tissues. The level of IAA within a given plant tissue may be determined consequently by the simultaneous operation of two enzyme systems, one capable of synthesizing this auxin and the other responsible for its oxidative degradation into inactive products. Since the typical physiological and morphological behavior of a growing plant are believed to depend upon the distribution of auxin among its various organs, this behavior may be related to a regulatory balance between the functioning of these two enzyme systems. For example, while IAA promotes the elongation of stem cells in the area immediately below the meristematic area, the same or somewhat higher concentrations are known to inhibit the elongation of root cells.[6] Tang and Bonner[7] have suggested that the relatively high

concentration of the IAA-oxidase system in the roots of many species may be related to the necessity of preventing an excessive accumulation of growth substance in this organ. The suggestion that the function of IAA-oxidase is to keep in check the IAA level produced by the synthetic system has been advanced by other workers.[8]

Beside occurring widely in roots, the IAA-oxidase system is known to occur in the etiolated tissues of many seedlings. Tang and Bonner[9] found breis of etiolated pea epicotyls to be an excellent practical source of this enzyme system. They showed the inactivation process to be an oxidative decarboxylation in which one molecule of oxygen is apparently taken up and one molecule of carbon dioxide liberated for every molecule of IAA inactivated (Equation (1))

(1) $O_2 + IAA \xrightarrow{\text{IAA oxidase}}$ oxidized IAA + CO_2

Wagenknecht and Burris[8] report the same stoichiometry for the destruction of IAA by the oxidase present in root homogenates of the yellow wax bean. They also found that 3-indolepropionic acid and 3-indolebutyric acid undergo oxidation in the presence of this enzyme but at rates much lower than that of IAA. Galston, Bonner, and Baker[10] have presented evidence that the IAA-oxidase system consists of a peroxidase coupled to a hydrogen peroxide generating flavoprotein in the following manner

(2a) Red. flavoprotein + O_2 ⟶ Oxid. flavoprotein + H_2O_2

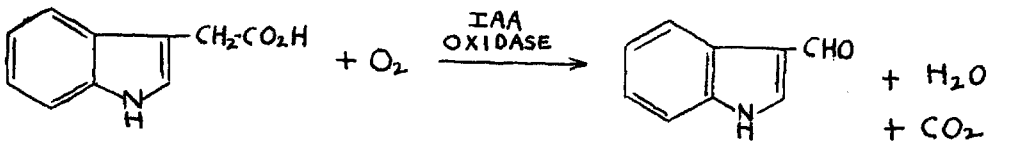
(unknown red. agent)

(2b) H_2O_2 + IAA $\xrightarrow{\text{peroxidase}}$ Oxid. IAA + CO_2 + H_2O

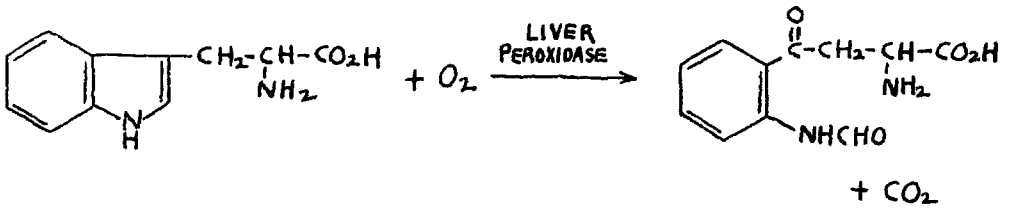
This oxidation is accelerated by light[11] by optimal amounts of manganous ion[8] and by certain phenolic type co-factors, particularly 2,4-dichlorophenol.[12] A somewhat similar enzyme system studied by Kenten and Mann[13-15] indicates a possible partial explanation for the actions of the two latter factors. The hydrogen peroxide-peroxidase component of their system was capable of oxidizing manganous ion via coupled oxidations of phenolic type co-factors. The high valency (+ 3, or + 4) manganese ion resulting is presumably a potential agent for further substrate oxidation and could be one of the responsible agents in the breakdown of IAA. Recently Siegel and Galston[16] have produced evidence that light, 2,4-dichlorophenol, and manganous ion enhance the peroxide producing capacity of pea tissue and also show that IAA itself is a peroxygenic agent. These agents can, therefore, boost the overall oxidation by intervening at an earlier stage of the oxidation chain as well.

While the general flavoprotein-peroxidase nature of the IAA-oxidase system seems fairly certain, the actual chemical transformations involved in the decarboxylative oxidation are obscure. Nothing definite has been disclosed regarding the fate of the IAA carbon skeleton. Tang and Bonner[9] have stated that the indole nucleus remains intact as evidenced by the application of the Hopkins-Cole test to the reaction products and furthermore that it is a neutral substance capable of extraction from the reaction mixture by ether at neutral or alkaline pH's. Wagenknecht and Burris[8] arrived at the same conclusions, basing their decision regarding the indole ring on the observation that

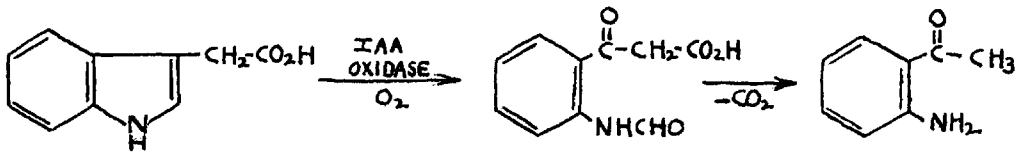
ether extracts of the reaction mixture gave a red color with Ehrlich's reagent. They also state that the product is a carbonyl compound as evidenced by its formation of a 2,4-dinitrophenylhydrazone derivative. Neither the method of preparation nor the physical properties of this derivative are described by these authors. This evidence, particularly the observed stoichiometry of the degradation prompted both of these groups to postulate the formation of 3-indolealdehyde as the oxidation product.

(3) 

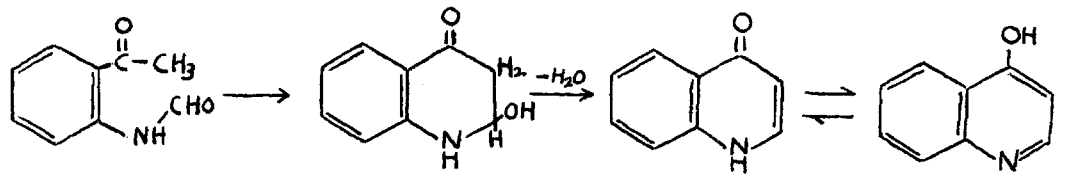
Wiltshire[17] agreed with this hypothesis on the basis of spectral evidence. This compound, resulting from exclusive attack on the side chain, is known, moreover, to exhibit no growth promoting action. An alternate possibility is initial oxidative attack upon the indole nucleus. This hypothesis derives support from two comparable instances of peroxidase-catalyzed tryptophane oxidation. Knox and Mehler[18] observed that a peroxidase-containing enzyme system of liver was capable of opening the indole ring of L-tryptophane to yield formyl kynurenine

(4) 

Like the IAA-oxidase of pea seedlings,[9] this oxidase is an adaptive enzyme capable of increasing in response to added substrate. Wiltshire[17] has reported the oxidation of tryptophane by a pea seedling enzyme which is identical, apparently, with the IAA-oxidase preparation used in the present investigation. One of his products was tentatively identified as 3-hydroxykynurenine apparently resulting from oxidation of both pyrrol and benzene nuclei. These reports, in combination with the above mentioned stoichiometry, led us to hypothesize the following tentative reaction sequence for IAA oxidation

(5) 

The o-formamidoacetophenone (OFA) resulting from nuclear oxidation might appear in the reaction mixture or it might be deformylated in the presence of a formylase in a manner parallel to that observed by Knox and Mehler[19] with formylkynurenine. Alternatively, the OFA could undergo ring-closure to give 4-hydroxyquinoline[a].

(6) 

In the hope of gaining information regarding the nature of the oxidation product (or products) it was decided to prepare a number of these proposed products and compare them with the actual products obtained from the oxidation of IAA by preparations of pea-epicotyl oxidase.

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[a] A suggestion by Dr. D.G. Crosby of the Carbide and Carbon Chemicals Company.

Materials and Methods

Plant Material and Crude Enzyme - Alaska peas were planted in flats of moist vermiculite and allowed to germinate and grow in a darkroom maintained at 25 ± 1°C for a period of 7-9 days. The yellowish-white epicotyls were harvested, by cutting approximately one inch above the ground line, the necessary illumination being supplied by a weak green light.[a] The epicotyls were cut into small sections and ground with Sorensen's phosphate buffer, pH 6.6, in a Waring blendor at 0-2°C. It was found convenient to use ca. 60-100ml. of the buffer solution per flat of peas planted in this operation. The resulting brei was pressed through cheesecloth to remove the larger particles and the filtrate centrifuged in the cold for 30 minutes at 10,000 x g. The milky supernatant was used as the crude enzyme preparation and was stored in a deep-freeze until such time as it was fractionated and employed in the oxidation experiments.

Fractionation of the Enzyme - In a typical fractionation 138 ml. of the crude enzyme preparation was carefully thawed and centrifuged in the cold for thirty minutes at 10,000 x g, discarding the sediment. To the supernatant, maintained at 0-5°C, was added solid reagent grade ammonium sulfate to effect the precipitation of protein fractions of decreasing average molecular weight. These fractions were allowed to precipitate over periods of ca. 5-12 hours at 0-2°C after which they were collected by centrifugation at 10,000 x g for 20-30 minutes. They were then resuspended, each in 50 ml. of phosphate buffer, pH 6.6,

[a] Photomorphogenically inactive.

and stored in the refrigerator at 0-2°C. Such preparations showed no deterioration in the cold over a period of at least two weeks.

The IAA-oxidase activity of each fraction was determined by incubation of a 1-ml. aliquot with 3-indoleacetic acid (Eastman) at an initial concentration of 130 γ/ml. for an interval of ca. 65 minutes after which the remaining IAA was determined by the colorimetric method with Salkowski's reagent described by Tang and Bonner[9]. In every case the reaction mixture contained 2,4-dichlorophenol as added co-factor at a concentration of 10^{-5} M.[12]

Table I summarizes the activity data for a typical series of enzyme fractions. In all cases the highest activity was found associated with the final supernatant remaining after saturation with ammonium sulfate. Although this was somewhat surprising in view of the known molecular weight of 44,000 of horseradish peroxidase[20], it is in agreement with the observation of Wiltshire[17] that 37 per cent, or the major portion, of the tryptophane oxidase activity of pea-seedling brei remained in the final supernatant, 2/3 saturated with respect to ammonium sulfate.

Oxidation Experiments - Although no careful study was made to compare the effects of the different enzyme fractions on IAA, there was no indication that different fractions of the enzyme led to different oxidation products.[a] In the oxidation experiments, therefore, either whole unfrozen enzyme, centrifuged 30 minutes at 10,000 x g to remove inactive fragments, or various combinations of the salt fractions were used.

[a] A difference was actually noted by Wiltshire[17].

TABLE I

Ammonium Sulfate Fractionation of IAA-oxidase from Breis of Etiolated Pea Epicotyls.

Frac. No.	Degree of Satn.[a,b]	Total Salt Added	Reac. Time	Colorim. Read.[c]	IAA Destroyed[d]
		grams	min.		γ/ml.
1	0-0.33	32.1	66	135	51.5
2	0.33-0.50	48.7	66	80	83.5
3	$0.50-0.58_5$	57.1	66	87	79.0
4	$0.58_5-0.65$	63.3	$65\frac{1}{2}$	119	60.0
5	$0.65-0.75_2$	73.3	65	137	50.0
Final supt.	0.75_2	73.3	65	70	89.0

a These figures are with respect to pure water at 0°C from the known solubility of ammonium sulfate. Actually the degrees of saturation are higher than above since the solubility of the salt is decreased by the presence of the protein.

b Total volume of original enzyme solution = 138 ml.

c Method of Tang and Bonner[9].

d It must be understood that the activities represented by these figures are total activities and do not represent enzymic activity per unit protein-nitrogen.

In a typical oxidation, 50-100 ml. of an aqueous solution[a] of IAA at an initial concentration of 500 γ/ml. was mixed with an equal volume if the enzyme solution in a 500-ml. Erlenmeyer flask and the mixture incubated at room temperature with gentle[b] shaking on an Eberbach shaker. In most instances the reaction mixtures contained 2,4-dichlorophenol at a concentration of 10^{-5} M.[12] The oxidations were allowed to proceed to about 75 per cent completion, as determined by colorimetric assay of withdrawn aliquots for IAA[9]. The time intervals required for this amount of reaction varied from ca. 30 to ca. 300 minutes. At the end of the incubation, such a reaction was stopped by bringing it to pH 3 or pH 9, by the addition of catalase[c], or by simply extracting the unreacted IAA and oxidation products away from the enzyme with reagent grade chloroform.

Extraction and Chromatography of the Oxidation Products - Reagent grade chloroform proved to be the best solvent for extraction of the oxidation products. Usually it was necessary to apply low speed (1500-2000 RPM) centrifugation to cause separation of the chloroform phases from the protein containing aqueous portions following equilibration in a separatory funnel. Contrary to the reports of previous workers[8,9], ether did not appear to be a satisfactory solvent for the oxidation products. The combined chloroform extracts from several equilibrations were partially dried by chilling to 0-5°C. and filtering through a

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[a] Containing a stoichiometric equivalent of sodium bicarbonate to dissolve the IAA.

[b] To avoid foaming and consequent denaturation of the enzyme.

[c] Armour (No. 30) Catalase was used.

chloroform wet filter paper and concentrated by evaporation to a few ml. volume *in vacuo*.

Chromatographic separation of the oxidation products was performed upon ascending chromatograms of Whatman No. 1 filter paper. The most satisfactory procedure was the following one. Rectangles of the paper, 29 cm. x 33 cm., were cut and a pencil line drawn parallel to the long dimension and ca. 3/4" from one edge. The oxidation product concentrates were spotted at intervals of a few inches along the line, each spot being made by repeated applications of the chloroform concentrate from a glass capillary, 0.5-1.0 mm. in diameter. Care was exercized to keep the size of the spots as small as possible. Synthetic compounds of known composition were similarly applied at different points along the line. It was found convenient to hasten the evaporation of solvent between each capillary application by means of a gentle compressed-air stream. A chromatogram thus prepared was wound into a cylinder, 29 cm. in length, and fastened at each end and at the central junction by stapling. Such a cylindrical chromatogram could be conveniently contained in a cylindrical glass developing tank, 15 cm. in diameter and 30 cm. high. The prepared chromatogram was then suspended over the developing solvent contained in the bottom of the tank. A rigid iron wire, bisecting the circular section of the chromatogram near the top, was caused to hold the paper cylinder firmly against the lower side of a glass plate, covering the open upper end of the tank, by means of a permanent magnet placed upon its upper surface. After the paper had been allowed to equilibrate in the solvent atmosphere for periods varying

from 90 to 180 minutes, the magnet was removed, causing the paper cylinder to drop gently into the developer. The chromatogram was allowed to remain in the developer until the solvent front had nearly reached the upper edge of the paper and was then removed and hung in the air to dry. The dried developed paper was then sprayed with an appropriate spray reagent and given a brief heat treatment to accelerate the development of the colored spots.

Developing Sovents - Two mixtures containing isopropanol, concentrated aqueous ammonia, and water were used. With a 10:1:1 volume ratio[21] the solvent front moved rapidly, nearing the upper end of the cylinder in 6 hours. A 2:1:1 volume ratio, however, proved superior for the separation of the oxidation products and was used in the development of the chromatograms discussed in the next section. This developer moved slowly, 15 hours being a common time for development.

Spray Reagents - Two spray reagents were used. A solution of Ehrlich's reagent[22] (p-dimethylaminobenzaldehyde), 1 per cent in 1N hydrochloric acid was found to be an effective color reagent for indole-type compounds and aromatic amines. A solution of ferric chloride, 1 per cent in water, was used as a spot reagent for 4-hydroxyquinoline and was later found to give color reactions with some of the other compounds involved.

Hopkins-Cole Reagent - This reagent,[23] containing magnesium glyoxylate as the active agent, was prepared according to Benedict.[24]

The test for indolic compounds is conducted as follows.[25] To a small amount of the material to be tested is added 1 ml. of the reagent plus 0.2 ml. of 0.01 M aqueous cupric sulfate and the constituents well mixed. 5 ml. of concentrated sulfuric acid is then added cautiously and finally mixed thoroughly with the original solution. The formation of a red to blue-violet color is characteristic of many compounds containing the indole nucleus.

Chromatography and Preparation of The Oxidation Products for U.V. Spectral Analysis - The entire concentrated chloroform extract of the particular fraction to be analyzed was applied in a strip along the bottom of a cylindrical paper chromatogram, the sides of which were graduated in centimeter-unit scales. Such a chromatogram was then developed and dried in the usual manner. It was then opened, placed flat upon a flat surface and parallel pencil lines drawn connecting the opposite corresponding centimeter marks. The horizontal zones containing the oxidation products and unreacted IAA were located by cutting two thin vertical sample strips from near the ends of a chromatogram and spraying them with Ehrlich's reagent. The entire chromatogram was then cut horizontally into the 19cm. wide strips defined by the pencil lines, each strip being labelled with the number corresponding to the distance in centimeters of its upper edge from the bottom of the chromatogram. Each strip was then cut into small fragments and these washed with 3 ml. of reagent chloroform in a labelled test tube to elute any soluble material present. 0.1 ml. of each chloroform eluate was added to 5 ml. of absolute methanol and the resulting solution analyzed with a Beckman U.V. Spectrophotometer.

3-Indoleacetic Acid - Eastman No.4271 was used without further purification.

3-Indolealdehyde - A sample donated by Mr. R.A. Bernhard was employed. It was recrystallized twice from water to give white blades, insoluble in aqueous soidum hydroxide, m.p. 193.5-195°. Lit[26], m.p. 194°.

o-Formamidoacetophenone - Prepared by the method of Witkop[27]. A solution of 4.3 g. of C.P. skatole (Eimer and Amend) in 50 ml. of formamide, contained in a 250-ml. round bottom flask, was chilled to 4°C by a beaker of water surrounded by an ice bath. A stream of oxygen-diluted ozone[a] was passed into the solution for a period of nine hours at which time the exit gas contained considerable ozone as evidenced by its ability to liberate iodine from an aqueous boric acid - sodium iodide solution. The dark solution was extracted with four 50-ml. portions of ethyl ether which were then combined and washed with water. The yellow ether solution was dried over anhydrous potassium carbonate and the ether removed by evaporation. The total weight of the crude product thus obtained was 3.11 g. Recrystallization of it from ether containing a small amount of ligroin gave sparkling prisms. m.p. 78.9-79.5°. Lit.[27] m.p. 78°.

o-Aminoacetophenone - 0.25 g. of crude o-formamidoacetophenone was heated under refluxing conditions with 20 ml. of 3N hydrochloric acid for 45 minutes. Upon cooling, the mixture was neutralized by the addition of 5-6 g. of sodium bicarbonate which caused the separation of o-amine-acetophenone as a dark oil. The oil was co-distilled from the neutral

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[a] I am indebted to Professor A.J. Haagen-Smit for the use of his ozone generator.

mixture with water and separated from the latter by ether extraction. Removal of ether upon the steam bath left the o-aminoacetophenone as a yellow, pleasant smelling oil.

4-Hydroxyquinoline - Prepared according to the procedure of Camps.[28]

1.0 g. of o-formamidoacetophenone was dissolved in a boiling mixture of 71.5 ml. of water and 6.4 ml. of 95 per cent ethanol. To this was added a solution of 0.43 g. of sodium hydroxide in 4.0 ml. of water and the yellow mixture heated under refluxing conditions for 3 hours. o-aminoacetophenone, liberated as a hydrolysis by-product, was removed by repeated co-distillation with water and the volume of the reaction mixture was reduced to 10 ml., allowed to cool, and filtered to remove the dark side-product, o-flavaniline (bimolecular condensation). 6N hydrochloric acid was added to the clear filtrate to neutrality and the precipitate which formed was collected by suction filtration. Gradual evaporation of the filtrate caused the formation of beautiful flat prismatic crystals of 4-hydroxyquinoline which were combined with the original precipitate, ground to a powder, and washed with ether to remove traces of o-aminoacetophenone. m.p. 198-205°. Lit.[28] m.p. 201°. Color with ferric chloride in aqueous solution, deep red at high concentrations, orange in dilute solution. Lit.[28] Color, Carmine.

Experimental Results

Color Reactions of Known Compounds

It was hoped that by means of parallel chromatography and the use of color reagents, the oxidation product (or products) would be identified among the test compounds employed. For this purpose, three color reagents were used. The Ehrlich's reagent and the ferric chloride solution[a] were sprayed separately on dried paper chromatograms, while the Hopkins-Cole tests[a] were carried out in test tubes.

Table II summarizes the average R_F values and colors given by the various known compounds with the different reagents. The observation that Hopkins-Cole reagent gives a pale yellowish color with 3-indolealdehyde indicates that this compound alone could not have accounted for the purple color observed by Tang and Bonner[9]. With the other indolic substances tested, this reagent gave various shades of red and purple. Apparently the presence of the indole nucleus is a necessary, but not sufficient, condition for an intense color reaction with this reagent. Table II indicates that the non-indolic compounds were negative to this test.

With respect to Ehrlich's reagent, the compounds tested fall into two general categories. Compounds containing the indole nucleus gave colors ranging from pink through lavender to blue-violet and required a definite development time characteristic of each substance. IAA, indole, and skatole gave their respective colors within about

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[a] See section on materials and methods.

TABLE II

R_F Values and Color Reactions of Various Compounds Related to The Enzymic Oxidation of IAA

Substance	R_F		Color With		
	1.[a]	2.[b]	H-Cole.	Ehrlich	$FeCl_3$
IAA	0.35	0.79	violet	violet in ca. 5 min.	reddish (slow)
3-indole-aldehyde	0.90	0.91	pale yellow	violet in ca. 10-15 min.	reddish (very slow)
OFA	0.87	0.92	none	immediate orange-yellow[c]	none
OAA	0.89	0.93	none	immediate orange-yellow	none
4-hydroxy quinoline		0.83		none	immediate orange
skatole	0.87			bl-violet in 5 min.	
indole	0.92			pink in ca. 5 min.	
kynurenine[d]				yellow	
anthranilic acid				immediate orange-yellow	pale yellow (slow)

[a] 10:1:1 developer.

[b] 2:1:1 developer.

[c] Becomes bluish on standing.

[d] From Knox and Mehler.(18)

4-6 minutes of drying with gentle heat while 3-indolealdehyde was much slower to appear, requiring 10-12 minutes to give a perceptible violet. Compounds containing an aromatic amino group give an immediate color with Ehrlich's reagent due to Schiff base formation. Thus o-amino-acetophenone (OAA) and o-formamidoacetophenone (OFA) both gave immediate intense orange-yellow coloration persisting indefinitely in the case of OAA and changing slowly to blue in the case of OFA. It seems probable that the immediate yellow given by OFA results from cleavage of the formyl group by the acid present to give OAA and that the blue may be characteristic of OFA itself. Knox and Mehler[18] found kynurenine to give a similar yellow color with Ehrlich's reagent.

Hopkins-Cole Test upon the Oxidation Products.

It is not clear from the report of Tang and Bonner[19] whether they carried out their Hopkins-Cole reactions upon aliquots of reaction mixture or upon ether extracts of the same. Since their results in this text are compared with the Salkowski assays, however, and the latter are performed directly upon the reaction mixture it is presumed that their Hopkins-Cole tests were performed in the same way. In the present experiments Hopkins-Cole tests were carried out on both a reaction mixture and upon a chloroform extract of the same at pH 9. Blank determinations upon an enzyme solution which had been treated as in an oxidation run but containing no IAA were also performed. The results of these tests are shown in Table III. When performed directly upon a reaction mixture, the Hopkins-Cole test gave a pinkish-brown color regardless of the presence or absence of IAA or of its oxidation products.

TABLE III

Hopkins-Cole Behavior of Various Components of an Oxidation Reaction Mixture.

Tested	Result
Directly on reaction mixture[a]	pinkish-brown
Directly on enzyme preparation	pinkish-brown
Upon a chloroform extract of a reaction mixture at pH 9	purple
Upon a chloroform extract of[a,b] the enzyme preparation	pale-yellow
Upon a chloroform extract of an IAA solution at pH 9	nothing

[a] 10^{-5} M in 2,4-dichlorophenol.(12)

[b] The preparation had been incubated in the manner of an oxidation run.

This color probably results from the high "blank" of tryptophane-containing proteins in the enzyme preparation. Table III indicates that the oxidation reaction mixture contained a substance, presumably indolic which could be extracted into chloroform[a] and gave a Hopkins-Cole color. This was shown to be neither present in nor formed from the enzyme preparation. That this was not unreacted IAA was indicated by a negative Hopkins-Cole test given by a chloroform extract of an IAA solution at a comparable concentration and pH 9.

Chromatographic Separation of the Oxidation Products

Most of the knowledge regarding the nature of the oxidation products was gained by paper chromatography of the chloroform extracts of oxidation reaction mixtures. A large number of paper chromatograms from the material of eight oxidation experiments indicated the presence of at least two principal oxidation products. These appeared, before any spray treatment, as yellowish spots having average R_F values of 0.94 and 0.87, respectively.[b] Upon spraying with Ehrlich's reagent both spots were immediately colored orange-yellow, resembling OFA and OAA in color and behavior. Upon standing, however, both spots, unlike either OFA or OAA, began to develop reddish tinges, the spot at R_F 0.94 becoming pinkish-orange and the lower one dark pinkish-orange to light red. These colors were dependent upon the concentrations of the oxidation products and deepened toward red with increasing concentration. That this color ripening did not result from a superposition of an aromatic amine and IAA was indicated by the presence of IAA as a clearly defined

a It was found necessary to remove the chloroform prior to the test as it gives an interfering dark color.

b These values varied between chromatograms. This variation seems to be due in part to time of equilibration before development and in part to other factors. The composition of the developer had little effect on these R_F's.

violet spot below the oxidation products.

Treatment with the ferric chloride spray revealed both products as rose colored spots, which appeared within three to five minutes and intensified with time, particularly the R_F 0.94 spot, which became deep rose upon standing. This is unlike the behavior of 4-hydroxyquinoline which gives an immediate and unchanging orange spot with ferric chloride. This and the fact that 4-hydroxyquinoline gives no color with Ehrlich's reagent eliminate it as an agent responsible for the observed spots. Beside failing to give Ehrlich tests that correspond to those of the oxidation products, 3-indolealdehyde, OFA, and OAA behave differently with ferric chloride as well. OFA and OAA give no color with this reagent even on standing and 3-indolealdehyde gives only a faint reddish spot on standing ten to twenty minutes (Table II).

The Accumulation of Oxidation Products with Time

To confirm the identity of the above spots as authentic reaction products, and to detect any possible change in the nature of these products or in the approximate ratios of their concentrations with time, a run was performed in which one-third of the reaction mixture was withdrawn at each of three successive time intervals and extracted with chloroform to remove the oxidation products. The results of a paper chromatogram of these chloroform extracts are indicated in Table IV. With each aliquot, three spots appeared upon treatment with Ehrlich spray. An upper pair, at R_F values of 0.94 and 0.91, appeared immediately as yellow spots which ripened slowly to orange in the case of R_F 0.94

[a] The extractions were performed upon the reaction mixture portions without any prior adjustment of pH. The natural pH of the reaction mixtures was close to 7.

TABLE IV

Accumulation of The Oxidation Products with Time[a]

Spot	R_F	Color devel. with Ehrlich	Order of color inten. I_t of time aliquot spots
IAA	0.80	violet in 5 min.	$I_{65} >> I_{100} > I_{276}$
Ox. prod. I	0.91	yellow → red	$I_{65} < I_{100} << I_{276}$
Ox. prod. II	0.94	yellow → orange	$I_{65} < I_{100} < I_{276}$

[a] Time in minutes.

and bright red at R_F 0.91. Unreacted IAA appeared gradually as a violet spot at R_F 0.80. It was clear that while the intensities of the IAA spots decreased regularly with time, the intensities of the two upper spots, and in particular the one at R_F 0.91, increased in the same order. The same evidence was obtained by spraying with ferric chloride. This indicates that as IAA is destroyed at least two oxidation products accumulate in the reaction mixture.

Acidic or Basic Natures of the Oxidation Products.

In the hope of gaining information about the natures of the oxidation products from their pH-solubility relationships, an oxidation run was performed following which one-half of the reaction mixture was adjusted to pH 3 and the other half to pH 9, and both extracted in identical manners with chloroform. The first indication of a difference in behavior of the oxidation products was given by the colors of the two chloroform extracts. The extract from aqueous phase at pH 3 was bright yellow and was found to contain a dark yellow-brown oily pigment upon evaporation of the chloroform. The extract from pH 9 solution was virtually colorless and contained a small amount of a yellowish-white solid. Table V summarizes the chromatographic data of this experiment. The chromatogram of the pH 3 extract showed, when sprayed with Ehrlich's reagent, two spots. At R_F 0.93 an immediate orange color appeared which ripened to red in twenty-four hours. At R_F 0.82 an immediate carmine spot appeared which faded over a twenty-

TABLE V

pH-Solubility Behavior of The Oxidation Products

Spot	R_F	Ext. at pH 3		Ext. at pH 9	
		Ehr. color	$FeCl_3$ color	Ehr. color	$FeCl_3$ color
IAA	0.74	violet in 5-10 min.	pale violet (v.slow)	none	none
Ox. prod. I	0.82	carmine	none	none	none
Ox. prod. II	0.93	orange-red	rose-pink	orange-red	vivid rose

four hour period. IAA appeared below at R_F 0.74 as a violet spot. Ehrlich treatment of the chromatographed extract from pH 9 solution yielded only the spot at R_F 0.93 which was practically identical with the upper spot on the other chromatogram. Only the compound responsible for the spots at R_F 0.93 gave a color with ferric chloride, and, of these, the rose color of the spot from the pH 9 extraction was considerably more intense than that of the corresponding spot on the pH 3 chromatogram. To check the possibility that any of these spots arose from the enzyme preparation, enzyme blanks were incubated in the usual manner, made pH 3 and pH 9, extracted as before and the concentrated extracts chromatographed. Neither Ehrlich's reagent nor ferric chloride indicated the presence of any interfering compounds in the enzyme preparation.

There is a possibility that the product causing the lower spot (R_F 0.82) may not be identical with the oxidation product observed at the lower R_F's of pairs seen on previous chromatograms. This is suggested by its R_F value of 0.82 which is considerably lower than the values of ca. 0.91 usually observed. The apparently neutral compound appearing at R_F 0.93 may actually, then, consist of two unresolved spots that have appeared as a resolved pair on some of the previous chromatograms.

OFA and 3-Indolealdehyde as Possible Oxidation Intermediates

Although the observations described in this section cannot be explained by the presence of either OFA or 3-indolealdehyde as such in the oxidation reaction mixtures, the possibility remained that either

or both of these compounds are initially formed from IAA and then converted into other final products. Thus, while neither of these substances may be the end product of the reaction, their occurrence as intermediates would be in accord with the observed stoichiometry provided that the remaining steps in the overall reaction did not involve O_2 -CO_2 gas exchange. To test this possibility, OFA and 3-indolealdehyde were incubated with preparations of IAA-oxidase in separate experiments. The conditions employed were the same as previously described. In each experiment the concentration of the hypothetical intermediate was comparable to the initial concentrations of IAA used in the other runs, and in each case chloroform extraction was performed at the natural pH of the reaction mixture following the incubation period. The Ehrlich's reagent and ferric chloride treatment of the chromatograms made from the chloroform extracts indicated clearly that neither OFA nor 3-indolealdehyde is an intermediate responsible for the observed oxidation products.

U.V. Spectra of The Oxidation Products

To ascertain[a] the possible presence of oxidation products having R_F's below ca. 0.75 and not responding to either of the spray reagents employed, the optical densities of the methanol solutions prepared from the paper strips (see page 85) cut from this region were determined at wavelengths of 220 mμ, 250 mμ, and 280 mμ. In Figure 1 the optical densities at these three wavelengths are plotted versus the R_F values corresponding to the sample strips. It is evident

[a] A suggestion of Prof. H.K. Mitchell.

that below the R_F region responsible for the color reactions observed the amount of material absorbing at these chosen wavelengths is negligible.

In Fig. 2 are shown the absorption spectra of the two oxidation products extracted from the reaction mixture at pH 3 and of the product extracted at pH 9. The product of R_F 0.93 extracted at pH 9 possesses maxima at 224 mμ and 280 mμ and a minimum at 255 mμ. In contrast, the material responsible for the R_F 0.93 spot extracted at pH 3 lacks the 255 mμ minimum and the maximum at 280 mμ. This difference is probably due to the presence of a second product, in one of the R_F 0.93 fractions, whose spectrum is superposed upon that of a common product.[a] Of course, this spectral modification could also result from the presence of a compound (such as a phenolic co-factor) in the original brei possessing a higher chloroform solubility at the lower pH. This observation is, however, more in agreement with the possibility stated earlier that the spot appearing at R_F 0.93 represents two unresolved oxidation products.

The U.V. spectrum of the acidic product (R_F 0.82) appears to have only the one maximum at ca. 218 mμ and a point of inflection near 270 mμ. It is possible, however, that the spectrum at this R_F is due, in part, to the presence of some IAA.

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[a] Although the spectrum of the material extracted at pH 9 (R_F 0.93) resembles that of the added co-factor, 2,4-dichlorophenol, the concentration of this substance which might be present in the final methanol solution (at the most, 3×10^{-5} M) is insufficient to account for the observed optical densities.

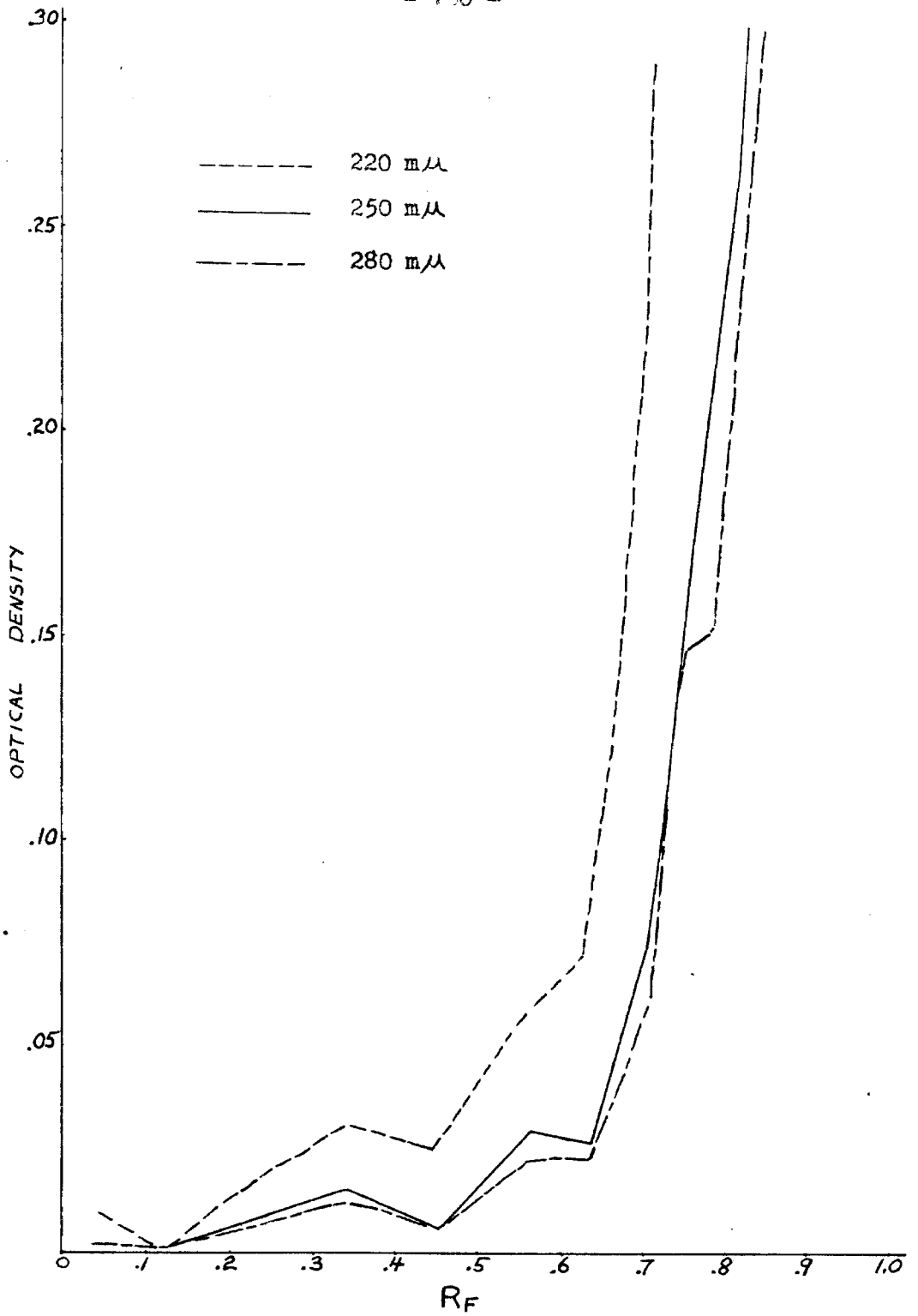

Fig. 1. Distribution of Material on a Chromatogram According to Absorption at Three Chosen Wavelengths.

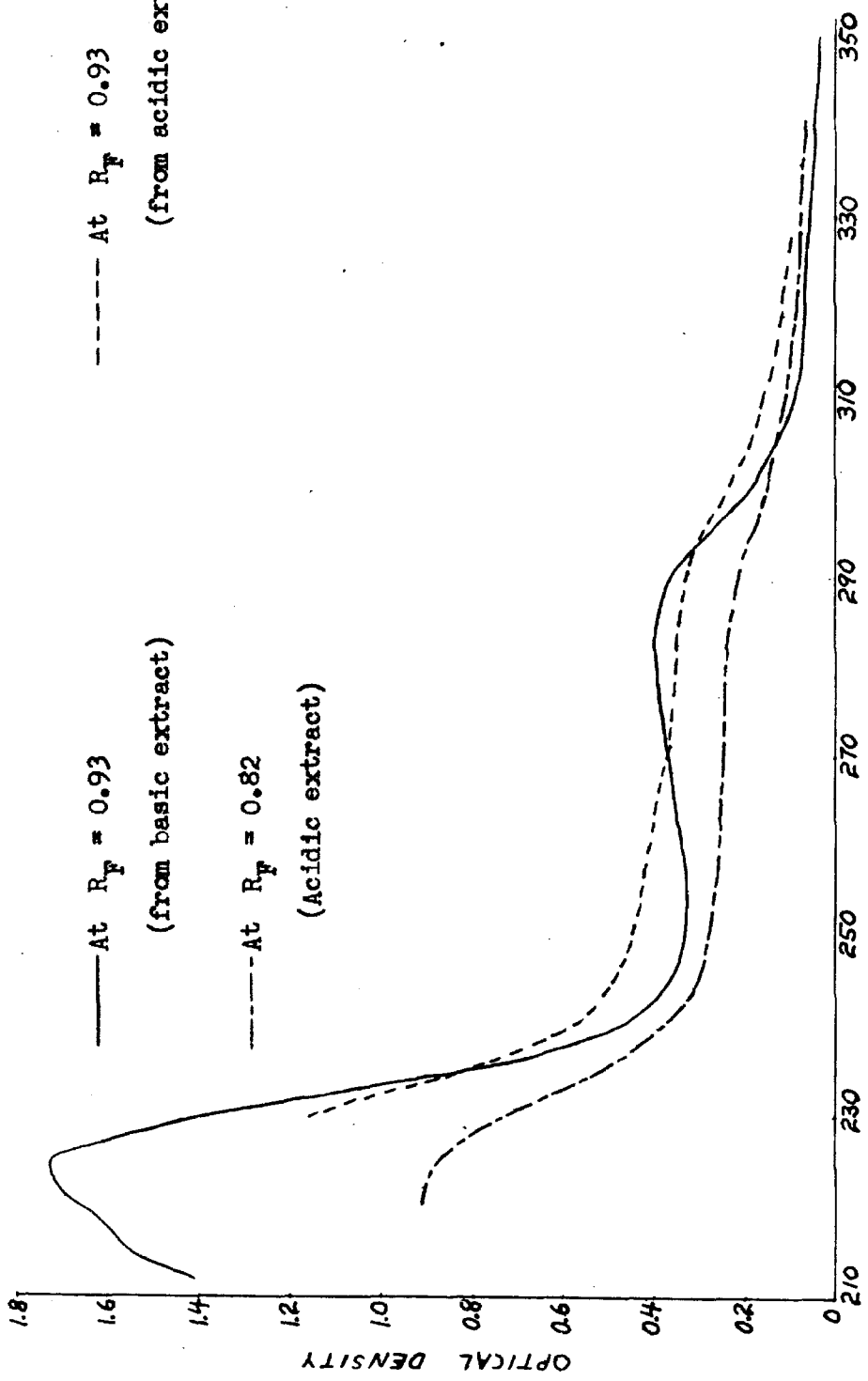

Fig. 2. U. V. Absorption Spectra of Some of the Oxidation Products of IAA.

Discussion

The evidence obtained in this investigation indicates that IAA undergoes oxidative degradation in the presence of the IAA-oxidase system, giving rise to several oxidation products. That at least one of these products contains an indole ring is suggested by the red Hopkins-Cole color given by a chloroform extract of a reaction mixture. That this color does not result, however, from the presence of 3-indolealdehyde (as was originally proposed[8,9]) is shown by the failure of this compound to give a similar color when treated with Hopkins-Cole reagent. At the same time, there is evidence (immediate formation of an orange-yellow color with Ehrlich's reagent) that at least two of these products contain aromatic amino groups, these necessarily resulting from cleavage of the indole nucleus. In the case of one experiment, one of the products (R_F 0.82) contained, in addition to a potential amino group, an acidic group causing it to be extractable into chloroform from aqueous solution at pH 3, but not at pH 9.

Although OFA, OAA, 3-indolealdehyde, and 4-hydroxyquinoline all possess R_F's within the range of the oxidation products, their behaviors with the spray reagents employed differ from those of the actual oxidation products. It does not seem likely that a combination of OAA (or OFA) and 3-indolealdehyde at the same position is responsible for the immediate orange-yellow ripening toward pinkish-orange which is observed on treatment with Ehrlich's reagent. In comparative chromatograms, the violet color of 3-indolealdehyde appeared at least five minutes after the color change of the oxidation products began. Moreover,

this color ripening occurred in every case with not just one, but with all spots representing oxidation products. 4-hydroxyquinoline is definitely excluded as an oxidation product due to the failure of any product to give an immediate permanent orange color with ferric chloride. It is further evident that neither 3-indolealdehyde nor OFA participate as intermediates inthe formation of the observed products. It is possible, however, that they are present to some extent and that their typical color reactions are masked by the presences of the major products. The absence of interference with these results by either the added co-factor, 2,4-dichlorophenol, or by any substances occurring naturally in the enzyme preparations was established by performing blank runs, identical with the oxidation runs, but containing no added IAA. Chromatograms of extracts from such blank reaction mixtures showed no color with either spray reagent provided that fresh enzyme preparations were used. On only one occasion, a chloroform extract of a brei which had been stored in the deep-freeze for several months yielded, at R_F 0.9, a slowly developing faint pink spot upon Ehrlich spray treatment.

While the present indications that the indole ring of IAA is, at least in part, destroyed are in opposition to the conclusions of previous workers[8,9,17] who have studied the IAA-oxidase system, some of the observations of these workers seem, nevertheless, to favor the concept of ring opening. The destruction of the indole ring of tryptophane reported by Knox and Mehler[18], and by Wiltshire[17] has already been mentioned. Wagenknecht and Burris[8] report that whereas the enzymic degradation of IAA results in the liberation of one molecule of carbon dioxide per molecule of oxygen consumed, the oxidation of

3-indolepropionic acid occurs with an R.Q. of only 0.25. Such a reduced yield of carbon dioxide in the case of 3-indolepropionic acid could be explained by an opening of the indole ring to give a gamma-keto acid, which would be more resistant toward decarboxylation than the corresponding beta-keto acid resulting from IAA.[a] Whereas the liver tryptophane oxidase studied by Knox and Mehler[18] was specific for the naturally occurring L isomer, the pea-seedling oxidase employed by Wiltshire[17] (possibly identical with our IAA oxidase) brought about the oxidation of both D and L tryptophane at the same rate. It is possible that such behavior could be related to the occurrence of an enzyme substrate complex involving modes of combination specific for the indole nucleus (or for its general structural outline) alone and more or less independent of the nature of the side chain.

Although the hypotheses of OFA and 3-indolealdehyde do not appear adequate in the light of the present experimental findings, it should be noted that other possible explanations for the observed stoichiometry exist. For example, the formation of any isomeric dihydroxy skatole would occur with the uptake of one molecule of oxygen and the liberation of one molecule of carbon dioxide per molecule of IAA activated. A compound of this type could not, of course, give a Schiff's base color with Ehrlich's reagent. Alternatively (or additionally), IAA inactivation may proceed via oxidative cleavage of the indole nucleus, and

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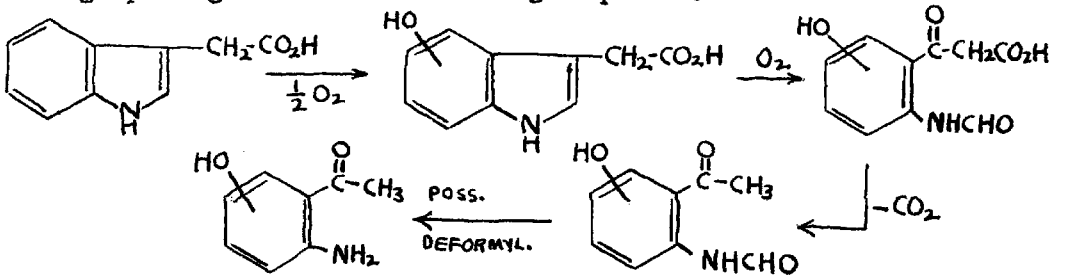
[a] A proposition (This Thesis).

the color reactions described earlier in the present paper seem to indicate that this process occurs. Excluding OFA (or anything arising from it with no further gas exchange) as an oxidation product, it is difficult to devise a hypothetical scheme involving ring opening and in harmony with the 1:1:1 stoichiometry. The present experiments have demonstrated, however, that the IAA-oxidase catalyzed degradation of IAA leads to not one, but to several products. Unless the formation of one of these products alone may be considered to be the predominant course of the inactivation, it is pointless to hypothesize a single product capable of explaining the stoichiometry when the net gas exchange observed is certainly the resultant of a number of degradation pathways. Such a multi-pathway destruction of IAA is hardly surprising in view of the known complexity of the enzyme system involved and it is particularly suggested by the fact that IAA plays a dual role in the enzymic sequence. Not only is it the substrate for the H_2O_2-peroxidase complex, but it may also react in some peroxygenic capacity to produce peroxide for its own further destruction.

If the stoichiometry of the inactivation process need not be accounted for by the formation of a single product it becomes possible to indulge in further speculation as to possible products resulting from oxidative cleavage of the indole nucleus. One possibility involves, in addition to opening of the pyrrol ring, hydroxylation of the benzene ring as well. Such a hypothesis derives some support from the apparent formation of 3-hydroxykynurenine from tryptophane by the same pea-seedling enzyme system.[17] Moreover, tryptophane is known to be converted

into 3-hydroxyanthranilic acid in the rat[29] and in Neurospora[30]. If a hydroxy OFA (or OAA) were to arise from the IAA-oxidase mediated degradation of IAA, the hydroxylation step would necessarily (since OFA is known not to be an intermediate) occur at some point before the actual ring opening as in the following sequence.[a]

(7)



A final product such as hydroxy OAA would have the advantages of being a near neutral compound (as is the product of R_F 0.93) capable of giving a Schiff color reaction and probably (by virtue of the phenolic hydroxyl) a color with ferric chloride as well.

Despite the numerous possibilities for further investigation of the chemistry of this degradation along the lines presently taken, the way appears open for a more direct assault upon the problem. Since the possibility of chromatographic separation of the oxidation products has been demonstrated it would seem advisable to carry out a large scale conversion of IAA to its oxidation products, separate these chromatographically, and attack the problem of their identities by the standard procedures of organic chemical characterization.

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[a] Actually, the hydroxylation would not have to occur as the first step, but it would probably have to occur before the ring opening.

Summary of Conclusions

In the presence of pea-seedling IAA-oxidase, IAA undergoes oxidative conversion into at least two, probably more, products.

Application of chromatographic techniques and color reagents indicates that some of the more obvious hypothetical products cannot account for the observed behavior of the authentic oxidation products. The compounds tested were 3-indolealdehyde, OFA, OAA, and 4-hydroxy-quinoline. It is also found that neither OFA nor 3-indolealdehyde participates as intermediates in the formation of the final oxidation products.

Color reactions with Ehrlich's reagent and ferric chloride solution further indicate that the two most prominent oxidation products contain aromatic amino groups resulting from the oxidative cleavage of the indole nucleus. At the same time, the additional presence of a compound containing an indole ring is suggested by the Hopkins-Cole reaction.

While the behavior of OFA with the various color reagents employed is not identical with the behaviors of the actual oxidation products there are, at the same time, certain similarities and it is felt that at least some of these products may be close to OFA in structure. It is suggested that the neutral oxidation product of R_F 0.93 may be a hydroxy-o-aminoacetophenone and a reaction sequence is proposed which involved hydroxylation of the benzene ring, followed by oxidation of the pyrrol nucleus in a manner similar to the ring openings of tryptophane observed by Knox and Mehler[18] and Wiltshire[17].

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SECTION III

THE SYNTHESIS OF SOME α-ALKYL α-AMINO ACIDS AND THEIR DERIVATIVES

Introduction

In Section I of this thesis was described the working hypothesis that alpha-acylamino acid amides, of the general formula $R_1\ CHR_2\ R_3$, capable of interacting with the enzyme alpha-chymotrypsin as specific substrates or competitive inhibitors do so by virtue of individual interactions of the three main structural features (R_1, R_2, and R_3) of the molecule with three complimentary centers, ρ_1, ρ_2, and ρ_3, presumed to exist at the active enzymic site. Since both members of an enantiomorphic pair of such an amino acid derivative appear, in general, to combine with the enzyme at the same active site, the question arises as to what particular characteristics of those combinations confer upon the L isomer alone the behavior of a substrate. One possible explanation is that whereas both D and L isomers of an alpha-acylamino acid amide are capable of meeting the active site in a three-point attachment, the failure of the D isomer to undergo hydrolysis may result from its inability to attain an orientation in which the hydrolyzable bond of R_3 is in a favorable position with respect to a particular enzymic entity presumed responsible for hydrolysis of the L substrates. Certain lines of evidence described in Section I of this thesis have led us to believe, however, that both D and L isomers of a great number of alpha-acylamino acid amides are capable of combination with the active site of alpha-chymotrypsin by virtue of the same $R_1 - \rho_1$, $R_2 - \rho_2$, $R_3 - \rho_3$ interaction. If this is the case, then we are required to explain the selective hydrolysis of the L isomers knowing that the carbamido group of the D isomer may enter into the same $R_3 - \rho_3$ relationship. A consequence of

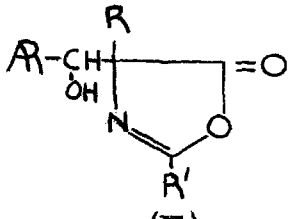
this hypothesis of similar modes of combination offers, however, a possible answer to the problem. Let us consider the R_1, R_2, and R_3 groups of an alpha-acylamino acid amide as situated at three of the vertices of a tetrahedron having the asymmetric alpha carbon at its center and the alpha-hydrogen atom at the remaining vertex. It is then clear that if both D and L isomers of such a derivative enter into similar modes of combination ($R_1 - \rho_1$, $R_2 - \rho_2$, $R_3 - \rho_3$), the alpha-hydrogen "vertex" of one isomer must be directed away from, and the other toward the enzymic site. Within the realm of this difference, therefore, we may seek the reason for the difference in behavior of the D and L isomers. Comparison of the enzyme-substrate and enzyme-inhibitor dissociation constants for a large number of enantiomorphic alpha-acylamino acid amides has shown that in every case studied, with but one exception, the D isomer of an enantiomorphic substrate-inhibitor pair is bound more tightly to the enzyme in its EI complex than is the L isomer in ES. From this, it is reasonable to assume that if both isomers of such a pair undergo similar modes of combination with the enzyme then the alpha hydrogen "vertex" is likely directed away from the enzymic site in the interaction of the D isomer (EI) and toward the site (impeding the union) in the case of the L isomer. The susceptibility to hydrolysis of the L isomers could then result from the introduction of strain into the amide linkage by the impeding orientation of the alpha-hydrogen. In the light of this interpretation the alpha-hydrogen atom of an L substrate plays a curious and vital role in supplying an amount of steric strain sufficient to provoke the

hydrolytic reaction without preventing the ES union altogether. If this interpretation is correct it would seem likely that replacement of the alpha-hydrogen of an L specific substrate by a bulkier group would render it incapable of forming an ES complex and hence preclude its subsequent hydrolysis. Such a "deformed" substrate molecule might, however, be able to function as a bifunctional competitive inhibitor making use of the strong $R_2 - \rho_2$ interaction and the remaining R group properly oriented. On the other hand, substitution of a bulky group for the alpha-hydrogen of a D inhibitor might not be expected to change, significantly, its K_I value from that of the original compound.

It was the purpose of the present investigation to test the adequacy of the above hypothesis by studying the behavior with chymotrypsin of several D and L alpha-acylamino acid amides, substituted in the alpha position by bulky alkyl groups. Specifically, it was decided to prepare the alpha-methyl and alpha-isopropyl derivatives of DL tyrosine, DL phenylalanine, and DL tryptophane, resolve these into their D and L isomers, and then to determine the enzymic interaction properties of these isomers (in the form of their acyl amides) by the methods of enzyme kinetics.

Synthetic Methods

Relatively little attention has been given, in the past, to the synthesis of alpha-alkyl alpha-amino acids. This is due in part, of course, to the greater importance of and interest in the naturally occurring amino acids having hydrogen in the alpha position which has stimulated the work of synthetic chemists along this line. To a lesser degree it is due, undoubtedly, to the somewhat greater difficulties attending the syntheses of the alkyl acids, particularly when the substituent alkyl group is larger than methyl. Not all of the standard methods of synthesis of amino acids containing hydrogen in the alpha position are applicable to the preparation of their alpha-alkyl homologues. Thus, the malonic ester type syntheses are ruled out due to the fact that alpha-hydrogen is automatically introduced during the final step of decarboxylation. The aldehyde condensation syntheses, including the hydantoin, diketopiperazine, and Erlenmeyer azlactone syntheses, all depend upon a Perkin type condensation at an active methylene group followed by elimination of water to give a stabile unsaturated conjugated intermediate which is then reduced and hydrolyzed to the amino acid. The use of an active methylene component substituted at the alpha position by alkyl would necessarily lead to a beta-hydroxy compound, illustrated by the azlactone which would be incapable of



(I)

losing water to give the unsaturated intermediate. Apparently, little is known concerning the possibility of preparing such compounds as (I) by this method and converting them into alpha-alkyl alpha-amino acids; a recent review[a] on the subject of azlactone chemistry mentions no such attempts.

The synthetic approaches to these alpha-alkyl compounds which have proved most successful are of two general types. Of these, the Strecker reaction and a modification of it in which the intermediate amino nitrile is converted into a 5,5-dialkyl hydantoin prior to the final hydrolytic step have been most widely employed. Thus, Herbst and Johnson[(1)] obtained a DL alpha-methylphenylalanine by vigorous hydrolysis[b] of 5-methyl-5'-benzylhydantoin. Their hydantoin was obtained by treating the amino nitrile hydrochloride of benzyl methyl ketone with potassium cyanate followed by a mild acid treatment to close the ring. Examples of the use of the conventional Strecker reaction include the conversion of the propiophenone into DL-alpha-ethylphenylalanine by treatment with ammonium cyanide and hydrolysis of the amino nitrile[(2)] and a similar synthesis of DL-alpha-phenylalanine from acetophenone[(2)]. Henze and Speer[(3)] have successfully converted a large number of ketones, including highly hindered ones, into the corresponding 5,5' dialkylhydantoins by treating the ketones in alcoholic solution with sodium cyanide and ammonium carbonate according to the method of Bucherer and Lieb[(4)]. All of these hydantoins are

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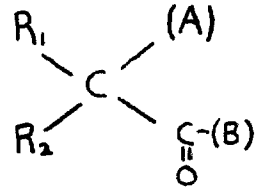
[a] Carter, H.E., Org. Reactions III, 198 (1947).

[b] By heating with aqueous barium hydroxide under reflux for thiry hours.

potential sources of their corresponding alpha-alkyl alpha-amino acids.

A second group of synthetic methods for alpha-alkyl alpha-amino acids may be considered as a class of molecular rearrangements involving electronically deficient nitrogen atoms. Thus the Curtius rearrangement of acid azides, the Schmidt reaction upon ketones, the Hoffman degradation of amides, and the Lossen rearrangement of hydroxamic acid derivatives are all potential methods of replacing the carbonyl function of (II) by an amino group to give the alpha-amino acid skeleton.



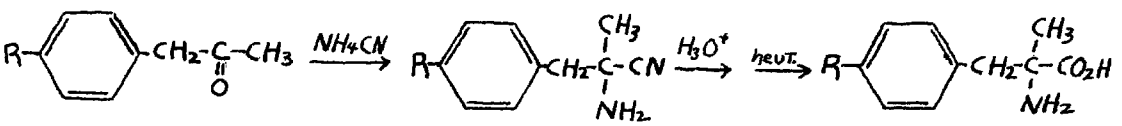
(II)

In (II), R_1 and R_2 are the potential alpha-amino acid sidechain and alpha-alkyl group respectively, $-\overset{O}{\overset{\|}{C}}-(B)$ represents the carbonyl function ($-\overset{O}{\overset{\|}{C}}-N_3$, $-\overset{O}{\overset{\|}{C}}-CH_3$, $-\overset{O}{\overset{\|}{C}}-NH_2$, and $-\overset{O}{\overset{\|}{C}}-NH-O-COR$ for the Curtius, Schmidt, Hoffman, and Lossen rearrangements, respectively) to be replaced by amino, and (A) is a hydrolyzable carboxyl function ($-C\equiv N$, , $-CO_2R$ etc.) which is inert toward the conditions of the particular method employed and may be hydrolyzed to the amino acid carboxyl group following the rearrangement. In each of these methods the amino acid side chain and alpha-alkyl substituent are initially introduced, generally as the halide, into ethyl acetoacetate or ethyl cyanoacetate. Lin, Li, and Huang[5] have reported the

syntheses of several alpha-alkyl alpha-amino acids, employing the Hoffman rearrangement of the appropriately substituted half-amides of malonic ester. The amides were obtained by the partial hydrolysis of the corresponding dialkyl cyanoacetic esters with concentrated sulfuric acid at 25-100°.

In the present investigation, both of the above mentioned classes of synthetic methods were explored as possible approaches to the desired alpha-alkyl alpha-amino acids. In the following description of the synthetic procedures, the methods employed for the various amino acids will be outlined, reserving the more detailed accounts of the important reactions for the experimental section. Also included are several interesting and rather anomalous reactions which complicated the experimental work and are considered worthy of mention.

The Strecker reaction (Equation (1)) proved to be a practical route to the alpha-methyl derivatives of DL-phenylalanine, and DL-tyrosine. The appropriate methyl ketone, in ethanol-water solution, was treated with sodium cyanide, ammonium chloride, and ammonium hydroxide in a pressure bottle at 40-50° for a two hour period. Following acid hydrolysis and neutralization, the crude amino acid was obtained in good yield.

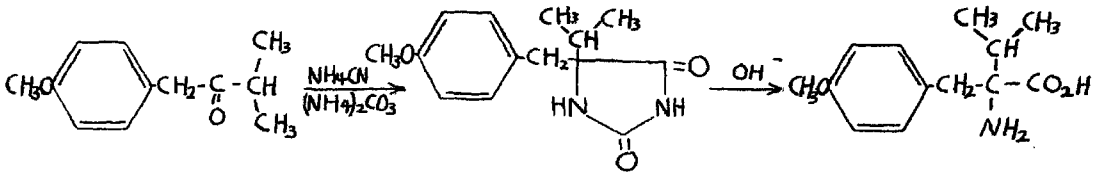
(1) 

R = H, CH_3O

In the case of the tyrosine derivative, cleavage of the nuclear methoxyl was accomplished with 30 per cent solution of hydrobromic acid

in glacial acetic acid and introduced no particular difficulties.

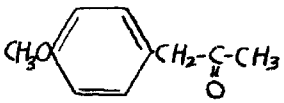
An attempt to convert p-methoxybenzyl isopropyl ketone into DL-O-methyl-alpha-isopropyltyrosine by the Strecker reaction failed, the ketone being recovered, apparently unchanged, at the end of a ten hour reaction period. In the case of this more sterically hindered ketone, however, it was found possible to convert it to 5-isopropyl-5'-p-methoxy benzylhydantoin, in 74 per cent yield, by the method of Henze and Speer.[3] Here the formation of the stable 5 membered ring overcomes the steric resistance of the ketone and drives the first step of hydantoin formation to completion.

(2) 

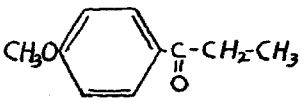
The hydantoin thus obtained was heated with aqueous barium hydroxide under refluxing conditions in an attempt to convert it to DL-O-methyl-alpha-isopropyltyrosine. Only the unreacted hydantoin was isolated at the end of this treatment despite an attempt to recover the amino acid by acetylation.

During the present work, considerable attention was given to the preparation of p-methoxybenzyl methyl ketone. Numerous accounts of the synthesis of this ketone are present in the early literature, most of which involve anethole as the starting material. p-Methoxybenzyl methyl ketone has reportedly been prepared by the dehydration of the isomeric glycols of anethole with 20 per cent sulfuric acid[6,7] and with zinc chloride,[6,8] and by treatment of the epoxide of anethole with heat or acid.[9] The epoxide of anethole is isomeric with p-methoxybenzyl methyl ketone and may be converted into it by a proton transfer

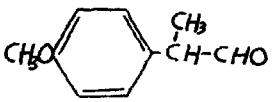
mechanism. This method of preparing the ketone has been employed as recently as 1938 by Auwers, Pötz, and Noll.[10] While evidence indicating the formation of p-methoxybenzyl methyl ketone (III) from the glycols of anethole has been adduced by at least one of the early workers,[a] somewhat similar syntheses beginning with anethole have been described as leading to the formation of the isomeric compounds p-methoxypropiophenone (IV) and p-methoxyhydratropaldehyde (V).



(III)



(IV)



(V)

Specifically, the preparation of p-methoxypropionphenone (IV) has been reported by the treatment of anethole dibromide with alcoholic potassium hydroxide[11] and by treatment with sodium methoxide followed by hydrolysis[12]. In this latter instance Wallach and Pond[12] supported their conclusion as to structure by performing a permanganate oxidation and identifying, among the oxidation products, both propionic acid and p-methoxyphenylglyoxalic acid.[b] While a quantitative study of the oxidation products was not made and it might be argued that the presence

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[a] Tiffeneau and Daufresne[7] reported the conversion of their product by hypiodite into iodoform and homoanisic acid, in agreement with the structure of p-methoxybenzyl methyl ketone.

[b] Each of these products could arise only from p-methoxypropiophenone and their empirical formulae were confirmed by analyses.

of p-methoxypropiophenone in a mixture of products could have accounted for their oxidation results, some indication of purity of their product was given by its melting point of 26-27°. In Table I is a list of the reported melting points of the various solid derivatives of the four isomeric p-methoxyphenyl-oxo-propanes. It is evident that on the basis of the oxímes and semicarbazones (the only solid derivatives reported for both compounds prior to the present communication) alone, p-methoxy benzyl methyl ketone cannot be distinguished from p-methoxy-propiophenone.[a]

p-Methoxyhydratropaldehyde has been reportedly prepared by treatment of anethole dibromide with aqueous sodium hydroxide followed by treatment of the resulting bromohydrin with hot alcoholic potassium hydroxide[13], by boiling the dibromide with an acetic acid solution of zinc acetate[7], by heating the iodohydrin of anethole with aqueous potassium hydroxide[14], and by oxidizing anethole with a mixture of iodine and mercuric oxide.[15] Table I indicates that the melting points of its known derivatives differ sufficiently from those of p-methoxybenzyl methyl ketone to prevent confusion of the two isomers. The same may be said for p-methoxyhydrocinnamaldehyde; moreover, neither of these compounds would be capable of the haloform reaction indicative of a methyl ketone.

An interesting synthesis of p-methoxybenzyl methyl ketone has been reported by Wallach and Muller.[16] Anethole was treated with nitrous acid and the resulting intermediates converted into

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[a] Unless, of course, samples of each were available for mixed melting point determination of identity or non-identity.

TABLE I

Melting Points of The Solid Derivatives of The Isomericp-Methoxyphenyl-oxo-propanes.

Compound	m.p. Oxime	m.p. Semicarbazone	m.p. 2,4-DNP
p-methoxypropio--phenone	74° a	173-4° b 172-3° a	192-3° b
p-methoxybenzyl methyl ketone	74° c 78-9° d 61-2° d 56-61° g 77.5°-78.0° h	175° c 175° d 175-6° e 182° f	107.8°-108.2° i 108.6°-109.5° j
p-methoxyhydra-tropaldehyde	96° k	135° e 207-8° e	
p-methoxyhydro-cinnamaldehyde	175-6° e	184° l	

a Wallach and Pond.(12)

b Auwers, Pötz, and Noll.(10)

c Tiffeneau and Daufresne.(7)

d Wallach and Muller.(16)

e Balbiano and Paolini.(8)

f Béhal and Tiffeneau.(14)

g Hoover and Hass.(17)

h Winstein.(18)

i Fert, to be published.

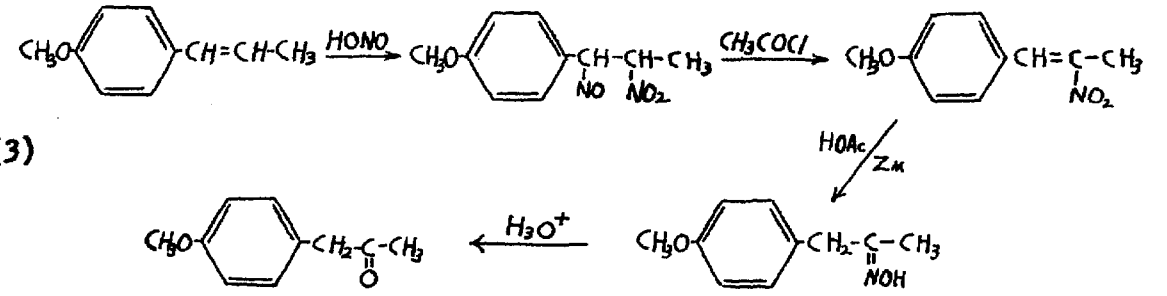
j Manning, This Thesis.

k Bougault.(19)

l Forneau and Tiffeneau.(20)

1-(p-methoxyphenyl)-2-nitro-1-propene by treatment with acetyl chloride.

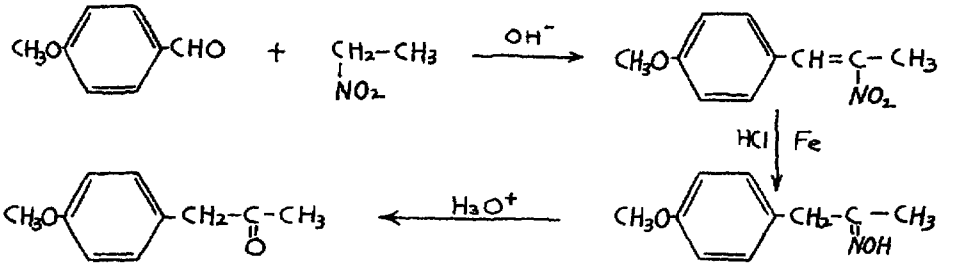
(3)



The unsaturated nitro compound, obtained in 90 per cent yield, was reduced to the oxime which was then hydrolyzed to the ketone. Wallach and Muller concluded that this ketone was p-methoxybenzyl methyl ketone rather than p-methoxypropiophenone on the basis of a hypobromite oxidation which yielded bromoform, anisic acid, and homoanisic acid.

Further support of the conclusion of Wallach and Muller has been given by Hoover and Hass[17] in their synthesis of p-methoxybenzyl methyl ketone from anisaldehyde and nitroethane. Here, the 1-(p-methoxyphenyl)-2-nitro-1-propene obtained from the initial condensation, melting at 43-44°, appears to be identical with the unsaturated nitro product of Wallach and Muller.

(4)



The most recently reported synthesis of p-methoxybenzyl methyl ketone is that of Winstein et al.[18] Anethole was treated with N-bromosuccinimide in aqueous solution and the resulting product, presumably the bromohydrin, converted to the epoxide by treatment with

sodium hydroxide. This was then rearranged to the ketone by boiling with a solution of p-toluenesulfonic acid. While this synthesis may possibly be criticized with regard to the uncertainty in the manner of ring-opening in the final step, the resulting ketone has been shown to give the bromoform reaction, to give a solid compound with sodium bisulfite, and possesses the IR absorption maximum at 5.83 microns characteristic[21] of aliphatic ketones. Moreover, the possible presence of p-methoxyhydratropaldehyde as a side product was legislated against by the failure of any part of the product to react with dimethone.[a]

That p-methoxybenzyl methyl ketone and not p-methoxypropiophenone[b] results from all syntheses involving the acid catalyzed rearrangement of anethole oxide has been further indicated by an independent synthesis of p-methoxypropiophenone by Auwers, Pötz, and Noll[10]. Phenyl propionate was converted into p-hydroxypropiophenone by the Fries rearrangement with boron trifluoride and the hydroxy ketone methylated to give the methoxy product. The melting point of their 2,4-dinitrophenylhydrazone[c] 192-193°, is in contrast with the value of 107.8-108.2[d] for the 2,4-dinitrophenylhydrazone of the ketone prepared by Winstein's method.

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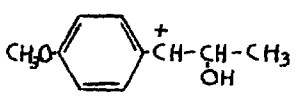
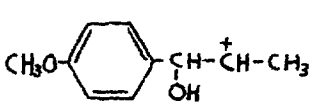
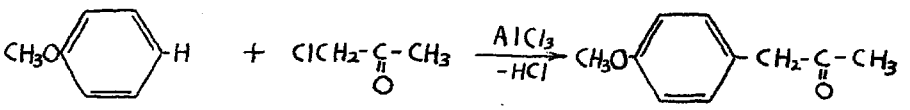
[a] Fort, A., and Roberts, J.D. to be published.

[b] A consideration of the relative energies of the two transition states  and  would predict formation of the methyl ketone.

[c] Empirical formula determined by analysis.[10]

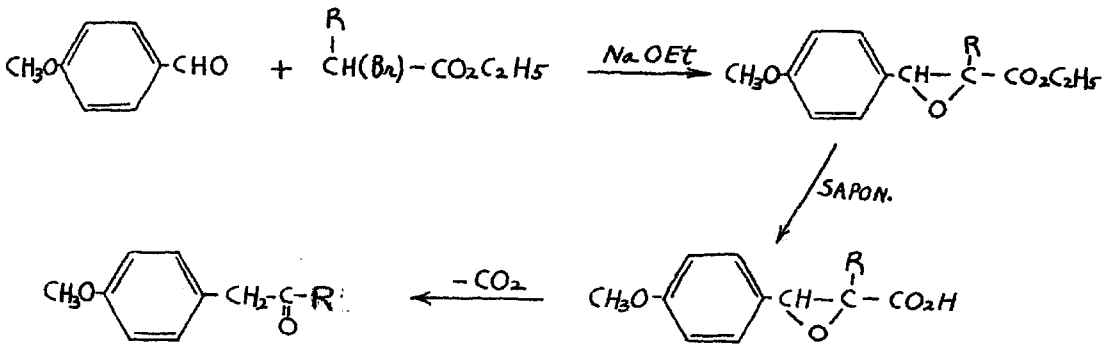
[d] Fort, A., and Roberts, J.D. to be published.

In the present investigation, several methods for the synthesis of p-methoxybenzyl methyl ketone were studied. A report by Mason and Terry[22] of the condensation of chloroacetone with benzene in the presence of anhydrous aluminum chloride to give benzyl methyl ketone suggested the possibility of replacing the benzene with anisole to obtain the methoxy ketone.

(5) 

This condensation was attempted employing both aluminum chloride and boron trifluoride as catalysts, with various solvent systems, and at temperatures from 0-100°C. In all attempts an intractable resinous product resulted.

Of the ketone syntheses attempted, the Darzens glycidic ester method proved the most successful and was used to prepare p-methoxybenzyl methyl ketone and p-methoxybenzyl isopropyl ketone according to the following reaction sequence.

(6) 

R = CH_3, iso-C_3H_7

Darzens[23] has reported the preparation of p-methoxybenzyl methyl ketone by this method, and, although this report does not contain the properties of the ketone's derivatives, the ketone prepared in the present study using this method was found to be identical[a,b] with the ketone obtained by the method of Winstein[18] and identified as p-methoxybenzyl methyl ketone.

The Grignard synthesis was investigated as a possible route to some of the ketones, the intention being to react a Grignard reagent of the desired alpha-alkyl group (methyl or isopropyl) with the nitrile of the amino acid side chain radical.

(7) $Ar-CH_2-CN + R-Mg-X \longrightarrow[H_3O^+] Ar-CH_2-\underset{\underset{O}{\|}}{C}-R$

$R = CH_3$, $iso\text{-}C_3H_7$

Ramart-Lucas[24] has reported the preparation, in 60 per cent yield, of benzyl methyl ketone from phenylacetonitrile and methyl magnesium bromide.

When phenylacetonitrile was reacted with a 20 per cent excess of isopropylmagnesiumbromide and the reaction mixture treated with aqueous acid, however, benzyl isopropyl ketone was not obtained. Instead, a syrupy high boiling mixture resulted from which, on prolonged standing, a substance crystallized out in compact lemon-yellow prisms, m.p. 179-181.2°. This substance is the trimer of phenylacetonitrile and was obtained by Rondou[25] by the action of methylmagnesium bromide on the monomer as a yellow crystalline material melting at 178.5-179°.

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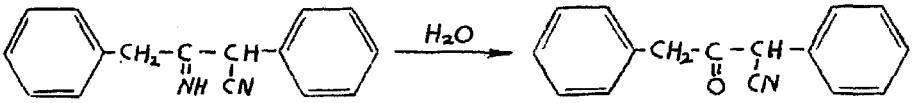
a See experimental section for details.

b I am indebted to A. Fort for a sample of p-methoxybenzyl methyl ketone prepared by the Winstein method.

Rondou[25] also isolated the nitrile dimer, m.p. 114.5-115°, which had previously been obtained by von Meyer[26] from the action of sodium metal on phenylacetonitrile. By treating the dimer with hydroxylamine under aqueous conditions, von Meyer[26] and Rondou[25] obtained an oxime, m.p. 107°, apparently resulting from the oximation of benzyl alpha-cyanobenzyl ketone (VII), the hydrolysis product of the imine dimer (VI).

(8) 

While the crystalline dimer (VI) was not isolated in the present study, treatment of the undistillable residue with hydroxylamine hydrochloride gave the oxime, m.p. 107.2-108.9°.

A further example of the polymerization of nitriles under the conditions of the Grignard reaction has been given by Baerts[27]. When propionitrile was treated with ethylmagnesium bromide, diethyl ketone was obtained in low yield accompanied by the dimer and trimer of propionitrile.

Ivanov and Paounoff[28] have demonstrated the ability of organomagnesium halides to react with an active alpha-methylene hydrogen of phenylacetonitrile producing a new organomagnesium compound.

(9) 

R= C_2H_5, iso-C_3H_7, ⊖

When the nitrile was reacted with a 25 per cent excess of the Grignard reagent and the resulting reaction mixture treated with carbon dioxide, phenyl cyanoacetic acid was obtained in 40 per cent yield.

(10) 

In the absence of added electrophilic reagents, the primarily formed cyano Grignard reagent may react with the cyano group of the nitrile itself to form the imino dimer. This condensation may be considered as an example of the Thorp reaction in which the aldol type dimerization of nitriles is prompted by, in addition to organometallic compounds, sodium, sodium alkoxides, and sodium and lithium amide.(29)

While the tendency of nitriles to undergo polymerization in the Grignard reaction may be decreased by the addition of a secondary reactant such as carbon dioxide(28) it may also be controlled by the presence of a large excess of the organomagnesium halide. Under these conditions, nitriles containing no extremely active methylene groups may react with the Grignard reagent at the cyano group to give a ketimine complex, convertible to the ketone by hydrolysis. Thus, whereas acetonitrile has a rather great tendency to undergo the polymerization[a], it, and a number of higher aliphatic nitriles, have been successfully converted into the corresponding phenyl alkyl ketones by treatment with a 300 per cent[b] excess of phenylmagnesium bromide.(30)

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[a] Presumably due to the steric freedom at the methyl carbon.

[b] Higher excesses of Grignard reagent lowered the yields of ketone due to formation of the tertiary carbinol.(30)

During the present study, several attempts were made to prepare p-methoxybenzyl methyl ketone by reacting homoanisonitrile with a 300 per cent excess of methylmagnesium iodide. Instead of the expected product, a ketonic substance was obtained which resembled p-methoxybenzyl methyl ketone in some respects but was clearly a different compound as indicated by the melting points of its derivatives and by its failure to react with aqueous ethanolic sodium bisulfite. In Table II is given a summary of its properties, including the melting points of its solid derivatives, compared with those of p-methoxybenzyl methyl ketone. A comparison of the IR spectra of the two substances was made and it was found that while both were essentially identical in the region below ca. 9 microns, including the aliphatic carbonyl peak at 5.83 microns, there were certain dissimilarities at the higher wavelengths.[a] The information presented in Table I and Table II indicates that the carbonyl compound arising from the Grignard synthesis cannot be any of the isomeric p-methoxyphenyl-oxo-propanes.

There is strong, if not conclusive, evidence favoring the identification of the principal carbonyl compound[b] resulting from the Grignard synthesis as 3-(p-methoxyphenyl)-2-butanone (VIII). This ketone could be formed during the Grignard synthesis according to the following sequences of reactions.

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[a] Of course, this might be attributable to impurities.

[b] The product as a whole appears to be quite impure as judged by the difficulty in obtaining even approximately pure derivatives.

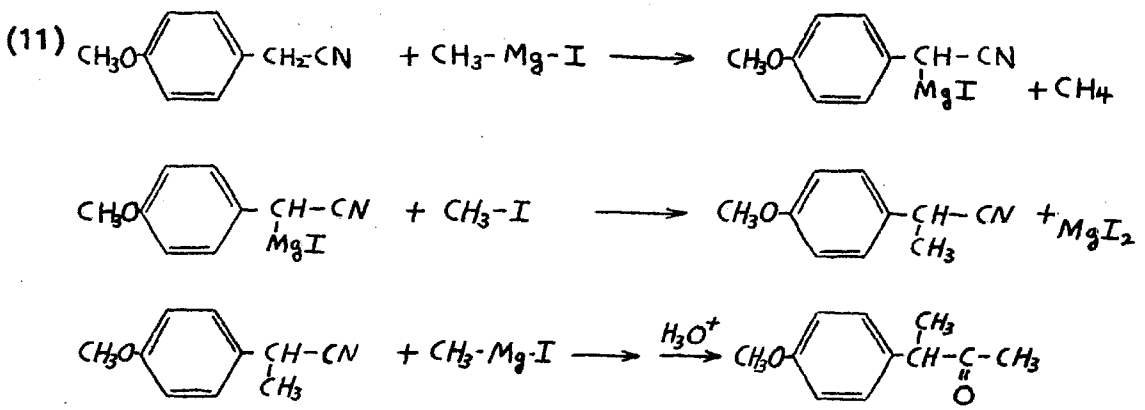
TABLE II

Property or Derivative	Ketone from Grignard	p-Methoxybenzyl methyl ketone
b.p.	95-100.5°/650-800 88-92° /450-500	101-104°/1500 [a] 88-93° /500 [a]
n_D	1.5176^{24}, 1.5185^{24}	$1.5223^{25.6}$, 1.5239^{23} $1.5236^{23.5}$ [a]
oxime m.p.	118-119.2°	78-79° [b] 61-62° for 2 forms
2,4-DNP m.p.	135.5-136.8°	108.6-109.5° [a] 107.8-108.2° [c]
semicarbazone m.p.	172.8-174.3°	175° [b]
reaction with bisulfite	negative	immediate solid formation
IR spectrum	max. at 5.83 microns	max. at 5.83 microns
reaction with dimethone	negative	negative
iodoform reaction	positive	positive[a,c]

[a] Darzens synthesis.

[b] Wallach and Muller.(16)

[c] Winstein synthesis.

(11) 

In view of the known tendencies of benzyl type nitriles to undergo the replacement of hydrogen at the active methylene carbon, and considering the present experimental conditions employed, 3-(p-methoxyphenyl)-2-butanone (VIII) indeed would seem to be the expected product. Thus, in the reaction mixtures, the components; nitrile, methylmagnesium iodide, and methyl iodide were present in the approximate molar proportions of 1:4:1, the excess of methyl iodide over magnesium metal (Ca. 5:4) being sufficient to convert the cyano magnesium halide formed in the first step into the alpha-methyl nitrile (coupling reaction).

3-(p-methoxyphenyl)-2-butanone is a known compound, having been prepared by Sosa[15] by an independent method. From Table III, in which the known properties and derivatives of Sosa's ketone are compared with those of the present Grignard product, it is evident that there is considerable similarity between the melting points of the oximes[a] and semicarbazones. The observation that 3-(p-methoxyphenyl)-2-butanone does not give a bisulfite compound (in agreement with the behavior of the Grignard product) is interesting and appears to reflect the increased steric hindrance introduced by the additional methyl group. In

---

[a] The lower m.p. of the presently obtained oxime may be due to the presence of other ketone oximes.

TABLE III

Property or Derivative	Ketone from Grignard	3-(p-methoxyphenyl)-2-butanone
η_D	1.5176^{24}	1.5287^{20}
oxime m.p.	118-119.2°	122-123° (125°)
semicarbazone m.p.	172.8-174.3°	170°
reaction with bisulfite	negative	negative

comparative, small scale (4 drops of ketone) iodoform tests, the product from the Grignard synthesis yielded much more iodoform than did p-methoxybenzyl methyl ketone. This would appear to be in agreement with the structure of 3-(p-methoxyphenyl)-2-butanone in which the methyl group upon the methylene carbon would block the iodination at this position with the concomitant unproductive (of iodoform) cleavage which occurs in the case of p-methoxybenzyl methyl ketone[a]. Further support for the identification as VIII of the Grignard product was given by analyses performed upon samples of its 2,4-dinitrophenylhydrazone. The analytical data is in fair agreement with that calculated for the butanone derivative (see experimental section). The data for the oxime, however, does not seem to correspond to that of any likely product and may result from the presence of impurities.

An attempted Strecker reaction upon a sample of the Grignard carbonyl product resulted in a very low yield of an amino acid which was isolated and purified as the acetyl derivative (experimental section). Although this acetyl derivative was found to possess analytical data corresponding to N-acetyl-O-methyl-alpha-methyltyrosine, its melting point (222-224°) is considerably higher than that of the alpha-methyltyrosine derivative (178-181°) and does not seem to represent a mere difference in crystalline modification. At the same time, the failure of the unknown acetyl derivative to give the ninhydrin test is some cause for believing that it is an alpha-alkyl amino acid.

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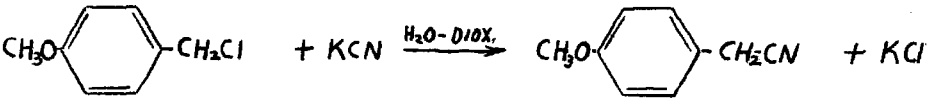
[a] That this occurs in the case of p-methoxybenzyl methyl ketone was shown by Wallach and Muller[16] who identified anisic acid as one of the reaction products.

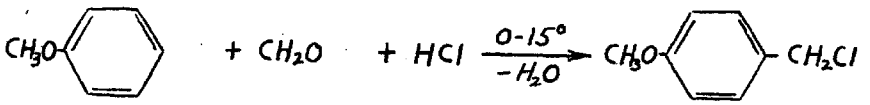
It seems likely that this amono acid results from some minor ketonic constituent in the Grignard product, especially in view of the low yield obtained.

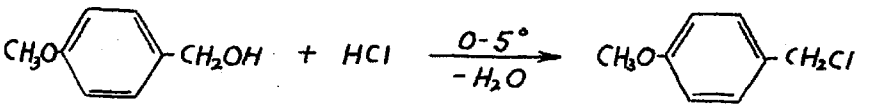
The homoanisonitrile, employed in the Grignard reaction, was best prepared by a method similar to that of Shriner and Hull,[31] reacting p-methoxybenzyl chloride with cyanide ion in boiling aqueous dioxane.

(12) 

When the dioxane-water solvent was replaced with aqueous ethanol, p-methoxybenzyl ethyl ether was formed as a major side-product. The yields of this ether were found to increase (at the expense of the nitrile) as the ratio of ethanol to cyanide ion was increased (see experimental section). In as much as aqueous ethanolic media have been successfully employed[32] for the conversion of benzyl chloride to benzyl cyanide in high yields this occurrence in the case of the p-methoxy derivative is somewhat surprising. A probable explanation lies in the enhanced reactivity (in either or both the S_{N_1} and S_{N_2} sense) of the chloride due to p-methoxy substitution with a resultant levelling effect upon its selectivity toward reaction with various nucleophilic agents.

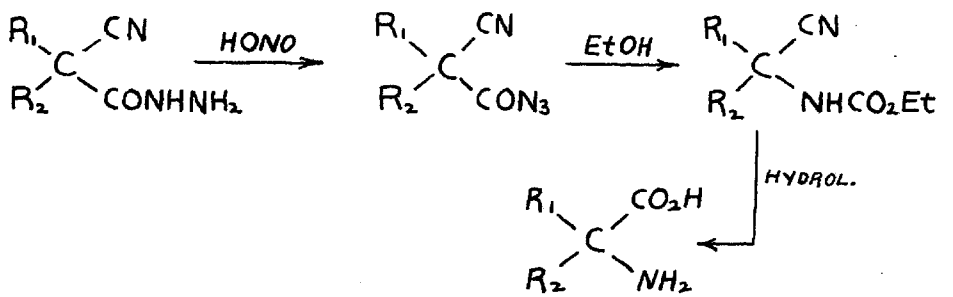
Two methods were employed in the preparation of p-methoxybenzyl chloride.

(13A) 

(13B) 

Of these methods, (13B) proved more satisfactory from the standpoint of yield than the chloromethylation procedure (13A) and since the quantitative oxidation of the anisaldehyde, used to prepare the alcohol, to anisic acid (see experimental section) left no doubt as to its isomeric purity method (13B) was used exclusively for the preparation of the p-methoxybenzyl chloride used in the syntheses of the presently described amino acid derivatives.

Preliminary investigations were made concerning the possibility of preparing the alpha-alkyl alpha-amino acids via the Curtius rearrangement of dialkylcyanoacethydrazides.

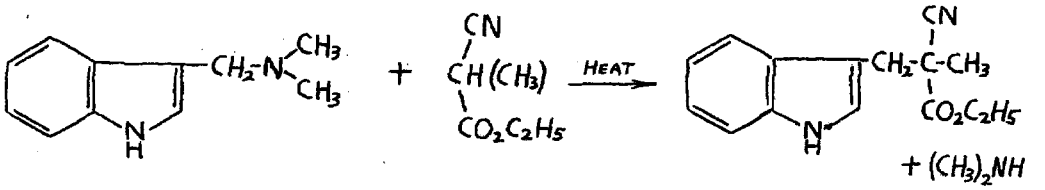
(14) 

While this method has been successfully employed[33] in the preparation of a number of amino acid derivatives unsubstituted at the alpha-carbon, there is no report of this reaction having been performed upon dialkylcyanoacethydrazides. In the present study attempts were made to convert isopropyl-p-methoxybenzylcyanoacethydrazide and methyl-3-indolylmethylcyanoacethydrazide into their respective amino acid

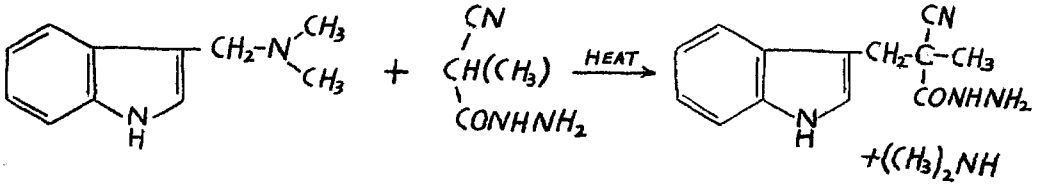
derivatives. In each case the reaction products appeared unpromising and the attempts were not pursued further. Nevertheless, it is felt that these two failures are insufficient to condemn this method and that a further study of the Curtius reaction as applied to these compounds would likely reveal more suitable experimental conditions.

Outside of the difficulties encountered in the Curtius rearrangement, a second problem exists in obtaining the dialkylcyanoacethydrazides themselves. Thus, while ethyl methylcyanoacetate was found to react readily enough with hydrazine, and Gagnon, Gaudry, and King[33] have reported a similar reactivity for the isopropyl derivative, the substitution of cyanoacetic ester by pairs of bulky groups (such as benzyl and isopropyl) brings about great resistance toward hydrazinolysis of the ester group. After eight days of treatment with a large excess of anhydrous hydrazine at 120-170°, ethyl isopropyl-p-methoxybenzylcyanoacetate underwent only 18.4 per cent conversion to the hydrazide.

The synthesis of alpha-alkyl alpha-amino acids via the dialkylcyanoacethydrazides appears to have great potential possibilities for the preparation of the alpha-alkyl tryptophanes. When, in the present study, an equimolar mixture of gramine and ethyl methylcyanoacetate was heated to 130-150° condensation occurred with the liberation of an amount of dimethylamine equivalent to a 61.6 per cent conversion of the gramine.

(15) 

When the difficulty of reacting hydrazine with highly hindered dialkylcyanoacetic ester derivatives became evident, the possibility of introducing the 3-indolylmethyl group into preformed monalkyl hydrazides was investigated. It was found that gramine and methylcyanoacethydrazide will also undergo condensation, upon fusion, liberating dimethylamine and forming the crude disubstituted hydrazide in high yield.

(16) 

Conclusions Regarding the Synthetic Methods

The synthetic studies described in the present paper enable certain conclusions to be drawn regarding the methods of preparation of the alpha-alkyl alpha-amino acids.

While the Strecker reaction per se is of limited scope in the preparation of these compounds, modification of the Strecker reaction by the addition of ammonium carbonate to form the 5,5'-dialkylhydantoin should greatly increase its synthetic capabilities. Thus, the application of the Strecker reaction is limited to ketones which are unbranched in the two positions adjacent to the keto group. Moreover, the remainder of the molecule must not be sensitive to the vigorous acid treatment used to hydrolyze the amino nitrile. More sterically hindered ketones

may be converted into their corresponding alpha-alkyl alpha-amino acids by preliminary formation of the 5,5'-dialkylhydantoins followed by hydrolysis.[a] In this case treatment with acid is avoided, the hydrolytic cleavage being effected with aqueous barium hydroxide.

The Curtius rearrangement remains an unproved but promising possibility for the synthesis of alpha-alkyl alpha-amino acids. While it is difficult to prepare the hydrazides of the highly hindered dialkyl derivatives of cyanoacetic ester it may be possible to improve the yields and convenience by carrying out the hydrazinolyses in sealed tubes at temperatures of ca. 130-160°.

The syntheses of the alpha-alkyl derivatives of DL-tryptophane involve special problems peculiar to the 3-indolylmethyl group. At the same time the properties of this group in the form of the Mannich-type base gramine open new synthetic possibilities. Gramine may be easily condensed with monoalkylcyanoacethydrazides, thus avoiding the problem of reacting hydrazine with a highly hindered disubstituted derivative. It seems possible that other active methylenic compounds will be found capable of condensing with gramine to give intermediates which can then be converted to the DL-alpha-alkyltryptophanes by methods superior to the Curtius rearrangement. It would be of interest to attempt the condensation of gramine with certain 4-alkyl oxazolones to give 4,4' disubstituted oxazolones convertible to the alpha-alkyl

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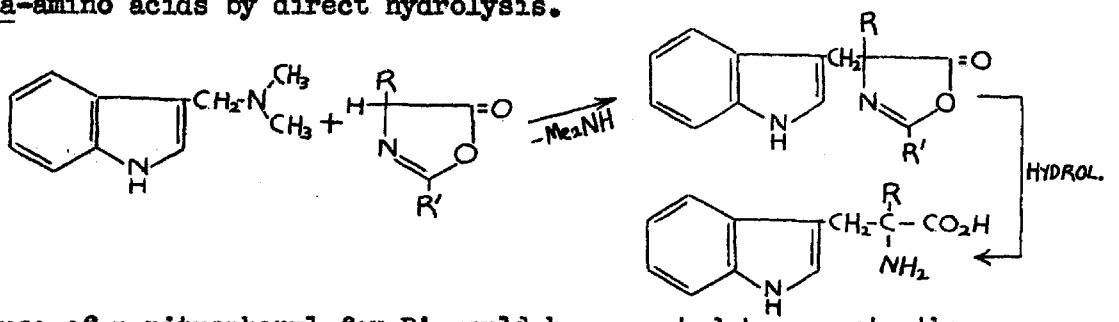
[a] No amino acid was isolated, however, in the present attempt to hydrolyze 5-isopropyl-5'-(P-methoxybenzyl)-hydantoin (pages 118, 161-2). Nevertheless, it may be possible to obtain better results by conducting the hydrolysis in a sealed vessel at higher temperature.

alpha-amino acids by direct hydrolysis.[a]

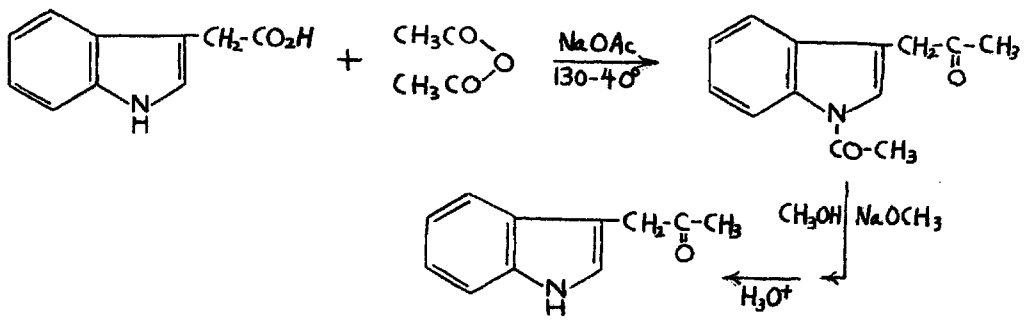
(17)



The use of p-nitrophenyl for R' would be expected to promote the condensation by increasing the lability of the active hydrogen atom.

An additional possibility for the preparation of the DL-alpha-alkyltryptophanes is, of course, by way of the Strecker reaction upon the appropriate 3-indolylmethyl alkyl ketones. These ketones may be prepared by the decarboxylative acylation of 3-indoleacetic acid with acid anhydrides in the presence of sodium acetate or pyridine. Brown, Henbest, and Jones[34] have prepared 3-indolylacetone by the prolonged action of acetic anhydride and fused sodium acetate upon 3-indoleacetic acid at 135-140°. The resulting N-acetyl-3-indolylacetone was saponified by treatment with methanolic sodium methoxide followed by aqueous acid.[b]

(18)



While there may be some question regarding the ability of the indole nucleus to withstand the acidic treatment involved in the Strecker

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[a] Proposition, This Thesis.

[b] A 24.6 per cent yield of 3-indolylacetone was obtained from 2.0 g. of 3-indoleacetic acid.[34]

reaction, it should be possible to employ the hydantoin modification with success. This modification will, of course, be the necessary route to the more sterically hindered 3-indolylmethyl alkyl ketones.

Attempted Resolutions of The DL-alpha-Alkyl alpha-amino Acid Derivatives.

Several enzymatic methods were employed in the attempted resolutions of the various derivatives of DL-alpha-methylphenylalanine and DL-alpha-methyltyrosine. Asymmetric hydrolyses attempted were: (1) N-acetyl-DL-O-methyl-alpha-methyltyrosine methyl ester by chymotrypsin in aqueous methanolic and in water solutions, (2) O-methyl-DL-alpha-methyltyrosine methyl ester by "Viobin" in an aqueous paste, and (3) N-acetyl-DL-alpha-methylphenylalanine methyl ester by chymotrypsin in aqueous methanol and in water. Also attempted was the papain catalyzed asymmetric synthesis of N-acetyl-L-alpha-methyltyrosine-p-toluidide. In only one instance, namely the incubation of N-acetyl-DL-alpha-methylphenylalanine methyl ester with an approximately equal weight of chymotrypsin in water solution, was there evidence of a small amount of ester hydrolysis. This evidence suggests that, at best, the enzymatic resolution of these compounds would be a very slow process requiring prohibitively costly amounts of chymotrypsin to resolve gram scale quantities of the alkyl amino acids.[a] A complication seems to exist, moreover, in the use of high concentrations of enzyme, namely the autolysis of the enzyme over long reaction periods with consequent contamination of the desired products by the products resulting from the breakdown of the enzyme.

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[a] This requires the assumption, not too well justified, that only the L-alpha-alkyl esters are split in the process.

The failure to effect enzymic resolutions of the alpha-alkyl esters led to the consideration of other separation methods. An attempt was made to fractionally crystallize the diastereomeric L-brucine salts of D and L N-acetyl-O-methyl-alpha-methyltyrosine, without success. Nevertheless, it is felt that future attempts to resolve the enantiomorphic alpha-alkyl alpha-amino acids should be directed along the lines of fractional crystallization of such diastereomeric mixtures. Particularly promising is the recent success of Ingersoll et al.[35] in resolving a number of acetylamino acids by the use of alpha-fenchylamine, and it would be worth while to attempt the application of this method to the present problem.

The resistance of the L-alpha-alkyl-alpha-acetylamino acid esters to enzymic hydrolysis is in great contrast to the behavior of L-alpha-acylamino acid esters possessing hydrogen in the alpha position. Despite the present inability to obtain the individual isomers of the alpha-alkyl alpha-amino acids for enzyme kinetic study it is clear that the presence of alpha-alkyl substituents in the alpha-acylamino acid derivatives impedes the ES interaction necessary for the occurrence of the hydrolytic and synthetic reactions. This observation is in accord with the hypothesis presented in the introduction to this section.

Experimental

DL-alpha-Methylphenylalanine - Adapted from the method of Steiger, Org. Synthesis 24, 9 (1944).

To a solution of 12.5 g. of sodium cyanide (powdered 96 per cent egg) and 14.7 g. of ammonium chloride in 60 ml. of water contained in a 500 ml. glass stoppered Erlenmeyer flask was added 33.5 ml. of concentrated aqueous ammonia (Sp. Gr. ca. 0.9) and the flask stoppered. To this was added a solution of 33.5 g. of benzyl methyl ketone (Eastman practical) in 75 ml. of 95 per cent ethanol, adding the solution in four portions with shaking. The stopper of the flask was then wired down and the mixture warmed in a water bath at 40-50° for a period of two and one quarter hours.

After standing overnight at room temperature the mixture was chilled to 0-5° and poured into 200 ml. of cold 12 N hydrochloric acid contained in a 1000 ml. 3-neck flask equipped with inlet bubble tube and reflux condenser, and then saturated, at 0-5°, with anhydrous hydrogen chloride.

After standing overnight, the reaction mixture was diluted with 250 ml. of water and heated under reflux for two and one-half hours in the hood. Water was then removed by vacuum evaporation and the resulting solid collected and stored over sodium hydroxide in a vacuum desiccator. A second crop of solid was obtained by evaporating the filtrate to dryness on the steam bath. The dry solid, freed of excess hydrogen chloride, was dissolved in 150 ml. of warm absolute ethanol,

the resulting solution cooled, and the precipitate of ammonium chloride removed by suction filtration. DL-alpha-methylphenylalanine crystallized from the filtrate upon the addition of 40 ml. of pyridine, chilling the mother liquor in an ice bath during the crystallization. The first crop weighed 23.0 g., m.p. 283-285° with decomposition. Reported[1] m.p. 293-294°. Three additional crude crops of total weight 14.1 g. were recovered from the mother liquor.

Acetyl-DL-alpha-methylphenylalanine - A solution of 3.0 g. of crude DL-alpha-methylphenylalanine in 70 ml. of water was chilled to 0-5° in an ice bath and treated with 3.4 g. of acetic anhydride and 1.34 g. of sodium hydroxide in aqueous solution, added in small portions with continued shaking and cooling in such a manner that the reaction mixture remained strongly basic throughout the addition. The reaction mixture was evaporated in vacuo to 40 ml., cooled to 0-5°, and acidified with hydrochloric acid. Acetyl-DL-alpha-methylphenylalanine was allowed to crystallize in the cold for several hours and was then collected and dried in a vacuum desiccator. Recrystallization from water gave 1.52 g. of white needles m.p. 194.8-197.7°.

Anal. Calcd. for $C_{12}H_{15}O_3N$: C, 65.1; H, 6.8; N, 6.3.
Found: C, 65.0; H, 6.9; N, 6.3.

DL-alpha-Methylphenylalanine Methyl Ester - A suspension of 5.0 g. of DL-alpha-methylphenylalanine in 100 ml. of absolute methanol was cooled to 0-5° and saturated with anhydrous hydrogen chloride. The resulting solution was heated under reflux for 16 hours and the excess methanol

then removed by evaporation *in vacuo*. The ester hydrochloride was neutralized with aqueous potassium carbonate and the resulting solution allowed to stand for four days in the refrigerator. The stout prismatic crystals which had formed by this time were separated from the mother liquor and the latter was evaporated to dryness under an air stream. The resulting solid was dried in a vacuum desiccator and extracted with hot acetone to recover the ester. 4.8 g. of crude ester was obtained, m.p. 108.5-122°.

Acetyl-DL-alpha-methylphenylalanine Methyl Ester - A solution of 4.0 g. of crude DL-alpha-methylphenylalanine methyl ester in 60 ml. of 10:1 water-acetone was cooled to 0-5° in an ice bath. To this was added 6.3 g. of acetic anhydride in small portions, shaking and cooling after each addition, together with sufficient aqueous sodium hydroxide to keep the reaction mixture above pH 8 during the addition of the first 4.2 g. of acetic anhydride. Addition of the remainder of the anhydride caused the acetyl ester to precipitate. Recrystallization from aqueous ethanol gave 1.3 g. of small lustrous blades, m.p. 118.1-119.0°.

Anal. Calcd. for $C_{13}H_{17}O_3N$: C, 66.4; H, 7.3; N, 5.9$_5$.
Found: C, 66.2; H, 7.2; N, 5.8.

p-Methoxybenzyl Alcohol - A procedure adapted from Org. Synthesis Coll. Vol. II, page 590 was employed.

To a solution of 434 g. of potassium hydroxide in 680 ml. of absolute methanol in a 3000-ml. 3-neck flask equipped with reflux

condenser and mechanical stirrer was added, with stirring, a solution of 260 ml. of aqueous formaldehyde (37 per cent Merck), 354.3 g. of redistilled anisaldehyde[a] (b.p. 129/18 mm.), and 270 ml. of absolute methanol over a period of 30 minutes. The temperature, initially at 58°, rose to 60-70° at the beginning of the addition and was maintained at 64-67° for three hours. 820 ml. of methanol was then removed from the reaction mixture by distillation, and 780 ml. of cold water added causing an upper organic phase to separate. The aqueous layer was extracted with benzene and the benzene extract combined with the original organic layer. The organic phase was washed with water and dried over anhydrous sodium sulfate. After removal of the benzene, the p-methoxybenzyl alcohol was distilled at reduced pressure through a short Vigreau column. Yield, 322 g. (90 per cent of theoretical) b.p. 91-93°/500μ. $n_D^{22.5}$ = 1.5440. For an authentic sample(Matheson)

n_D^{21} = 1.5450.

p-Methoxybenzyl Chloride

Method I - Adapted from Org. Reactions Vol. I, page 63.

216.5 g. of anisole, 194.0 g. of aqueous formaldehyde (37 per cent Merck), and 1200 ml. of concentrated hydrochloric acid (assay 37.6 per cent) were cooled to 0-10°, and shaken for 14 hours in a 2000-ml. RB flask. The shakingwas interrupted and cooling in an ice bath applied at intervals to maintain a temperature below 15°.

The reaction mixture was diluted with water and extracted with ether and the ether extract dried over anhydrous sodium sulfate.

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[a] Basic permanganate oxidation of 1.0 g. of the aldehyde yielded 1.3 g. of crude anisic acid, m.p. 179.5-181° (Reported[36] m.p. 184°).

After removing the ether by vacuum evaporation, hydrogen chloride was removed by pumping at room temperature with a vacuum pump for 15 min. The product was distilled rapidly through a short path distilling head into a fraction cutter-condenser. The fraction boiling at 100.5-183°/6-123 mm. weighed 128 g. (41 per cent of theoretical). During the distillation hydrogen chloride was evolved with the resulting pressure increase noted.

A higher boiling fraction, b.p. 176-180°/5-6.5 mm., crystallized in the receiver. Recrystallization of this material from 95 per cent ethanol gave plate like crystals, m.p. 46.6-50.8°, Tentatively identified as bis-(p-methoxyphenyl)-methane.[a]

The p-methoxybenzyl chloride yielded, upon permanganate oxidation, anisic acid in fine yellowish-white needles. m.p. 180-182.2°. Lit.[36] m.p. 184°.

Method II - Essentially the method of Shriner and Hull.[31]

A solution of 210 g. of p-methoxybenzyl alcohol in 725 ml. of absolute ether was cooled to 0-5° and saturated with anhydrous hydrogen chloride. After standing in the cold for 20 hours, excess hydrogen chloride and ether were removed by vacuum evaporation, and the organic phase washed successively with water, aqueous sodium bicarbonate, dilute aqueous sodium chloride, and saturated aqueous sodium chloride. The organic phase was dried over anhydrous sodium sulfate and the ether removed by evaporation in vacuo. Prior to distillation the organic

[a] In the preparation of p-methoxybenzyl chloride by this method Sosa[15] reported the formation of this compound (m.p. 52-53°) as a side product.

material was pumped on with a vacuum pump for two hours to remove hydrogen chloride. The p-methoxybenzyl chloride was then distilled rapidly through a short path system in the manner described above. The fraction collected at 117-123°/16 mm. weighed 205 g. (86 per cent of theoretical).[a] $n_D^{26} = 1.5461$. Reported[15] $n_D^{20} = 1.5491$.

p-Methoxybenzyl chloride, obtained by either method, is unstable, undergoing eventual polymerization with concurrent liberation of hydrogen chloride. This decomposition is accelerated by heat, hydrogen chloride, ground glass surfaces, and ferric ion, and may occur very rapidly during distillation. The liberation of hydrogen chloride during distillation (particularly of the crude product) had the adverse effect of increasing the pressure in a system evacuated by a vacuum pump and thereby increasing the temperature and rate of decomposition. This difficulty was overcome to a large extent by evacuating the systems with a water aspirator during distillation. Although incapable of evacuation much below 15 mm., a water aspirator prevents the hydrogen chloride accumulation.

Pure redistilled (b. p. 91-93°/500 μ) p-methoxybenzyl chloride decomposes slowly and it is necessary to add solid calcium carbonate occasionally to preserve a sample over a period of several months.

Homoanisonitrile - A method adapted from Shriner and Hull[31] was employed.

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[a] Sosa[15] obtained a similar yield (80 per cent) by this method.

To a boiling solution of 182 g. of potassium cyanide (Mallinckrodt Analytical Reagent) in 153 ml. of water, contained in a 3000-ml. 3-neck flask equipped with reflux condenser and mechanical stirrer, was added, with stirring, a solution of 109.4 g. of p-methoxybenzyl chloride in 220 ml. 1,4 dioxane (redistilled from sodium) over a 45 minute period. Refluxing and stirring were continued for an additional three hours after which the dark reaction mixture was cooled, filtered through "hyflo" by water aspiration, and the excess solvents removed by evaporation in vacuo. The dark reaction mixture was again filtered, then washed with water and dried over anhydrous calcium chloride. The crude nitrile was distilled first at reduced pressure from a Claisen flask containing a few cm. of packing (Raschig rings). The pale yellow fraction boiling at 101-115°/500-1200 was collected and refractionated through a short Vigreau column. Yield, 79.8 g. (77.5 per cent of theoretical) of homoanisonitrile. b.p. 101-106°/800 μ. $\eta_D^{21.5} = 1.5320$. Reported[36] $\eta_D^{16.8} = 1.5317$.

5.0 g. of the above nitrile was hydrolyzed by heating under reflux for ca. 50 hours with concentrated aqueous sodium hydroxide.[a] After filtering the cooled reaction mixture, precipitation of the acid by acidification, and recrystallization from hot water, 1.82 g. of lustrous platy crystals, m.p. 85.9-87.9°, was obtained. Reported[37] for homoanisic acid, m.p. 85-87°.

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[a] In performing the basic hydrolysis of homoanisonitrile, Sosa[15] obtained only 40 per cent of the expected homoanisic acid. A side reaction was dimerization of the nitrile and hydrolysis of the dimer to give

Attempts to convert p-methoxybenzyl chloride to the nitrile by treatment with potassium cyanide in aqueous ethanol led to the formation of p-methoxybenzyl ethyl ether as a major by product. The yield of the ether was found to increase (and that of the nitrile to decrease) as the ratio of ethanol to cyanide ion in the reaction mixture was increased. This is illustrated by theresults of the following two experiments.

Trial	Molar ratio $(CN^-)/(ETOH)$	Yield (on basis of chloride) of ether	of nitrile
1	0.01	22.2 per cent	31.3 per cent
2	0.0028	40.1 per cent	insig.

For p-methoxybenzyl cyanide obtained from Trial I, b.p. 97-100.5°/620-650μ, η_D^{22} = 1.5304.

Anal Calcd. for C_9H_9ON: C, 73.4; H, 6.2.

Found: C, 73.1; H, 6.3.

For p-methoxybenzyl ethyl ether obtained from Trial II, b.p. 75.5-76.2°/930μ, η_D^{21} = 1.5060

Anal. Calcd. for $C_{10}H_{14}O_2$: C, 72.3; H, 8.5.

Found: C, 72.3; H, 8.5.

Carbonyl Product from the Reaction of Homoanisonitrile with Methylmagnesiumiodide

4.92 g. of ether washed magnesium turnings (99.8 per cent Mg) was placed under 60 ml. of dry absolute ethyl ether in a dry 500-ml.

3-neck flask equipped with mechanical stirrer, reflux condenser, and dropping funnel. A few ml. of methyl iodide (Mathesen) was added to start the reaction and, with stirring, a mixture of 34.3 g. of methyl iodide and 100 ml. of dry absolute ethyl ether was added dropwise over a 45-minute period.[a] At the end of this time thereaction slackened and a warmwater bath was applied for 15 min. to effect complete reaction of the magnesium.

To the reaction mixture, cooled to < 0° in an ice-sodium chloride mixture, was added a solution of 7.43 g. of homoanisonitrile in 20 ml. of dry absolute ethyl ether with vigorous stirring over a period of 15 minutes. Following addition of the nitrile, stirring was continued for one hour and the reaction mixture was allowed to stand overnight.

The reaction mixture was carefully poured onto a mixture of 60 ml. of concentrated hydrochloric acid and 100 g. of ice and the resulting organic and aqueous phases separated. The acidic aqueous phase was heated under reflux for one hour and the resulting dark oil extracted with ether. The ether extract was washed first with aqueous sodium bicarbonate, then with aqueous sodium thiosulfate to remove the iodine liberated during the reflux. The original ethereal phase was combined with the ether extract and the ether removed by evaporation. To insure recovery of any ketone converted to the bisulfite compound by the thiosulfate treatment, the organic phase was steam distilled with the aqueous thiosulfate extracts (which had been acidified with

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[a] This rate of addition provided a rapid but controlled reaction rate as evidenced by the rate of reflux.

dilute hydrochloric acid) and the nearly colorless oily component of the distillate recovered by ether extraction, washed with aqueous bicarbonate, and dried over anhydrous sodium sulfate. Following removal of ether by vacuum evaporation, the product was distilled from a Claisen flask. A yellow fraction boiling at 97-105°/700-800 μ was collected and refractionated to give 3.8 g. pale yellow oil, b.p. 95-100.5°/650-800 μ. $n_D^{22.5}$ = 1.5190.

2,4-Dinitrophenylhydrazone

Sample A.- Prepared according to the procedure of Lucas and Pressman[38]. Recrystallization 3 times from absolute ethanol and twice from 60-70° ligroin gave golden needles, m.p. 135.5-136.8°.

Sample B.- Prepared according to the procedure of Shriner and Fuson[39]. 6 recrystallizations from absolute ethanol and ligroin-ethanol mixtures gave flattened golden needles, m.p. 133.4-136.1°.

Anal. Calcd. for $C_{16}H_{16}O_5N_4$ (p-methoxybenzyl methyl ketone 2,4-DNP)

C, 55.8; H, 4.7; N, 16.3.

Calcd. for $C_{17}H_{18}O_5N_4$ (3-(p-methoxyphenyl)-2-butanone 2,4-DNP)

C, 57.0; H, 5.1; N, 15.6.

Found:

Sample A - C, 57.9; H, 5.0; N, 15.4.

Sample B - C, 56.8; H, 5.0; N, 16.0.

Oxime - A solution of 0.5 g. of the ketone, 0.7 g. of hydroxylamine hydrochloride, and 20 ml. of 1:1 ethanol-water (v per v) was made pH 7

with aqueous sodium hydroxide and heated under reflux for 10 minutes. Evaporation of the ethanol and cooling led to the separation of the oxime as an oil which crystallized upon scratching. 4 recrystallizations from 60-70 ligroin gave colorless needles, m.p. 118-119.2°.

Anal. Calcd. for $C_{10}H_{13}O_2N$ (p-methoxybenzyl methyl ketoxime)

C, 67.0; H, 7.3; N, 7.8.

Calcd. for $C_{11}H_{15}O_2N$ (3-(p-methoxyphenyl)-2-butanone oxime)

C, 68.3; H, 7.8; N, 7.2.

Found:

C, 65.0; H, 7.8; N, 7.8.

Behavior with Sodium Bisulfite - Under conditions in which benzyl methyl ketone and p-methoxybenzyl methyl ketone gave rapid formation of solid derivatives the Grignard product failed to react to any extent with saturated aqueous-ethanolic sodium bisulfite.

Dimethone Test - 0.1 g. of the product was boiled for 5-10 minutes with an excess of dimethone in aqueous ethanol. Upon cooling in the refrigerator, unreacted dimethone, m.p. 144.7-150.9°, was obtained.

Iodoform Test - Applied as directed by Shriner and Fuson[39]. The Grignard product gave a relatively large amount of golden lamellar iodoform, melting point of crude product, ca. 110-117° with charring. An equal amount of p-methoxybenzyl methyl ketone gave a much smaller yield of iodoform.

Strecker Reaction upon the Grignard Ketonic Product

20.1 g. of the ketone was treated with potassium cyanide, ammonium chloride, and ammonium hydroxide under the same conditions as described for the preparation of DL-O-methyl-alpha-methyltyrosine. After the hydrochloric acid treatment, the pH of the solution was adjusted to ca. 7 causing the separation of 1.05 g. of a brownish-white solid.

The product was then acetylated with acetic anhydride in the usual manner. 0.83 g. of brownish crystals were obtained and these were recrystallized from ethanol-water to give 0.44 g. of short white needles, m.p. 222-224°.

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

Found: C, 62.0; H, 6.8; N, 5.5.

Ninhydrin Test - A small sample of the acetyl derivative was heated with dilute hydrochloric acid at 90-100° for ca. 10 minutes to liberate the free amino acid, the pH adjusted to ca. 7, and 4-5 drops of 1 per cent ninhydrin solution added. No color was produced even after 25 minutes of heating at 90-100°. Similar treatment of acetyl-DL-tyrosine produced an intense blue-violet coloration in 5 minutes.

Reaction of Isopropylmagnesium Bromide with Phenylacetonitrile

Procedure adapted from Org. Synthesis 21, 79 (1941).

To 14.6 g. of clean magnesium turnings in a dry 1000-ml. 3-neck flask equipped with reflux condenser, mechanical stirrer, and dropping funnel was added a small amount of isopropyl bromide to start

the reaction followed by 125 ml. of dry absolute ethyl ether. A solution of 61.5 g. of isopropyl bromide in 200 ml. of absolute ether was then added dropwise, with stirring, at a rate causing the mixture to reflux gently. When the action slackened, heating under reflux was applied for an additional hour.

The reaction mixture was cooled in an ice-salt bath and, with stirring, a solution of 48.8 g. of redistilled phenylacetonitrile (b.p. 108-108.9°/14 mm.) in 100 ml. of absolute ethyl ether was added dropwise. After the addition of the nitrile the reaction mixture was allowed to stand for two hours.

The reaction mixture was decomposed in the cold with excess dilute sulfuric acid, the mixture extracted with ether, and the ether extract washed with aqueous potassium carbonate, water, and saturated aqueous sodium chloride, and dried over anhydrous sodium sulfate. After removing the ether by vacuum evaporation an attempt was made to distill the syrupy remainder at reduced pressure. When it became evident that no significant amount of material boiling in the range expected for benzyl isopropyl ketone was present, the attempt at distillation was abandoned and the high boiling material stored in the refrigerator.

In the hope of identifying any ketonic materials present in the undistillable material, a small amount of it was treated with hydroxylamine hydrochloride according to the directions of Shriner and Fuson[39] for the preparation of oximes. A white solid product was obtained which was recrystallized from aqueous ethanol to give compact prisms melting at 104.6-107.4°. Two recrystallizations from absolute

ethanol gave flat colorless prisms, m.p. 107.2-108.9°. Reported[25] for the oxime of dimeric phenylacetonitrile m.p. 107°.

Anal. Calcd. for $C_{16}H_{13}O N_2$: C, 77.1; H, 5.3; N, 11.2.

Found: C, 76.9; H, 5.5; N, 11.1.

Upon standing in the cold for two weeks a yellow crystalline material, m.p. 178-180.2°, separated from the undistillable reaction product. Recrystallization from absolute ethanol gave compact lemon-yellow prisms, m.p. 179-181.2°. Reported[25] for the trimer of phenylacetonitrile, m.p. 178.5-179°.

Anal. Calcd. for $C_{24}H_{21}N_3$: C, 82.0; H, 6.0; N, 12.0.

Found: C 81.8; H, 5.9; N, 12.2.

p-Methoxybenzyl Methyl Ketone - By the Darzens glycidic ester method, adapted from Org. Reactions Vol. V, page 427.

59.8 g. of ethyl alpha-bromopropionate (Eastman) and 45.1 g. of redistilled anisaldehyde (129°/18 mm.) were placed in a dry 500-ml. 3-neck flask equipped with reflux condenser, mechanical stirrer, and dropping funnel. The mixture was cooled to ca. -10-11° in an ice-salt bath and the system was swept out with a stream of dry nitrogen. Then, with stirring and continued cooling a solution of 7.6 g. of sodium metal in 160 ml. of dry absolute ethanol was added dropwise over a three hour period. Stirring at the low temperature was continued for 12 hours and then continued, warming to room temperature, for two hours longer. Finally, the mixture was heated under reflux, with stirring, for a period of six hours.

The reaction mixture was treated with cold water, acidified with acetic acid, and solid sodium chloride added to cause separation of the organic phase. The glycidic ester was extracted with ether and the ether extract washed with aqueous sodium bicarbonate, water, and saturated aqueous sodium chloride , and then dried in two stages, first with anhydrous sodium sulfate then with drierite. After removal of ether by vacuum evaporation the material was distilled at reduced pressure from a Claisen flask. 47.2 g. (60.4 per cent of theoretical) of glycidic ester was collected over the b.p. range 120-135°/500μ. $n_D^{26.7}$ = 1.5114 (for a sample prepared in a similar preparation as the above and boiling at 122-131°/500 μ).

With stirring, the glycidic ester was then added to a solution of 4.8 g. of sodium metal in 100 ml. of dry absolute ethanol and the resulting deep yellow solution cooled in an ice bath. Maintaining the temperature at 10-20°, 5 ml. of water was carefully added to precipitate the sodium salt of the glycidic acid. After standing overnight in the refrigerator the sodium salt was collected by suction filtration and washed with 15 ml. of cold ethanol and 35 ml. of ether successively.

The sodium salt was heated under reflux with a mixture of 93.5 ml. of water and 17.5 ml. of concentrated hydrochloric acid for two and one-half hours. After cooling, the yellow oily ketone, liberated by the acidic reflux, was extracted with benzene and the benzene extract washed successively with aqueous sodium bicarbonate, water, and saturated with aqueous sodium chloride and dried over

anhydrous sodium sulfate. After removing the benzene by vacuum evaporation, the ketone was distilled under reduced pressure from a Claisen flask. The fraction boiling at 88-93°/500 μ weighed 17.6 g. (32.5 per cent of theoretical for overall conversion). n_D^{23} = 1.5239. Reported[16] n_D^{22} = 1.5253. A somewhat purer sample prepared in a subsequent attempt boiled at 101-104°/1500 μ with $n_D^{25.6}$ = 1.5223. The ketone is a faintly yellowish oil with a slight butterlike odor.

p-Methoxybenzyl Methyl Ketone 2,4-Dinitrophenylhydrazone

Prepared according to the directions of Shriner and Fuson[39]. 0.5 g. of the ketone yielded 0.66 g. of crude product which was recrystallized twice from absolute ethanol to give golden-orange needles m.p. 108.6-109.5°. Reported[a] m.p. 107.8-108.2°. A mixed melting point with a sample of the 2,4-DNP of the ketone prepared by the method of Winstein[18] showed no depression (m.p. 108.6-109.4°).

Anal. Calcd. for $C_{16}H_{16}O_5N_4$: C, 55.8; H, 4.7; N, 16.3.

Found: C, 55.7; H, 4.8; N, 16.2.

In several instances when the 2,4-DNP's of the ketone samples were prepared by an alternative procedure in glacial acetic acid solution containing a few drops of concentrated hydrochloric acid, yellow needles melting at 198.7-200° were isolated in addition to the 2,4-dinitrophenylhydrazones. This compound, identified as acetyl-2,4-dinitrophenylhydrazine, was also obtained by Strain[40] during the preparation of 2,4-DNP's in alcoholic solutions containing sulfuric

[a] Fort, A., and Roberts, J.D., to be published.

and acetic acid. Strain reports a melting point of 199-201°.

Anal. Calcd. for $C_8H_8O_5N_4$: C, 40.0; H, 3.3; N, 23.3.

Found: C, 39.9; H, 3.3; N, 23.2.

DL-O-Methyl-alpha-methyltyrosine - was prepared from p-methoxybenzyl methyl ketone by the Strecker reaction in the same manner as described for DL-alpha-methylphenylalanine. The initial reaction mixture contained, in addition to the usual components, ca. 1 ml. of methanol per gram of ketone employed. Following hydrolysis of the amino nitrile, the amino acid was precipitated by adjusting the aqueous solution of its hydrochloride to pH 7 with sodium hydroxide. Nine days standing in the refrigerator was necessary to completely crystallize the amino acid. 56.6 g. of the ketone yielded 63.9 g. of crude amino acid.

DL-alpha-methyltyrosine - To 12.6 g. of crude DL-O-methyl-alpha-methyltyrosine in a pressure bottle was added 50 ml. of a 30-32 per cent solution of hydrobromic acid in glacial acetic acid solution, the bottle stoppered, and the mixture heated in a boiling water bath for one hour. After allowing the reaction mixture to cool and stand overnight, the excess acids were removed by evaporation in vacuo and the mixture was diluted to ca. 300 ml. with water. Upon cooling in an ice bath and adjusting the pH to ca. 7 with aqueous sodium hydroxide, a grayish precipitate of DL-alpha-methyltyrosine was formed. The dry weight of the crude material was 10.4 g.

N-Acetyl-DL-alpha-methyltyrosine - 10.0 g. of crude DL-alpha-methyltyrosine was acetylated with 13 ml. of acetic anhydride in a manner essentially the same as described for N-acetyl-DL-alpha-methylphenylalanine. Upon acidification of the reaction mixture, 5.9 g. of the crude acetyl derivative was obtained. The acetyl derivative proved to be very difficult to crystallize from aqueous ethanol as it entered solution slowly and then tended to supersaturate. 1.44 g. of small flat needles were obtained from ethanol-water, m.p. 207.5-211.5° with decomposition.

N-Acetyl-DL-alpha-methyltyrosine Methyl Ester - A solution of 1.37 g. of acetyl-DL-alpha-methyltyrosine in 20 ml. of absolute methanol was cooled in an ice-salt bath and saturated with anhydrous hydrogen chloride (Matheson). After warming to room temperature and standing for four days the mixture was again chilled and saturated with hydrogen chloride. After standing overnight at room temperature the volatile material was removed by vacuum evaporation and the remaining acid removed from the resulting brown syrup by vacuum dessication over sodium hydroxide. Upon dissolving the syrup in a little methanol, adding water, and cooling in an ice bath, 0.45 g. of crystals were obtained. Recrystallization from water containing a little ethanol gave fine flat prisms, m.p. 121-122°,

Anal. Calcd. for $C_{13}H_{17}O_4N$: C, 62.6; H, 6.8; N, 5.6.

Found: C, 62.9; H, 7.1; N, 5.3.

N-Acetyl-DL-O-methyl-alpha-methyltyrosine - 58.8 g. of the crude amino acid, acetylated with acetic anhydride in the usual manner, yielded 46.7 g. of the crude acetyl derivative melting at 176-178°. Recrystallization of 10 g. of the above acetyl derivative from aqueous ethanol gave 8.6 g. of flat prisms, m.p. 178-181°.

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

Found: C, 62.2; H, 6.8; N, 5.5.

Acetyl-DL-O-methyl-alpha-methyltyrosine Methyl Ester -

A suspension of 10.0 g. of acetyl-DL-O-methyl-alpha-methyltyrosine in 200 ml. of absolute methanol was cooled to 0-5° and saturated with anhydrous hydrogen chloride. After standing overnight, volatile material was removed by vacuum evaporation and remaining traces of water removed by repeated evaporation under benzene. The resulting dark syrup was again treated with methanolic hydrogen chloride and the benzene evaporation process repeated. Upon stirring the resulting syrup with aqueous methanol, 7.3 g. of a crystalline product was obtained. One recrystallization from aqueous ethanol gave 4.7 g. of fine stunted prisms, m.p. 120.7-121.7°.

Anal. Calcd. for $C_{14}H_{19}O_4N$: C, 63.4; H, 7.2; N, 5.3.

Found: C, 63.2; H, 7.2; N, 5.3.

p-Methoxybenzyl Isopropyl Ketone - Via the Darzens glycidic ester condensation.

Equimolar amounts of anisaldehyde, ethyl alpha-bromo-isovalerate (Matheson), and sodium ethoxide were reacted in a manner

entirely similar to that described for the preparation of the methyl ketone (see page 157). In this case, 135 g. of anisaldehyde and 207. 5 g. of the bromo ester yielded 147.6 g. (56.4 per cent of theoretical) of the glycidic ester, b.p. 123.5-127°/500-600 μ.

Saponification of the glycidic ester gave 112.2 g. of the sodium salt (77.8 per cent on the basis of the glycidic ester). The sodium glycidate was acidified and decarboxylated to give 67.9 g. (81.3 per cent conversion of the sodium salt; 45.8 per cent for the overall process) of p-methoxybenzyl isopropyl ketone, b.p. 95-96°/550 μ $n_D^{23} = 1.5115$. The ketone is a pale yellow, nearly colorless liquid having a delicate anise odor.

5-isopropyl-5'-(p-methoxybenzyl)-hydantoin- Adapted from the procedure of Henze and Speer.(3)

To a suspension of 13.2 g. of ammonium carbonate and 2.8 g. of sodium cyanide (powdered egg) in a mixture of 46 ml. of water and 10 ml. of 95 per cent ethanol, contained in a 300-ml. RB flask, was added a solution of 4.7 g. of p-methoxybenzyl isopropyl ketone in 36 ml. of 95 per cent ethanol. The resulting mixture was heated for two hours in a water bath maintained at 60° ± 2°.

Upon cooling, 50 ml. of water was added to the yellow reaction mixture whereupon a certain amount of unreacted ketone separated out as an oil. 2.0 g. of potassium cyanide (Mallinckrodt AR) and 50 ml. of 95 per cent ethanol were then added and the heating resumed and continued for two hours. Removal of ethanol by vacuum evaporation

caused the separation of the hydantoin as a white solid. The crude product weighed 4.74 g. (74 per cent of theoretical). 5-isopropyl-5'-(p-methoxybenzyl)-hydantoin crystallized from water-ethanol in bunches of colorless needles. m.p. 218.8°-221°.

Anal. Calcd. for $C_{14}H_{18}O_3N_2$: C, 64.1; H, 6.9; N, 10.7.

Found: C, 63.6; H, 6.8, N, 10.9.

Attempted Conversion to N-Acetyl-O-methyl-alpha-isopropyltyrosine - 4.0 g. of crude 5-isopropyl-5'-(p-methoxybenzyl)-hydantoin was heated under reflux with a solution of 20 g. of barium hydroxide in 40 ml. of water for 52 hours. The mixture was cooled and an excess of dilute sulfuric acid added to precipitate barium sulfate. The latter was allowed to precipitate over a 40 minute period at ca. 70° and was then removed by suction filtration.

When, after bringing the filtrate to pH 5-7 and allowing to stand, it appeared that the amino acid would not crystallize it was decided to recover it by converting it into its acetyl derivative. The solution was reduced in volume to 100 ml., made basic, cooled in an ice bath, and treated with 7 ml. of acetic anhydride under basic conditions in the usual manner. The reaction mixture was filtered and the filtrate brought to pH 3 and stored in the refrigerator. When, on standing, the acidic filtrate failed to yield crystals it was evaporated to dryness, combined with the insoluble residue formed during the acetylation, and extracted with hot acetone in a Soxhlet extractor. Evaporation of acetone left a solid which was recrystallized 3 times

from aqueous ethanol to give colorless blades, m.p. 218.2-220.1.

Anal. Calcd. for $C_{15}H_{21}O_4N$: C, 64.5; H, 7.6; N, 5.0.

Found: C, 70.4; H, 6.7.

Mixed m.p. with 5-isopropyl-5'-(p-methoxybenzyl)-hydantoin 218.7-220.2°.

Apparently, unreacted hydantoin has been isolated.

Ethyl isopropylcyanoacetate - Procedure adapted from the method of Hessler and Henderson[41].

To a solution of 12.2 g. of sodium metal in 500 ml. of absolute ethanol, contained in a 3000-ml. 3-neck flask equipped with reflux condenser and mechanical stirrer, was added, rapidly and with stirring, 59.9 g. of ethyl cyanoacetate (Matheson). To the resulting mixture, isopropyl iodide (Matheson) was added dropwise, with continued stirring, over a one hour period. The reaction mixture was then heated under reflux for ca. two hours until it had become ca. pH 7 at which time 350 ml. of ethanol was removed from the cherry-red mixture by distillation. After removing the remaining ethanol by vacuum evaporation, the reaction mixture was equilibrated with ether and water and the ether layer separated and shaken, first with dilute aqueous sodium thiosulfate, then with saturated aqueous sodium chloride and dried over anhydrous sodium sulfate. The ether was then removed by vacuum evaporation and ethyl isopropylcyanoacetate distilled through a short Claisen column packed with Raschig rings. The fraction collected at 68-78°/2200-3100 μ weighed 48.9 g. (60 per cent theoretical).

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Ethyl isopropyl-benzylcyanoacetate - To a solution of 5.9 g. of sodium metal in 370 ml. of dry absolute ethanol, contained in a 1000-ml. 3-neck flask equipped with reflux condenser and stirring motor was added 39.7 g. of ethyl isopropylcyanoacetate over a ten minute period. 36.7 g. of benzyl chloride was then added with stirring over a 15-20 minute period. As the stirring continued the reaction mixture began to reflux gently and after one and three-quarter hours, when the action had begun to slacken, heat was applied and the mixture stirred under reflux for an additional four hours. 300 ml. of ethanol was then removed by distillation and, after cooling, the reaction mixture was shaken with ether and ice water. The ether layer was dried, first over anhydrous sodium sulfate then with anhydrous calcium chloride and the ether removed by evaporation in vacuo. Ethyl isopropyl-benzylcyanoacetate was distilled through a 30 cm. Claisen column without packing. The fraction collected boiled at 130-136°/1300-1500μ and weighed 43.3 g. (69 per cent of theoretical).

Reaction of Ethyl Isopropyl-Benzylcyanoacetate with Hydrazine Hydrate

A mixture of 43.3 g. of ethyl isopropyl-benzylcyanoacetate, 12.2 g. of 100 per cent hydrazine hydrate (Matheson), and 150 ml. of n-hexanol was heated under reflux for 5 days. At the end of this time the reaction mixture was reduced to a small volume by vacuum evaporation and placed in the refrigerator. Upon standing in the cold for 12 days a small amount of a white precipitate formed and was collected by suction filtration. This substance was amphoteric in behavior, soluble in both acid and base and could be precipitated out of aqueous solution at pH 6-7. The material was dissolved in 3N HCl and the solution extracted with ether to remove the suspended high boiling liquids. The acidic aqueous phase was then filtered and the pH adjusted to ca. 6 with aqueous sodium hydroxide. After standing overnight in the ice box, the amphoteric material was collected and dried over sodium hydroxide in vacuo. The compound melted at 149.2-150.2°.

Anal. Found: C, 67.6 ; H, 7.6 ; N, 12.5.

Ethyl isopropyl-p-methoxybenzylcyanoacetate- 48.9 g. of ethyl isopropylcyanoacetate was converted to its sodium derivative with a solution of 7.25 g. of sodium metal in 400 ml. of dry absolute ethanol, and reacted with 49.4 g. of p-methoxybenzyl chloride under conditions identical with those described for the preparation of ethyl isopropyl-benzylcyanoacetate. Following removal of ether

the ethyl isopropyl-p-methoxybenzylcyanoacetate was distilled, collecting 52.7 g. (60.8 per cent of theoretical) over the range 138.5-146° / 300-400μ.

Anhydrous Hydrazine- Prepared according to the directions of Audrieth[42].

200 g. of 100 per cent hydrazine hydrate was stored with 200 g. of sodium hydroxide (C. P. pellets) under a nitrogen atmosphere for a period of 7 days. At this time the mixture, which had become a solid mass, was warmed in a water bath causing it to melt and form two liquid phases. The hydrazine rich upper phase was carefully decanted and distilled under a nitrogen atmosphere at reduced pressure. The most volatile material, boiling over a one degree range, was collected and used as the hydrazine preparation. A second fraction boiling over a ca. 7 degree range was collected, treated with sodium hydroxide as before, and fractionated to give an additional batch of nearly anhydrous hydrazine.

Isopropyl-p-methoxybenzylcyanoacethydrazide- 27.5 g. of ethyl isopropyl-p-methoxybenzylcyanoacetate and 6.4 g. of anhydrous hydrazine were placed in a 100-ml. 3-neck flask equipped with reflux condenser and nitrogen inlet tube. The mixture was heated in an oil bath, initially maintained at 145-155°, while passing a slow stream of nitrogen through the system. After heating for ca. 2 hours, the mixture was allowed to cool, 4.0 g. of anhydrous hydrazine and 30-40 ml. of n-hexanol added, and the heating resumed. The mixture was

heated intermittently for a period of approximately 8 days at a bath temperature of 120-170°, cooling from time to time to add small portions of anhydrous hydrazine and sweep the system out with nitrogen (the continuous nitrogen stream was abandoned since it tended to carry hydrazine out of the reflux condenser). Over the 8 day period, ca. 10 g. of additional hydrazine was added. Finally the bulk of the volatile material was removed by evaporation in vacuo and the oily residue stored overnight in the refrigerator. A white solid separated out and was collected by suction filtration, washed with ether and dried.

The crude product thus obtained weighed 5.0 g. and was fairly water soluble. The solid behaved in a peculiar manner upon heating in a capillary tube, clumping (without becoming moist) at 110° and evolving a volatile substance and finally melting over the range 147.5-161°. Upon recrystallizing from 95 per cent ethanol 4.8 g. (18.4 per cent of theoretical) of material was obtained, m.p. 176.5-180.9°, without the preliminary gas evolution.

Anal. Calcd. for $C_{14}H_{19}O_2N_3$: C, 64.4 ; H, 7.3 ; N, 16.1.

Found: C, 63.5 ; H, 7.3 ; N, 16.7.

Attempted Curtius Rearrangement of Isopropyl-p-methoxybenzyl-cyanoacethydrazide

A solution of 4.8 g. of the hydrazide in 5 ml. of concentrated hydrochloric acid and 50 ml. of water was placed in a 200-ml. 3-neck flask equipped with mechanical stirrer, thermometer, and dropping

funnel. 40 ml. of ether was then added, and the two phase system cooled to 0-5° in an ice bath. With vigorous stirring, a solution of 1.28 g. of sodium nitrite in a few ml. of water was run into the mixture over a period of 5 minutes. The temperature of the mixture increased by ca. 4° and a solid separated out. As soon as the addition of the nitrite was completed the stirring was ceased, and the yellow ether layer separated. The aqueous phase was extracted with ether and this extract combined with the original ether layer. The ether extract was washed with cold aqueous sodium bicarbonate, dried over anhydrous magnesium sulfate and added to a 300 ml. RB flask containing 50 ml. of absolute ethanol. A distilling head and condenser were attached to the flask and the ether was removed from the mixture by distillation. 100 ml. of absolute ethanol was then added and the solution heated under reflux for one hour. Finally, the ethanol was removed from the residue by vacuum evaporation.

100 ml. of concentrated hydrochloric acid was added to the residue and the mixture heated under reflux. After 15 minutes, a dark oil had begun to separate out. After refluxing for ca. one hour, the mixture was cooled in an ice bath, saturated with anhydrous hydrogen chloride, and heating under reflux recommenced and continued for two and one-half hours.

Upon reducing the volume of the reaction mixture and adjusting the pH to 7 a precipitate began to form. After standing in the cold room overnight the precipitate was collected by suction and dried. The yield of the crude material was extremely small.

The solid material appearing during the addition of the nitrite solution weighed 1.1 g. and is presumably the symmetrical dihydrazide.

Gramine- Prepared according to the method of Kuhn and Stein[43].

A mixture of 60.8 g. of 33 per cent aqueous dimethylamine and 62.4 g. of glacial acetic acid was chilled to 0-5°C and added to 46.5 g. of indole (Eastman) contained in a 2000-ml. beaker. As the reaction mixture became warm, cooling was applied by a water bath to keep the temperature below 45°. The clear light yellow mixture was allowed to stand at room temperature for one hour and then made strongly basic with aqueous sodium hydroxide causing the gramine to precipitate. The product was collected by suction, washed thoroughly with water, and dried over sodium hydroxide in vacuo. The crude material weighed 63.0 g.

Ethyl methylcyanoacetate- 103.0 g. of ethyl cyanoacetate (Matheson) was converted into the sodium salt by treatment with a solution of 20.9 g. of sodium metal in 1100 ml. of dry absolute methanol and alkylated with 142.2 g. of methyl iodide in the same manner as previously described for isopropylcyanoacetic ester. In this case, however, the reaction was run entirely at room temperature, cooling in early stages with a water bath. As in the previously described alkylation with isopropyl iodide it was important to remove the iodine from the organic phase prior to distillation by washing with aqueous sodium thiosulfate. The ethyl methylcyanoacetate was collected over the range 43-50°/ 1000μ and weighed 76.0 g. (65.7 per cent of theoretical).

Condensation of Gramine with Ethyl methylcyanoacetate

17.4 g. of gramine and 12.7 g. of ethyl methylcyanoacetate were placed in a 200-ml. RB flask equipped with a side arm. From a one-hole rubber stopper in the mouth of the flask a pyrex tube led to a simple trap consisting of a 250-ml. Erlenmeyer flask equipped with a 2-hole rubber stopper. From the trap a similar tube led to a third flask where it terminated below the surface of a solution of picric acid in benzene. A slow stream of nitrogen was started through the apparatus and the reaction mixture was heated by an oil bath maintained at 130-160°. The reaction mixture liquefied and as the reaction proceeded the liberated dimethylamine was swept into the picric acid solution where it was converted instantly into its yellow crystalline picrate. The heating was continued for ca. 3 hours after which the reaction mixture was allowed to cool and the dimethylamine picrate collected and dried in vacuo. The weight of the picrate, 16.9 g., corresponded to 61.6 per cent conversion of the gramine.

The cold reaction mixture was taken up in ether and extracted with cold dilute hydrochloric acid to remove unreacted gramine and then with dilute aqueous potassium carbonate and water. After removing the ether by evaporation, unreacted ethyl methylcyanoacetate (1.6 g.) was removed by distillation at reduced pressure. The product, presumably ethyl 3-indolylmethyl-methylcyanoacetate, remained as a dark viscous syrup.

Methylcyanoacethydrazide - To a solution of 35.4 g. of ethyl methylcyanoacetate in 100 ml. of n-amyl alcohol, contained in a 300-ml.

RB flask equipped with reflux condenser, was added 16 ml. of anhydrous hydrazine and the system swept out with nitrogen. The mixture was heated under reflux for ca. four and one-half hours and at the end of this time the excess solvent was removed by evaporation in vacuo. Upon cooling the residue, the hydrazide separated as a dark solid and was collected by suction filtration, washed with ether, and dried in vacuo. Remaining traces of solvent were removed from the hydrazide after several days storage in vacuo by grinding it to a fine powder and washing it repeatedly with ether. The dry dark red crude product weighed 29.5 g.

Condensation of Gramine with Methylcyanoacethydrazide

20.3 g. of gramine and 13.2 g. of crude methylcyano-acethydrazide were ground together in a mortar and the mixture placed in a 250-ml. wide mouth Erlenmeyer flask. Upon heating the mixture in an oil bath maintained at $135^{\circ} \pm 3^{\circ}$, dimethylamine was vigorously evolved and the heating was continued for two and one-half hours until the evolution of dimethylamine had virtually ceased.

The cool solidified mixture was ground to a powder and washed several times with water to remove unreacted methylcyanoacethydrazide, then with cold dilute hydrochloric acid to remove gramine. Finally it was washed with aqueous sodium bicarbonate and water and dried in vacuo. The crude product, presumed to be 3-indolylmethyl-methyl-cyanoacethydrazide, weighed 21.0 g.

Attempted Curtius Rearrangement of 3-Indolylmethyl-methylcyanoacethydrazide

To a solution of 9.13 g. of the crude hydrazide in 50 ml. of cold glacial acetic acid, contained in a 500-ml. 3-neck flask equipped with dropping funnel and mechanical stirrer, was added a cold mixture of 6.7 ml. of concentrated hydrochloric acid and 50 ml. of water. Addition of this solution caused at least partial precipitation of the hydrazide. Immediately following this, a solution of 2.76 g. of sodium nitrite in 7 ml. of water was rapidly added. A tarry substance separated and the reaction mixture was extracted with chloroform and the chloroform extract washed with aqueous sodium bicarbonate and dried over anhydrous magnesium sulfate. The extract was then added to 200 ml. of ethanol and the mixture refluxed for six and one-half hours. Removal of the volatile material left a dark red syrupy substance.

The tar originally formed in the reaction mixture had been observed to evolve a gas and change into a porous solid. The original aqueous acidic phase, containing the tar, was made neutral and heated under reflux for 4-5 hours. The porous solid, resembling peat moss, was collected, washed with water, and dried. This substance weighed 7.33 g. and appears to be the major reaction product. Neither the porous product, nor the red syrup obtained from the chloroform phase after the ethanol treatment was identified.

Action of Chymotrypsin upon N-Acetyl-DL-alpha-methylphenylalanine Methyl Ester

I- 0.0235 g. of N-acetyl-DL-alpha-methylphenylalanine methyl ester and 0.020 g. of alpha-chymotrypsin (Armour lot no. 10705) were incubated for a period of one hour at 25° in 10 ml. of aqueous solution. A formol titration[a] with aqueous 0.01 F sodium hydroxide indicated that approximately 14.5 per cent of the total ester had been hydrolyzed in this time interval.

II- 7.08 g. of N-acetyl-DL-alpha-methylphenylalanine methyl ester was dissolved in ca. 500 ml. of 30 per cent aqueous methanol and treated with 50 mg. of alpha-chymotrypsin and a small amount of "Viobin" paste at 30-35°. Following a small initial drop in pH upon the addition of the enzyme, no significant additional hydrolysis occurred over a 6-hour period. The methanol was then removed from the reaction mixture by evaporation and the attempt at resolution continued in 1600 ml. of aqueous solution. 8 days of treatment with "Viobin", added at intervals, caused no apparent hydrolysis beyond that accounted for by the autolysis of the enzyme.

Attempted Papain Catalyzed Synthesis of N-Acetyl-L-alpha-methyltyrosine-p-toluidide- The method employed was that of Huang and Niemann(44).

To a solution of 0.73 g. of N-acetyl-DL-alpha-methyl-tyrosine in 28 ml. of 0.5 M acetic acid-0.5 M sodium acetate buffer was added 0.037 g. of L-cysteine hydrochloride and 0.33 g. of

[a] See Section I, experimental, of this thesis.

p-toluidine (Merck). The resulting solution was warmed to ca. 40°, 0.75 g. of an aqueous papain solution[44] added, and the mixture incubated in an oven maintained at 40°. After 7 days of storage at this temperature no significant amount of solid material had been formed.

As a check, a control experiment under the above conditions was performed upon N-acetyl-DL-phenylalanine. After standing one day at 40° a crystalline precipitate of N-acetyl-L-phenylalanine-p-toluidide was clearly evident.

Attempted "Viobin" Resolution of O-Methyl-DL-alpha-methyltyrosine Methyl Ester

2.73 g. of O-methyl-DL-alpha-methyltyrosine methyl ester, 0.2 g. of "Viobin", and 0.22 g. of water were mixed into a paste, stoppered in a 50-ml. flask, and stored at room temperature. After standing for 5 days, the mixture was apparently unchanged and tested pH 7 with indicator paper.

Attempted Chymotryptic Resolution of N-Acetyl-DL-O-methyl-alpha-methyltyrosine Methyl Ester

I- 2.65 g. of the DL ester was partially dissolved in a mixture of 45 ml. of methanol and 150 ml. of water, the mixture adjusted to pH 8, and treated with 100 ml. of alpha-chymotrypsin (Armour lot no. 10705) at 30°. No significant amount of hydrolysis occurred on 12-15 hours standing.

A solution of 1.0 g. of papain and 0.3 g. of L-cysteine hydrochloride in 5 ml. of water was added to the reaction mixture and the pH adjusted to 5.5. Five hours of incubation at 25-30° failed to effect any resolution.

II- 0.25 g. of the DL ester and 0.25 g. of Armour alpha-chymotrypsin, in 78 ml. of aqueous solution, were incubated overnight at room temperature. No decrease in the pH of the reaction mixture was observed to occur.

Attempted Resolution of N-Acetyl-DL-O-methyl-alpha-methyltyrosine by Fractional Crystallization of the L-Brucine Salts

The method of Sealock[45] was employed.

10.0 g. of N-acetyl-DL-O-methyl-alpha-methyltyrosine and 15.7 g. of L-brucine were dissolved in 90 ml. of boiling absolute ethanol and the resulting solution filtered. The filtrate was then stored in the refrigerator. No crystallization had occurred after standing for four days. Nor did replacing the ethanol solvent by ethanol-ether, ethanol-water, or butanol-chloroform cause the crystallization of any material.

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Propositions

1. Alpha-amino nitriles undergo decomposition in the presence of base to give a carbonyl compound having one less carbon atom. Since alpha-amino acids may be converted into their corresponding alpha-amino nitriles by conversion first to the N-phthaloyl derivatives, reaction with thionyl chloride followed by ammonia to give the amides, dehydration of these to the nitriles by phosphorus pentoxide, and finally removal of phthaloyl with hydrazine, I propose the use of this reaction sequence as a method of degradation of alpha-amino acids into their corresponding carbonyl products of one less carbon atoms.

Lucas, H.J., and Pressman, D., "Principles and Practice in Organic Chemistry", Wiley, New York, 1950.

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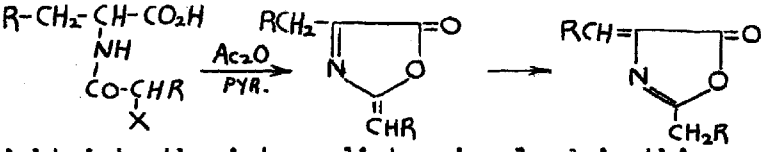
2. Tryptamine has been suggested as a possible intermediate in the conversion of tryptophane to IAA in plants. Recently, Jones et al. have isolated 3-indoleacetonitrile from cabbage leaves. Since the nitrile may arise from the same imino precursor proposed for 3-indoleacetaldehyde and IAA, I propose that the occurrence of the nitrile in plants strengthens the hypothesis that IAA may arise from tryptophane via tryptamine.

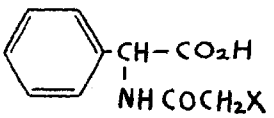
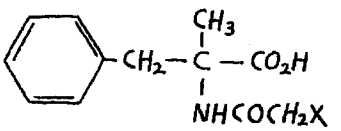
Bonner, J., "Plant Biochemistry", Academic Press, New York, 1950.

Jones, E.R.H., et al., Nature, 169, 485 (1952).

3. Bergmann and Stern have proposed the following mechanism for the dehydrohalogenation of an alpha-(alpha'-haloacyl)-amino acid by acetic anhydride.



I propose that insight into the intermediates involved in this reaction might be gained by treating the following types of compounds with acetic anhydride and pyridine.

(1)  and (2) 

Bergmann, M., and Stern, F., Ann. 448, 20 (1926).

4. D alpha-acylamino acid esters and amides have been considered to combine with alpha-chymotrypsin in the same mode as do their L enantiomorphs. This implies that the hydrolyzable bond of a D ester or amide makes a potentially hydrolytic interaction in the EI complex. I propose that this hypothesis be further investigated by studying, as possible substrates of chymotrypsin, D and L alpha-acyl amino acid esters substituted at the alpha-carbon by strongly electron-attracting groups.

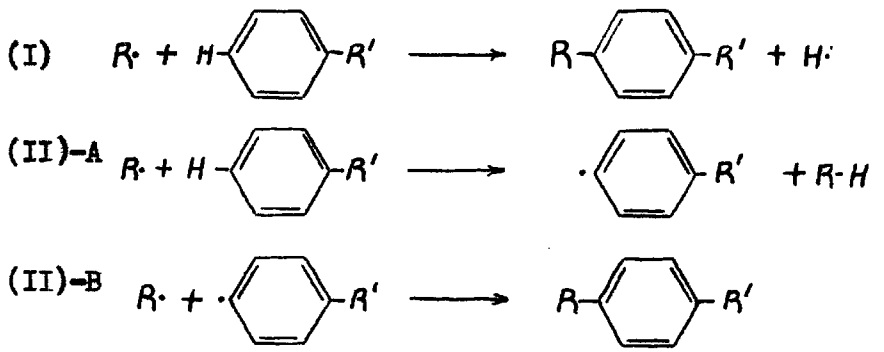
Huang, H.T. and Niemann, C., J.A.C.S. 74, 4634 (1952).

5. Wagenknecht and Burris report that IAA is oxidatively degraded by IAA oxidase, releasing one molecule of carbon dioxide per molecule of oxygen taken up whereas 3-indolepropionic acid releases only

one-quarter mole of carbon dioxide per mole of oxygen. While these authors maintain that the oxidation is limited to the side chain in IAA, I propose that the above observations are in better agreement with an opening of the indole nucleus to give, in the case of IAA, a beta-keto acid.

Wagenknecht, A.C., and Burris, R.H., Arch. Biochem. 25, 30 (1950).

6. Two principal mechanisms have been advanced for homolytic aromatic substitution.



In the case of R = CH_3 , pathway II would proceed with formation of methane. I propose that a measurement of such evolved hydrocarbon might be used to estimate the relative contributions of the two mechanisms. The formation of methane by coupling of R· and H· could be eliminated by the proper experimental conditions.

Hey, D.H., J. Chem. Soc. 1974 (1952).

Johnson, G.R.A., Stein, G., and Weiss, J., J. Chem. Soc. 3275 (1951).

7. Nitrobenzene, chlorobenzene, and phenol have been hydroxylated in aqueous solution by either the action of ionizing radiations or by an acidic ferric ion-hydrogen peroxide solution. The isomer distributions resulting from these hydroxylations have been found to

depend upon (1) the method of hydroxylation, and (2) the pH of the reaction mixture. I propose that this behavior is inconsistent with the simple free radical mechanism accepted by these authors.

Loebl, H., Stein, G., and Weiss, J., J. Chem. Soc. 2074 (1949); 2704 (1950).

Johnson, G.R.A., Stein, G., and Weiss, J., J. Chem. Soc. 3275 (1951).

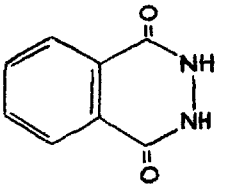
Stein, G., and Weiss, J., J. Chem. Soc. 3265 (1951).

8. I propose that the withdrawal symptoms characteristic of narcotic habituation may be the result of an adaptive enzyme mechanism.

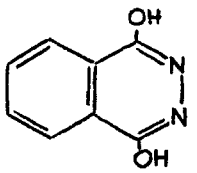
9. Gramine may be condensed with methylcyanoacethydrazide and ethyl methylcyanoacetate to give the corresponding 3-indolylmethyl substituted compounds. I propose that an attempt be made to condense gramine with the 4-alkyl derivatives of 2-(p-nitrophenyl)-oxazolone-5 as a direct route to the DL-alpha-alkyltryptophanes. The possibility of generalizing the use of these oxazolones to prepare other alpha-alkyl amino acids should also be investigated.

This Thesis, pages 137-138.

10. Sheehan and Frank, Barber and Wragg, and Ing and Manske give the following structure for phthalhydrazide



I propose that the acidic nature of this compound as evidenced by its solubility in dilute aqueous base and by its ability to form a salt with n-propylamine are in better accord with the tautomeric structure



Moreover, the formation of such a resonance stabilized compound would account for the great tendency for hydrazine to remove the phthaloyl group from phthalimido acid type compounds.

Ing, H.R., and Manske, R.H.F., J. Chem. Soc. 2348 (1926).

Barber, H.J., and Wragg, W.R., Nature 158, 514 (1946).

Sheehan, J.C., and Frank, V.S., J.A.C.S. 71, 1856 (1949).