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I N T R O D U C T I O N

α-Amino acid acylases are peptidases capable of hydrolyzing the peptide bonds of N-acylated amino acid derivatives. The enzymic hydrolysis of such amino acid derivatives was first observed by Schmiedeberg (1881) in aqueous extracts of animal tissues. The enzyme was originally called "histozyme" (Schmiedeberg, 1881); and later "hippuricase" (Clementi, 1923) since hippuric acid was used as the test substrate in the early investigations. The more general notation "aminoacylase" was suggested by Smorodinzev (1923). The nomenclature "amino acid acylase", which is now generally accepted, was suggested by Greenstein and coworkers since an enzyme concentrate from hog kidney was shown to hydrolyse most N-acylated L-amino acids (Levintow, Greenstein and Kingsley, 1951; Birnbaum, Levintow, Kingsley and Greenstein, 1952).

I. DISTRIBUTION

Enzymes that hydrolyse N-acylated α-amino acids are widely distributed. They occur in bacteria (Reis and Swensson, 1931; Imaizumi, 1938) in fungi (Dox, 1910; Kossowicz, 1912; Neuberg and Rosenthal, 1924; Grassmann and Basu, 1931; Michi and Nonaka, 1954a) and in the organs of higher animals (Smorodinzev, 1923), the kidney being particularly rich (Neuberg and Linhardt, 1924; Price and Greenstein, 1948; Fodor, Price and Greenstein, 1949).

II. STUDIES ON ACYLASES OF ANIMAL TISSUES

Smorodinzev (1923) observed that the enzyme of animal tissues possessed marked optical specificity in that it hydrolysed N-benzoyl α-L-aminobutyric acid and failed to act on the N-benzoyl-D-aminobutyric acid. Further he found that the enzyme acted only on N-acyl derivatives of α-amino acids while N-acylated β-amino acids like benzoyl-β-alanine and benzoyl-β-aminobutyric acid were not attacked.

Extensive studies on the properties of renal acylases were carried out by Greenstein and his associates

(cf. Greenstein, 1954). The observation that N-acylated DL-alanine is asymmetrically hydrolyzed by crude hog kidney preparation (Price and Greenstein, 1948; Fodor, Price & Greenstein, 1949) led this group of workers to the development of a general and simple procedure for the resolution of racemic amino acids. The procedure was based upon the action of a concentrated enzyme preparation obtained from hog kidney which asymmetrically hydrolyzed most N-acylated-DL-amino acids rapidly to give rise to N-acyl-D-amino acid, L-amino acid and the acyl acid. The L-isomer of the amino acid was then separated from acyl-D-amino acid either by virtue of the solubility of the derivative in organic solvent and the insolubility of the free amino acid in the solvent or in special cases by chromatographic separation on ion exchange resins (Baker and Sober, 1953). The D-amino acid was obtained from the N-acyl-D-amino acid by acid hydrolysis. Such asymmetric hydrolysis of N-acylated-DL-amino acids has in recent years been extensively used in the preparation of both enantiomorphs of some 40 α-amino acids.

A) <u>Purification of renal acylases</u> :

The observation of Price, Gilbert & Greenstein (1949) that the rates of hydrolysis of acetyl-DL-alanine by

crude homogenates of rat and hog kidneys were 24 μM and 350 μM per hr. per mg. protein nitrogen respectively, led to the use of hog kidneys for the preparation of acylase. A six- to ten-fold increase in activity towards N-acylated-DL-alanine over the crude tissue homogenate was obtained by alcohol fractionation at low temperature (Price, Gilbert & Greenstein, 1949). This preparation was used for the resolution of racemic methionine, valine, threonine, isoleucine, serine, leucine, aspartic and glutamic acids (Price, Gilbert & Greenstein, 1949), norvaline, norleucine, α-amino butyric acid and lysine (Greenstein, Gilbert & Fodor, 1950; Levintow, Greenstein & Kingsley, 1951) allothreonine (Greenstein and Levintow, 1950) and ornithine (Levintow and Greenstein, 1951) α-amino caprylic and α-amino lauric acids (Levintow, Price and Greenstein, 1950).

(i) Acylases I and II :

A more active acylase from hog kidney was obtained by Birnbaum, Levintow, Kingsley and Greenstein (1952) by a procedure involving fractionation with ammonium sulphate and acetone. Two fractions, acylase I and acylase II were obtained by this modified method of fractionation, comprising of the following steps : The kidney homogenate was adjusted to pH 4.7 at 0°C. and the

inactive precipitate discarded after centrifugation. The supernatant was adjusted to pH 6.5 and treated with 266 g. solid ammonium sulphate per litre of solution. The resulting precipitate, which contained most of the acylase I fraction, was removed after centrifugation and the solution preserved for the preparation of acylase II. The precipitate containing acylase I activity was suspended in ice-water and dialysed against water to remove ammonium sulphate. The dialysed solution was adjusted to pH 5.9 - 6.0 and fractionated with acetone at -10° to -15° when acylase I separated between 0.4 to 0.6 vol. acetone concentrations. Acylase I contained about one-half of the original activity of the crude homogenate towards acetyl-DL-methionine. Acylase I hydrolysed all the N-acylated amino acids which were studied at a rate roughly 30 times greater than that observed with the crude homogenate, with the exception of acylated aspartic acid. This substance was hydrolysed at a much slower rate than by the crude homogenate.

Acylase II fraction was precipitated from the supernatant fluid after the initial treatment with solid ammonium sulphate by the further addition of 150 g. solid ammonium sulphate per litre of solution. Acylase II showed an activity against N-acetylated aspartic acid derivatives some three to five times greater than the crude homogenate.

Acylase II in turn had little or no effect on N-acylated glutamic acid and other N-acylated amino acids.

Acylase I preparation has been used for the resolution of racemic alanine, α-aminobutyric acid, valine, norvaline, leucine, norleucine, isoleucine, alloisoleucine, serine, threonine, allothreonine, methionine, ethionine, lysine, ornithine, phenylalanine and glutamic acid (Birnbaum, Levintow, Kingsley and Greenstein, 1952) isovaline (Baker, Fu, Birnbaum, Sober and Greenstein, 1952), arginine, histidine and S-benzyl-cysteine (Birnbaum and Greenstein, 1952), α-aminoheptylic, α-aminocaprylic, α-aminononylic, α-aminodecylic, α-aminoundecylic and α-aminododecylic acids (Birnbaum, Fu and Greenstein, 1953; Fu and Birnbaum, 1953), homoserine and S-benzyl-homocysteine (Birnbaum and Greenstein, 1953), α-amino-cyclohexylacetic acid, α-amino-β-cyclohexylpropionic acid and α-amino-phenylacetic acid (Rudman, Meister and Greenstein, 1952), isovaline (Baker, Fu, Birnbaum, Sober and Greenstein, 1952) and α, γ-diamino-butyric acid (Fu, Rao, Birnbaum and Greenstein, 1952).

(ii) <u>ϵ-lysine acylase</u> :

Recently Paik, Bloch-Frankenthal, Birnbaum, Winitz and Greenstein (1957) reported an enzymic system in rat kidney which hydrolyzes ϵ-acetyl-L-lysine. The

enzyme was purified 100-fold by adsorption on calcium phosphate gel, elution with 0.1 M phosphate buffer at pH 7.2 and precipitation from the eluate with ammonium sulphate. ϵ-lysine acylase was distinct from acylase I as ϵ-acylated lysine derivatives are resistant to hydrolysis by acylase I. ϵ-Lysine acylase was more active towards ϵ-chloroacetyl-L-lysine than towards ϵ-acetyl-L-lysine and completely inactive towards ϵ-acetyl-D-lysine.

B) Relationship of the structure of N-acylated amino acids to hydrolysis by renal acylases :

Data concerning the relationship of the structure of N-acylated amino acids to hydrolysis are summarized below :

(a) Acylase I activity :

(1) Susceptibility of derivatives of α-amino acids :

The enzyme acts only on the N-acyl derivatives of α-amino acids, acyl groups in other positions being resistant to hydrolysis. Thus α,γ - α,δ , - and α,ϵ - acylated derivatives of the L-forms of α,γ di-amino butyric acid, ornithine and lysine, respectively, yield on hydrolysis the corresponding ω-acyl-L-amino acid (Fu, Rao, Birnbaum

and Greenstein, 1952; Levintow and Greenstein, 1951; Birnbaum, Levintow, Kingsley and Greenstein, 1952).

(2) <u>Susceptibility of derivatives of α-amino acids with free - COOH group</u> :

Free α-carboxyl group on the terminal amino acid was found to be essential for the enzymatic action of acylase I. Thus N-chloroacetyl-DL-alanine amide was found to be completely resistant to hydrolysis and the corresponding ethyl ester was hydrolysed at a very slow rate, the slow hydrolysis of the latter compound being due probably to to esterases present as impurity in the acylase preparation (Fu, Birnbaum and Greenstein, 1954).

(3) <u>Influence of the nature of the amino acid residue and the acyl radical</u> :

Studies on the relative susceptibility of a wide variety of N-acylated amino acids to the hydrolytic action of hog kidney acylase I have shown that : (a) with the same acyl radical the hydrolysis rates vary widely with the nature of the amino acid residue, and (b) with the same amino acid the rates vary with the nature of the N-acyl group.

(i) <u>Optical configuration of the terminal amino acid.</u>

Most N-acylated DL-amino acids are asymmetrically hydrolysed by the enzyme, the derivative of the L-amino acid being the susceptible substrate. In actual isolation procedures the level of possible contamination of one optical isomer by the other have been shown to be less than 1 part in 1000 (Meister, Levintow, Kingsley and Greenstein, 1951; Greenstein, 1954). Birnbaum, Levintow, Kingsley and Greenstein (1952) observed that with very high concentrations of acylase I and with long periods of incubation, however, appreciable hydrolysis of certain N-acylated D-amino acids like chloroacetyl D-methionine occurred, the pure acylated D-form being hydrolysed 10000 times more slowly than the corresponding acyl-L-isomer. In the presence of the corresponding free amino acids the hydrolysis of the acylated D-form was even slower, being inhibited to about one-fourth. The presence of the acylated D-amino acid component of the racemate on the other hand, had little or no effect on the rate of hydrolysis of the susceptible acylated L-amino acid component. Fones and Lee (1954) observed, however, that the optical specificity of the enzyme was markedly reduced by the introduction of the N-trifluoroacetyl group in place of the usual acetyl or chloroacetyl radical. Thus the ratio of the rate of hydrolysis of the trifluoroacetyl L-

and -D-alanines was about 90 : 1 which is in marked contrast to the ratio 20,000 : 1 found for the corresponding chloroacetyl derivatives while the substitution of the trifluoroacetyl group for acetyl or chloroacetyl in the case of L- and D-methionines lowered the ratio of the rates of hydrolysis of the L- to D-isomer from about 10,000 : 1 to 3 : 1. Only in the case of the branched chain amino acids, valine and leucine and ω-benzoyl lysine were the trifluoroacetyl derivatives of the D-enantiomorphs not hydrolysed at a measurable rate.

(ii) <u>Influence of the nature of the terminal amino acid</u> :

Kidney acylase I acts on a broad range of substrates. Birnbaum, Levintow, Kingsley and Greenstein (1952) observed that the relative susceptibility of a number of N-acylated amino acids varied widely, the rates decreasing in the following order : methionine > norvaline > amino butyric acid > norleucine > leucine > glutamic acid > alanine or serine > valine > glycine > threonine > phenylalanine > tyrosine > lysine > cysteine > tryptophane > proline. The aromatic substituted amino acids phenylalanine, tyrosine and particularly tryptophane were acted on by the renal enzyme at a markedly slow rate (Price, Gilbert and Greenstein, 1949). The range of the rates of hydrolysis will be evident

from the observed rates of 88000, 14800, 460, 33 and 12 for chloroacetyl derivatives of methionine, alanine, phenylalanine, tyrosine and tryptophane, respectively. In contrast with all the N-acylated amino acids studied, chloroacetyl-L-tryptophane was not completely hydrolysed by the enzyme, the maximum extent of hydrolysis increasing with increasing dilution of the substrate, the values being 48 per cent at 0.1 M substrate concentration and a maximum of 90 per cent at 0.03 M level (Rao, Birnbaum, Kingsley and Greenstein, 1952).

In the straight chain aliphatic, homologous series, i.e. glycine, alanine, α-amino-n-butyric acid, α-amino-n-heptylic acid, α-amino-n-caprylic acid, α-amino-n-nonylic acid, α-amino-n-decylic acid, α-amino-n-undecylic acid and α-amino-n-dodecylic acid the N-acylated derivatives were found to be split at regularly increasing rates to a maximum at n-valine, the rate of hydrolysis decreasing with the longer chain derivatives (Baker and Meister, 1951; Fu and Birnbaum, 1953; Birnbaum, Fu and Greenstein, 1953). Branching of the amino acid chain invariably depressed the rate below that of the corresponding straight chain isomer, the rates for chloroacetyl derivatives of norvaline and valine being 40500 and 4970 respectively, and for norleucine, leucine and isoleucine, 30400, 16500 and 376, respectively. Chloroacetyl-L-*tert*-leucine, which possesses a completely

substituted β-carbon was not attacked at all (Izumiya, Fu, Birnbaum and Greenstein, 1953).

When the α-hydrogen atom of the homologous series of straight chain α-amino acids was replaced by a methyl group, as in N-chloroacetyl isovaline (Baker, Fu, Birnbaum, Sober and Greenstein, 1952), N-chloroacetyl-DL-α-amino isobutyric acid, N-chloroacetyl-DL-α-amino-α-methyl-n-butyric acid, N-chloroacetyl-DL-α-amino-α-methyl-n-valeric acid and N-chloroacetyl-DL-α-amino-α-methyl isovaleric acid (Fu and Birnbaum, 1953) markedly lowered rates of hydrolysis were observed. Substitution by an ethyl or larger group e.g. N-chloroacetyl-α-amino-α-ethyl-n-butyric acid completely inhibited enzymatic hydrolysis (Fu and Birnbaum, 1953).

Replacement of the peptide hydrogen by an alkyl group e.g. N-chloroacetyl-N-methyl-DL-alanine and N-chloroacetyl-N-ethyl-DL-alanine, was more critical and no detectable splitting occurred (Fu and Birnbaum, 1953).

In the case of allostereoisomers e.g. of threonine and isoleucine, the rates of hydrolysis were found to be different from that of the corresponding normal forms (Birnbaum, Levintow, Kingsley and Greenstein, 1952).

(iii) Influence of the nature of the acyl radical :

The influence of the nature of the acyl radical on the rate of hydrolysis of an acylated amino acid by acylase I is expressible in terms of three properties, namely : (a) electronic (b) steric and (c) optical (Fu, Birnbaum and Greenstein, 1954).

For a study of the enzymatic susceptibility of several types of N-acyl substituents a variety of such derivatives of alanine and glycine were prepared by Fodor, Price and Greenstein (1950). Of the formyl, acetyl, chloroacetyl, propionyl, dl-chloropropionyl and benzoyl derivatives of alanine and glycine the most susceptible was the chloroacetyl followed by the acetyl or propionyl, formyl, dl-chloropropionyl and benzoyl

These studies were extended and the relation of the acyl structure in the substrate to enzyme susceptibility elucidated further by Fones and Lee (1953). The data obtained by these workers showed that the rate of hydrolysis of the monohalogen acetyl-alanines and -phenylalanines was influenced by electronic factors, the rate decreasing qualitatively in the order of decreasing electronegativity of the halogen atom, that is in the order fluoroacetyl $>$ chloroacetyl $>$ bromoacetyl $>$ iodoacetyl. However, the

rate of decrease was much greater in the case of the bromoacetyl and iodoacetyl compounds than would be expected from decreases in electronegativity of the halogen atom alone; in fact these two derivatives were hydrolysed slower than the less electronegative acetyl and propionyl derivatives. Fones and Lee (1953) suggested the possible operation of a steric effect in the approach of the N-acyl derivatives containing the bulkier bromine and iodine atoms to the enzyme surface. Further support for this hypothesis was the finding that dichloroacetyl derivatives of alanine and phenylalanine were hydrolysed at markedly slower rates than the amino acids containing the less electronegative, monohalogen group, while the trichloroacetyl derivatives were actually resistant to hydrolysis. The derivatives with the trifluoroacetyl group, which was the most electronegative group studied, were hydrolysed at the fastest rate, apparently due to a minimal steric hindrance in this compound on account of the diameter of the fluorine atom being nearer to that of hydrogen than to that of chlorine. The data suggested that both electronic and steric factors are involved in the influence of the acyl group on the susceptibility of the substrate. Further data obtained by Fones and Lee (1954) on the rates of hydrolysis of N-trifluoroacetyl derivatives of several L- and D-amino acids indicated that the two factors : (a) nature of the amino acid and (b) nature of the acyl radical which influence susceptibility of a particular amino

acid are not independent but are interrelated. Thus when the acyl group was trifluoroacetyl, the effect on the rates of hydrolysis of the L- and D-isomers of methionine is much greater than for those amino acids containing only carbon. Further if the carbon chain was branched as in valine or leucine, the ability of the trifluoroacetyl group to lower the optical specificity of the enzyme was lost.

Rao, Birnbaum, Kingsley and Greenstein (1952) observed that renal acylase I acts upon N-acylated amino acids with a free α-amino group on the acyl radical and that the relative order of hydrolytic rates among the glycyl amino acids was parallel to that among the corresponding chloroacetyl amino acids. When the acyl radical was the alanyl group, the L-alanyl amino acids were found to be more susceptible than the D-alanyl acids, the ratio of hydrolytic rates of pure L-alanyl to D-alanyl derivatives being about 300 to 500 : 1 (Fu, Birnbaum and Greenstein, 1954). Substitution of the amino group by halogen in the alanyl radical markedly reduced the influence of optical configuration in the acyl group on the hydrolytic rates, the L-chloropropionyl amino acids being hydrolysed at rates only 2 to 12 times that of the corresponding D-chloropropionyl derivatives.

(b) <u>Acylase II activity</u> :

Like acylase I the enzyme is optically specific

and hydrolyzes only N-acylated derivatives of L-aspartic acid (Birnbaum, Levintow, Kingsley and Greenstein, 1952). Acylase II is specifically an aspartic acid acylase and acts upon acylated aspartic acid derivatives such as N-chloroacetyl-L-aspartic acid in which an acyl radical does not possess a free α-amino group. Glycyl-L-aspartic acid is hydrolyzed at a very slow rate. Substitution of the β-carboxyl group of acylated aspartic acid, as in chloroacetyl-L-asparagine also renders the substrate resistant to acylase II (Rao, Birnbaum, Kingsley and Greenstein, 1952). N-acylated-L-asparagine, however, is readily hydrolyzed by acylase I.

C) Comparison of Kidney acylase I to pancreas carboxypeptidase :

Renal acylase I, like pancreatic carboxypeptidase, requires the presence of a free α-carboxyl group (Fu and Birnbaum, 1953). The two enzymes are distinct however with regard to optical specificity and the general nature of the amino acids and acyl groups in the susceptible N-acylated amino acids. Renal acylase I has been shown to be more effective towards acylated derivates of aliphatic amino acids whereas pancreatic carboxypeptidase is more active towards derivatives of aromatic amino acids (Birnbaum, Levintow, Kingsley and Greenstein, 1952). Further, kidney acylase I hydrolyzes N-chloroacetyl amino-cyclohexyl-propionic

acid at about the same rate as the corresponding aromatic derivative, N-chloroacetylphenylalanine, while carboxypeptidase readily hydrolyzes only the aromatic derivative.

Renal acylase also hydrolyzes N-acylated-L-dehydroalanine derivatives (Rao, Birnbaum and Greenstein, 1953) while carboxypeptidase is ineffective towards this class of compounds (Bergmann and Schleich, 1932).

Renal acylase I is less sensitive to the nature of the acyl group in the substrate than pancreatic carboxypeptidase (Birnbaum, Levintow, Kingsley and Greenstein, 1952; Fones and Lee, 1953). Thus the ratios of the hydrolytic rates of chloroacetylphenylalanine to acetyl-phenylalanine and of trifluoroacetylphenylalanine to acetyl-phenylalanine are 3 to 4 : 1 and 40 : 1, respectively, the corresponding ratios with the carboxypeptidase being 150 : 1 and 5000 : 1, respectively.

As described earlier, the optical specificity of acylase I is reduced markedly in N-trifluoroacetyl derivatives of several amino acids. On the other hand, the attachment of the trifluoroacetyl radical to aromatic amino acids has no effect on the optical specificity of pancreatic carboxypeptidase (Fones and Lee, 1953).

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D) Identity of diisopropylfluorophosphatases to acylase I :

Mounter (1956) has reported recently the identity of the enzyme (DFPase), which hydrolyzes diisopropylfluorophosphate and related compounds, and hog kidney acylase I. Similarity in solubility, stability to temperature, pH and ethanol, activation by metal ions and pH optima indicated that a single enzyme was involved. Both hydrolyses were similarly affected by a number of noncompetitive inhibitors.

E) Cobalt ion activation of renal acylase I :

Rao, Birnbaum, Kingsley and Greenstein (1952) noticed that added Co^{++} influences the hydrolysis of a number of acylated amino acid derivatives by renal acylase I. Added Co^{++} accelerated the hydrolytic rates of chloroacetyl and glycyl derivatives of the less susceptible L-amino acid substrates but inhibited hydrolysis of the more susceptible L-amino acid substrates even at very low concentrations (Rao, Birnbaum, Kingsley and Greenstein, 1952). Thus at $1 \times 10^{-3}M$ Co^{++} concentration, the hydrolytic rate of N-chloroacetyl-L-aspartic acid was increased by 610 per cent and of N-chloroacetyl-L-threonine, 33 per cent, while that of chloroacetyl-L-methionine was reduced by 62 per cent. Versene at $1 \times 10^{-3}M$ concentration was found to have no effect on

the activity of acylase I (Marshall, Birnbaum and Greenstein, 1955). All acetyl-D-amino acids susceptible to the action of acylase I were hydrolyzed at a greatly accelerated rate by this enzyme in the presence of added Co^{++} (Marshall, Birnbaum and Greenstein, 1956). Marshall, Birnbaum and Greenstein (1956) also observed that acylase was fully active after dialysis and contained no demonstrable Co^{++}. However when Co^{++} was added to acylase I and the mixture dialysed, centrifuged and lyophilized, the residual protein contained Co^{++} and hydrolyzed all substrates at a rate higher than that of native acylase I. The activity of the cobalt - treated acylase was not affected by versene at 1×10^{-3}M concentration.

Co^{++} has no effect on the activity of acylase II (Rao, Birnbaum, Kingsley and Greenstein, 1952).

III. STUDIES ON MOULD AND BACTERIAL ACYLASES

Although the isolation and properties of acylases of animal tissues have been extensively studied in recent years, very little is known about the enzyme from mould and bacterial sources.

The presence of an enzyme that hydrolyzes N-acetylated amino-acids has been shown in several mould

strains. Dox (1910) showed the presence of a hippuric acid hydrolyzing enzyme in strains of *Penicillium camemberti*, *P. chrysogenum*, *P. brevicaula* and *Aspergillus niger* grown on czapek medium. Grassmann and Basu (1931) found acylase activity in extracts of bran cultures of *Aspergillus oryzae*.

Neuberg and Linhardt (1924) observed that the acylase ("hippuricase") in takadiastase, a crude commercial enzyme product from mould bran cultures of *A. oryzae*, hydrolyzed benzoyl-DL-alanine asymmetrically, the benzoyl-D-alanine being isolated from the enzyme digest. The isolation of L-alanine from such an enzymic digest was reported by Hoppert (1924). More recently the isolation of D- and L-amino acids obtained by enzymatic resolution of N-acylated DL-amino acid derivatives has been described by Neuberg and Mandl (1950). Several commercially available enzyme preparations from mould takadiastase were used in this work in amounts varying from one-fourth to an equal part by weight of the N-acylated-DL-amino acid substrate. The digestion periods were 4 to 10 days at 37°C and the pH was maintained at 7 by adding excess calcium carbonate. Optically pure isomers of methionine, tryptophane, phenylalanine, isoleucine, aspartic acid, lysine and tyrosine were prepared by this procedure.

Ellis and Walker (1942) studied the rates of hydrolysis of hippuric acid and various isomeric ring substituted derivatives of hippuric acid by the acylase

("hippuricase") of takadiastase. The nature of the substituents in the benzene ring was found to have little influence on the hydrolytic rates, but the position of the substituent was found to be important. Ortho substitution of the groups was found to inhibit hydrolysis, meta substitution to accelerate hydrolysis and para substitution to have no measurable effect. It is interesting to contrast the results reported by these authors with those obtained by Fones and Lee (1953) using renal acylase I. With the renal enzyme the nature of the substituent was found to affect the hydrolysis rate markedly.

Michi and Nonaka (1954a) surveyed several mould strains for acylase activity towards N-acylated-DL-glutamic acid. Extracts of mould bran cultures were found to have acylase activity, the highest activities being observed in strains of *A. tamarii*, *A. oryzae*, *P. vinaceous*, *P. oxalicum* and *P. corymbiferum*. Enzyme preparations from some of these strains were used for the resolution of DL-glutamic acid and DL-valine (Michi and Nonaka, 1954b). The pH optimum of the enzyme towards N-acetyl-DL-glutamic acid was about 5.5 and towards N-acetyl-DL-alanine and N-chloroacetyl-DL-valine, 7 to 8. Michi and Nonaka (1954b) also reported the use of an acylase concentrate obtained by ammonium sulphate and acetone fractionation in the resolution of these amino acids, although no data on the degree of purity or the properties

of the concentrate appear to have been reported.

Except for reports on the presence of the enzyme in some bacterial strains (Leuthardt, 1951) no work appears to have been done on the acylases from this source.

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PRESENT INVESTIGATION

PRESENT INVESTIGATION

N-Acylated derivatives of α-DL-amino acids are asymmetrically cleaved by the group of enzymes, amino acid acylases. Besides the great ease of manipulation the procedure allows the preparation of both enantiomorphs of almost all α-amino acids investigated. Although the enzymes extracted from animal sources have been studied extensively as will be evident from the foregoing introduction no systematic studies have been reported so far on the concentration and properties of these enzymes from other sources. The present investigation describes the purification and properties of a mould enzyme.

Among 15 different fungi screened for acylase activity towards N-formyl-DL-tryptophane and N-acetyl-DL-alanine, Aspergillus flavus-oryzae NRRL 536 was found to be the best enzyme-producer. No acylase activity was obtained when the moulds were grown in synthetic media. The yield of the enzyme was highest from 62 hour semi-solid mould bran preparations, while yields from liquid media containing bran extract were low, both in stationary and shake cultures. No measurable activity was observed in the culture fluid from the liquid media, all the activity being present in the mycelium.

The enzyme from aqueous extracts of the mould bran was concentrated about 400-fold by the following fractionation procedure :-

i) solvent fractionation with acetone at pH 5.6

ii) ammonium sulphate fractionation at pH 6.0

iii) adsorption on tricalcium phosphate gel followed by elution with ammonium sulphate solution.

iv) ammonium sulphate fractionation of the eluate at pH 6.0

and v) refractionation with ammonium sulphate at pH 7.4

The mould enzyme hydrolyzed N-acylated tryptophane and phenyl alanine derivatives more rapidly than the corresponding alanine derivatives unlike the usual animal tissue preparations which are known to act extremely slow on N-acylated derivatives of aromatic amino acids, particularly of tryptophane. The mould preparation was active towards chloroacetyl, acetyl, and formyl derivatives of tryptophane, phenylalanine, alanine, methionine, histidine, glutamic and aspartic acids; the formyl derivatives being most susceptible and acetyl the least. The susceptibility of N-acyl derivatives of amino acids to the enzyme increased in the following order : aspartic acid, histidine, glutamic acid, alanine, tryptophane, methionine, and phenylalanine.

The influence of pH, temperature and substrate

concentration on the activity of the enzyme was determined.

The acylase from Aspergillus flavus-oryzae was found to be a metal activated enzyme being inhibited by reagents which combine with metals, such as ethylene diamine tetraacetic acid (EDTA), $\alpha\alpha'$-dipyridyl, o-phenanthroline, cyanide and pyrophosphate. The enzyme was completely inactive after treatment with EDTA and dialysis and was reactivated by Fe^{++} and Zn^{++} whereas Co^{++}, Mn^{++}, Mg^{++}, Ca^{++} and Fe^{+++} were without effect.

The isolation of the pure enantiomorphs of tryptophane from the hydrolysate of acetyl-DL-tryptophane was also carried out by using purified preparations of the enzyme.

Part I of this thesis deals with the screening of several strains of fungi for acylase activity and the conditions for optimal yield of the enzyme;

Part II deals with the purification and properties of an enzyme from bran cultures of Aspergillus flavus-oryzae (NRRL 536);

and Part III deals with the preparation of the optical enantiomorphs of tryptophane by the use of the mould enzyme concentrate.

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EXPERIMENTAL

PART - I

SCREENING OF STRAINS OF FUNGI FOR ACYLASE ACTIVITY AND CONDITIONS FOR OPTIMAL YIELD OF ENZYME

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Methods and Materials

Organisms tested :

15 fungal strains were screened for acylase activity. The strains tested were *Aspergillus aureus* NCIM 592, *Aspergillus parasiticus* NCIM 547, *Aspergillus flavus-oryzae* NRRL 536, *Aspergillus oryzae* NRRL 1919, *Aspergillus oryzae* NCIM 428, *Aspergillus wentii* NCIM 591, *Aspergillus niger* NRRL 337, *Aspergillus niger* NRRL 67, *Aspergillus niger* NRRL 330, *Aspergillus niger* NRRL 597, *Penicillium notatum* NCIM 640 and *Penicillium chrysogenum* NCIM 643 and three strains of *Aspergillus niger* isolated in the Laboratory (Isolates NCIM 703, 704, 705).

Maintenance of the organism :

The moulds were maintained at $0^{\circ}C$ on potato-dextrose agar slants which were prepared as follows :

200 g. peeled and cut potatoes were steamed with 700 ml. distilled water for one hour and the extract filtered through cheese cloth. To this extract 20 g. glucose and 30 g. shredded agar were added and the volume was made to 1 litre with distilled water. The medium was steamed for about 1 hour till the agar completely dissolved.

About 10 ml. portions of the medium were taken in pyrex test tubes (6" x 1") and autoclaved at 15 lbs pressure for 20 minutes and slants prepared as usual.

Studies on Synthetic media

Growth of organism for studies on synthetic media :

Active spores were obtained by two successive transfers on Czapek-Dox agar slants of the following composition :

	g./litre
Sodium Nitrate	2.0
Potassium dihydrogen phosphate	0.5
Di-potassium monohydrogen phosphate	0.5
Potassium chloride	0.5
Magnesium sulphate heptahydrate	0.8
Ferous sulphate heptahydrate	0.01
Glucose ('Dextrosol', Corn Products Co.)	40

The pH was adjusted to 6.7 to 7.

The medium was steamed for about one hour with 30 g. shredded agar per litre till the agar dissolved completely.

About 10 ml. portions of the medium were taken in pyrex test tube (6" x 1") and autoclaved at 15 lbs pressure for twenty minutes and the slants prepared as usual.

The Czapek-Dox agar slants were inoculated with a loop of spores from the potato-dextrose-agar slants and incubated at 30°C. Subcultures were made every 5 days.

Studies on acylase production on synthetic media :

All the organisms, except the strains of *Aspergillus niger* and *Aspergillus wentii* were grown on Czapek-Dox liquid medium of the composition given above as well as on a medium of the following composition :

	g./litre
Glucose ('Dextrosol')	120
Ammonium sulphate	2.0
Ammonium nitrate	2.0
Potassium dihydrogen phosphate	1.0
Potassium monohydrogen phosphate	1.0
Magnesium sulphate (heptahydrate)	0.5
Zinc sulphate (heptahydrate)	0.01
Ferrous sulphate (heptahydrate)	0.0005
Manganous sulphate (tetrahydrate)	0.001

pH 5.6 to 5.7

75 ml. portions of the medium were transferred to flasks of 250 ml. capacity and were autoclaved at 15 lbs pressure for 15 minutes.

Spores obtained from five day old cultures on Czapek-Dox agar were suspended in 10 ml. sterile water. 2 ml. of this spore suspension were added to the medium in the flasks. The flasks were incubated at 30°C and the mycelium and culture liquid from 3 days to 9 days old cultures were collected and were assayed separately for acylase activity.

The strains of *Aspergillus niger* and *Aspergillus wentii* were grown on Curries medium of the following Composition :

	g./litre
Glucose ('Dextrosol')	120
Ammonium Nitrate	2
Potassium acid phosphate	1
Magnesium sulphate	0.2

pH 1.8 to 2.0

75 ml. portions were distributed in 250 ml. flasks and the flasks were autoclaved at 15 lbs. pressure for 15 minutes.

The medium was inoculated with 2 ml. spore suspension obtained from Czapek-Dox slants as described

above. The flasks were incubated at 30°C and the mycelia and culture liquid from 3 to 9 days old cultures were separately assayed for acylase activity.

Synthetic media supplemented with bran extract :

The effect of supplements of bran extract was tried since earlier workers have reported acylase ("hippuricase") activity in extracts of mouldy bran (Neuberg and Linhardt, 1924).

Preparation of bran extract :

800 g. wheat bran were ground in a Wiley mill and passed through a 50-mesh sieve. The powdered bran was suspended in 4 litre water and autoclaved 1 hour at 15 lbs pressure. The extract was cooled and strained through cheese cloth.

Supplemented Czapek-Dox Medium :

5 litre of Czapek-Dox medium of the composition given above was mixed with 2.5 litre of wheat bran extract, the pH adjusted to 5.6 and autoclaved at 15 lbs for 10 minutes. The clear supernatant was decanted from the sediment which formed and 75 ml. portions were distributed

in 250 ml. flasks and were autoclaved at 15 lbs. pressure for 10 minutes. All organisms except the strains of A.niger and A.wentii were grown on the supplemented Czapek-Dox media. 2 ml. spore suspension from Czapek-Dox agar slants were added to each flask and were incubated at 30°C. Mat and culture fluid were separately assayed for acylase activity.

Supplemented Curries medium :

5 litre of Curries medium of the composition given above was mixed with 2.5 litre of wheat bran extract. The pH was adjusted to 1.8 to 2.0 and the solution autoclaved at 15 lbs for 5 minutes. 75 ml. portions of the clear supernatant were distributed in 250 ml. flasks and were autoclaved at 15 lbs pressure for 10 minutes. Strains of A. niger and A. wentii were grown on this medium. 2 ml. spore suspension were added to each flask and incubated at 30°C. Mycelia and culture fluid were separately assayed for acylase activity as described already.

Preparation of Bran Cultures

Maintenance of organism :

For bran cultures the organism was grown on malt agar slants prepared as follows :

Difco malt extract was dissolved in 1 litre distilled water to give a solution of specific gravity 1.02 (corresponding to 5 per cent solids). The pH was adjusted to 6.7 and 25 g. shredded agar added to the solution and steamed till the agar dissolved.

10 ml. portions of the medium were taken in pyrex test tubes (6" x 1") and autoclaved at 15 lbs. pressure for 20 minutes and slants were prepared as usual.

The malt agar slants were inoculated with a loop of spores from the stock cultures on potato-dextrose agar and were grown for 10 days at 30°C. Two successive subcultures were made on malt agar slants to obtain active spores.

Preparation of semi-solid bran medium :

450 g. fresh powdered wheat bran was mixed with 550 ml. water and sterilized in an enamelled vessel by autoclaving for 2 hours at 20 lbs pressure. The quantity of water used was just sufficient to moisten the bran. The sterilized bran was spread under sterile conditions in a sterilized shallow enamel tray (18"x 15"x 2") in a uniform layer of about ½" thickness.

Preparation of mouldy bran :

Spores from 10 day old cultures on malt agar slants were used for the mouldy bran preparation, spores from eight malt agar slants being used for each tray containing 1 Kg. moist bran. Spore suspension from each slant was obtained by addition of 10 ml. sterile water and teasing the spores with a sterile platinum loop. The bran was inoculated uniformly, on the trays under sterile conditions. The trays were covered with sterilized filter paper sheets and allowed to stand in a sterile chamber in which the humidity was maintained with shallow trays containing water and the temperature was controlled at 28° to 30°C.

Extraction of mouldy bran :

Mouldy bran after 52, 62 and 72 hours growth was extracted and the extract was assayed for acylase activity. The extraction was as follows :

Approximately 1 Kg. mouldy bran was broken up and taken in a porcelain vessel of about 10-litre capacity, previously cooled to 0°C. 1.5 litre of ice cold toluene-water was added to the mouldy bran and the contents ground in a ball mill with porcelain balls for 1 hour. After the end of this period the wet mouldy bran was a uniform paste

and at a temperature of about 15°C. The pasty mass was strained through cheese-cloth and the aqueous extract filtered overnight at 0°C. Approximately 1.1 litre extract was obtained from 1 Kg. of mouldy bran.

Determination of acylase activity

Preparation of extracts of mycelia :

Mat extracts were obtained by grinding the mycelia with 1 gm. of sand and 20 ml. of toluene-water for 5 minutes at 0°C. The extract was centrifuged at 0°C and the supernatant was used for estimation of acylase activity.

Test system :

N-Acetyl-DL-alanine and N-formyl-DL-tryptophane were used as test substrates. In the screening studies, the test system consisted of 2 ml. $\frac{M}{10}$ neutralized substrate, 0.5 ml. $\frac{M}{15}$ phosphate buffer (pH 6.3) and 0.5 ml. of mat extract (or 1 ml. culture fluid). The digestion was carried for 3 hours at 37°C and 1 ml. aliquots assayed by Sörensen's formol titration for liberated amino acid using formalin at a dilution of 1 part reagent : 2 parts water as recommended by Sisco, Cunningham and Kirk (1941). Recoveries of free

tryptophane and alanine from the test system according to this modified Sörensen's formol titration was uniformly 97 to 100 per cent.

The test system for assay of mouldy bran extracts consisted of 2 ml. $\frac{M}{10}$ neutralized N-acetyl-DL-alanine or N-formyl-DL-tryptophane, 0.6 ml. $\frac{M}{15}$ phosphate buffer (pH 6.3) and 0.4 ml. diluted extract. Readings were taken at 20 minutes by the modified Sörensen's formol titration procedure already described and at levels of less than 10 per cent splitting of the racemate.

RESULTS AND DISCUSSION

Organisms grown on synthetic media :

Both mycelia and culture fluid were inactive in all the organisms tested under the conditions of assay.

Organisms grown on synthetic media supplemented with wheat bran extract :

A measurable activity was obtained only in the case of *A. flavus-oryzae* strain NRRL 536. The results are tabulated below (Table I).

TABLE - I

Acylase activity of *A. flavus-oryzae* 536 grown on Czapek-Dox medium supplemented with wheat bran extract. 75 ml. media incubated at 30°C.

Stationary cultures

Age of culture (days)	Mat weight (g)	N-acetyl-DL-alanine (mM/hr.)		N-formyl-DL-tryptophane (mM/hr.)	
		Mat	Culture fluid	Mat	Culture fluid
3	3.7	0.028	0	0.048	0
4	3.7	0.032	0	0.052	0
5	3.7	0.052	0	0.076	0
6	4.2	0.064	0	0.096	0
7	4.1	0.052	0	0.076	0
8	3.9	0.052	0	0.076	0
9	3.5	0.040	0	0.056	0

Shake culture conditions did not improve yields of acylase.

All the other organisms tested gave no acylase activity.

Bran Cultures :

Heavy growth of *A. flavus-oryzae* NRRL 536 was

obtained by 52 hours in bran cultures. The mouldy bran was light yellow green in colour and growth was obtained throughout the bran mass as a heavy carpet. Bran cultures of A.flavus-oryzae NRRL 536 gave the same total activity after 62 hours and 72 hours growth. The results obtained after 52 hours growth were not uniform and the total activity at this period was lower than after 62 hours of growth. The results obtained after 62 hours of growth are tabulated below (Table II).

TABLE - II

Bran cultures of A.flavus-oryzae NRRL 536 grown at 28° to 30°C for 62 hours.

pH of toluene-water extract, 5.6

Mouldy bran (Wt.)	Extract Vol.	Total activity	
		N-acetyl-DL alanine (mM/hr.)	N-formyl-DL tryptophane (mM/hr.)
1 Kg.	1.1 L	50	80

Comparison of enzyme yields from the supplemented Czapek-Dox medium and bran cultures :

It will be evident from Table I that the calculated weight of mycelium of A.flavus-oryzae NRRL 536 grown on Czapek-Dox medium supplemented with wheat bran extract to yield a

total activity of 1 M/hr. towards N-formyl-DL-tryptophane is approximately 42 Kg. of mat on the sixth day and for a total activity of 1 M/hr. towards N-acetyl-DL-alanine, approximately 64 Kg. mat on the sixth day, the corresponding weight of mouldy bran for a total activity of 1 M/hr. towards N-formyl-DL-tryptophane being approximately 12 Kg. and for a total activity of 1 M/hr. towards N-acetyl-DL-alanine, approximately 20 Kg. (Table II). For these reasons bran cultures were considered a more convenient source of the enzyme. Bran cultures of A. flavus-oryzae NRRL 536 were therefore used as source of the enzyme for the purification and specificity studies reported in Part II.

SUMMARY

(1) Among 15 fungal strains screened for acylase activity towards N-formyl-DL-tryptophane and N-acetyl-DL-alanine, Aspergillus flavus-oryzae NRRL 536 was found to be the best enzyme-producer.

(2) No acylase activity was obtained when the mould was grown in synthetic media.

(3) The yield of enzyme was highest from 62 hour semi-solid mould bran preparations, while yields from liquid media containing bran extract were low, both in stationary

and shake cultures. No measurable activity was observed in the culture fluid from the liquid media, all the activity being present in the mycelium.

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E X P E R I M E N T A L

P A R T - II

PURIFICATION AND PROPERTIES

OF ACYLASE OF ASPERGILLUS FLAVUS-ORYZAE NRRL 536

EXPERIMENTAL

PART - II

PURIFICATION AND PROPERTIES OF ACYLASE OF ASPERGILLUS FLAVUS-ORYZAE NRRL 536

Methods and Materials

SUBSTRATES

N-Formyl amino acids :

The formyl derivatives were prepared according to the procedure described by du Vigneaud and Meyer (1932). The amino acid (0.1 mole,) was dissolved in 90 per cent formic acid (200 ml.). The solution was warmed to 45°C and acetic anhydride (75 ml,) added in small portions. The temperature rose to 70°C during the addition. The solution was allowed to stand at this temperature for 15 minutes and was then diluted with water (200 ml.). The solution was evaporated to dryness. The residue was extracted with ethyl acetate and the extract filtered. The ethyl acetate was removed in vacuo and the residue crystallized on chilling. N-Formyl-DL-tryptophane and N-formyl-DL-phenylalanine were recrystallized from hot water whereas the N-formyl-derivatives of DL-methionine, DL-alanine, DL-aspartic acid and DL-glutamic

acid were recrystallized from acetone.

N-Acetyl amino acids :

The acetylation of DL-alanine, DL-phenylalanine, DL-methionine and DL-histidine was carried out according to the procedure of Knoop and Blanco (1925). The mixture of DL-amino acid (1 mole), glacial acetic acid (12 moles) and redistilled acetic anhydride (1.5 moles) was heated on a boiling water bath for 2 hours. The solution was then evaporated to dryness in vacuo and washed thrice with ice water. The residual oil crystallized on chilling. N-acetyl derivatives of the amino acids were dissolved in ethyl acetate and filtered to remove unreacted material. The ethyl acetate was removed under reduced pressure. The residues crystallized on chilling. N-Acetyl-DL-phenylalanine was recrystallized from hot water and acetone and the remaining derivatives were recrystallized from acetone. N-Acetyl-DL-tryptophane was prepared by the method described by du Vigneaud and Sealock (1932) and N-acetyl-DL-aspartic acid and N-acetyl-DL-glutamic acid were prepared according to Harrington and Overhoff (1930). The methods depend on treating the solution of DL-amino acid (1 mole) in 2N alkali with acetic anhydride (3 moles) and 2 equivalents of 2N alkali. On acidifying the reaction mixture with 6N sulphuric acid, the N-acetyl-DL-tryptophane precipitated and was filtered and washed with

water. The tryptophane derivative was recrystallized from acetone and hot water.

In the case of N-acetyl-DL-aspartic acid and N-acetyl-DL-glutamic acid the acidified solution was evaporated to dryness. The residue was extracted five times with 95 per cent boiling alcohol and the extracts filtered. The alcohol was removed under reduced pressure. The residue which was kept at 0°C in a desiccator under vacuo crystallized after several days.

N-Chloroacetyl amino acids :

N-Chloroacetyl derivatives of DL-alanine, DL-tryptophane and DL-phenylalanine were prepared according to the method described by Fischer and Schoeller (1907) by the treatment of the DL-amino acid (0.56 mole) solution in one equivalent of N alkali with 1.2 mole ethereal solution of chloroacetyl chloride and 1.2 equivalent of N alkali. After the reaction was over the mixture was acidified with 5N hydrochloric acid. N-Chloroacetyl derivatives of DL-tryptophane and DL-phenylalanine separated as oils which were extracted with ether. The ether layer was separated, washed with ice cold water till free from chloride and dried over anhydrous sodium sulphate. The ether layer was then filtered and concentrated to a small volume. The

derivatives were precipitated with petroleum ether and were recrystallized from hot water. The acidified solution in the case of DL-alanine was evaporated to dryness in vacuo. The residue was then extracted seven to eight times with boiling acetone (Fischer and Schulze, 1907). The acetone extracts were cooled and filtered. The acetone was removed under reduced pressure and the residual oil crystallized on chilling.

The N-chloroacetyl derivatives of DL-methionine, DL-aspartic acid and DL-glutamic acid were prepared according to the method described by Birnbaum, Levintow, Kingsley and Greenstein (1952). The DL-methionine (1 mole) in one equivalent of 4N NaOH or DL-aspartic acid (1 mole) or DL-glutamic acid (1 mole) in 2 equivalents of 4N NaOH was treated with 2 moles of chloroacetic anhydride and 2 equivalents of 4N NaOH in small portions. When addition was complete the reaction mixture was adjusted to pH 1.7 with concentrated hydrochloric acid. The N-chloroacetyl-DL-methionine was extracted from the acidified solution with ethyl acetate. The ethyl acetate was removed in vacuo to give an oily residue of the amino acid derivative. The residual oil was dissolved twice in acetone and then evaporated to dryness. The residue was then washed with petroleum ether to remove monochloroacetic acid. The residue of N-chloroacetyl-DL-aspartic acid crystallized from acetone on chilling. A small portion of N-chloroacetyl-DL-glutamic acid crystallized from chloroform and acetone at $0^{\circ}C$. whereas the rest of it remained as an oil.

The properties of the various N-acylated derivatives are summarized in Table III.

TABLE - III

Summary of properties of the N-acylated amino acids tested with the mould acylase.

Derivative	M.P. °C.	N-Cal. per cent	N-found per cent	Yield per cent	Recrystallizing solvent.
N-Formyl-DL-alanine	135	11.97	11.90	90	Acetone
N-Acetyl-DL-alanine	136	10.70	10.81	62	Acetone
N-Chloroacetyl-DL-alanine	92	8.50	8.43	85	Acetone
N-Formyl-DL-methionine	99	7.91	7.72	85	Acetone
N-Acetyl-DL-methionine	110	7.40	7.53	60	Acetone
N-Chloroacetyl-DL-methionine	011*	6.20	6.03	50	
N-Formyl-DL-tryptophane	166	12.07	12.13	65	Hot Water
N-Acetyl-DL-tryptophane	204	11.40	11.19	90	Hot Water
N-Chloroacetyl-DL-tryptophane	152	9.90	9.67	75	Hot Water
N-Formyl-DL-phenylalanine	168	7.25	7.00	86	Hot Water
N-Acetyl-DL-phenylalanine	145	6.80	6.92	80	Acetone
N-Chloroacetyl-DL-phenylalanine	120	5.80	5.68	82	Acetone
N-Formyl-DL-aspartic acid	132	8.70	8.51	80	Ethyl acetate & acetone
N-Acetyl-DL-aspartic acid	140	7.40	7.29	70	Alcohol & acetone
N-Chloroacetyl-DL-aspartic acid	148	6.70	6.64	55	Ethyl acetate & acetone
N-Formyl-DL-glutamic acid	109	8.00	7.83	75	Acetone
N-Acetyl-DL-glutamic acid	170	7.40	7.29	60	Alcohol & Water
N-Chloroacetyl-DL-glutamic acid	122	6.30	6.33	72	Chloroform & Petroleum ether
N-Formyl-DL-histidine	202	22.99	22.61	80	Acetone
N-Acetyl-DL-histidine	142	19.50	19.32	72	Acetone

* N-Chloroacetyl-DL-methionine did not crystallize even after standing for several days at 0°C.

N-Acetyl-DL-alanine and N-formyl-DL-tryptophane were used as test substrates for following enzyme purification during the fractionation procedure.

Enzyme Measurements

The test system consisted of 2 ml. 0.1 M neutralized substrate, 0.6 ml. $\frac{M}{15}$ phosphate buffer (pH 6.3) and 0.4 ml. enzyme solution. The enzyme measurements were made at 37°C.

Acylase activity was determined by Sörensen's formol titration of the liberated amino acid using formalin at a dilution 1 part reagent : 2 parts water as recommended by Sisco, Cunningham and Kirk (1941). The determination of the amount of hydrolysis of the acyl amino acids was also made by the ninhydrin-CO_2 method of Van Slyke, Dillon, MacFadyen and Hamilton (1941) and the Van Slyke nitrous acid procedure (Van Slyke, 1929). Values obtained by the titration procedure were in good agreement with those obtained by the manometric methods.

Protein Measurements

Protein determinations in crude extracts and concentrates were made with the Folin phenol reagent according

to the procedure described by Lowry, Rosebrough, Farr and Randall (1951). Crystalline ovalbumin (Armour and Co.) was used as the working standard and colorimetric measurement were made on a Klett-summerson photometric colorimeter using a 660 mμ filter. Enzyme solutions were diluted to contain 25 to 250 μg. of protein per ml.

Protein measurements in the enzyme concentrates were also carried out according to the optical method of Warburg and Christian (1941). The following empirical formula was used to correct for light absorption due to nucleic acid;

$$\text{mg. protein per ml. solution} = \frac{4}{7}(2.3 \times \text{optical density at } 280\ m\mu - \text{optical density at } 260\ m\mu),$$

the light path being 1 cm. Values obtained by the use of this equation were found to be in good agreement with those obtained by the phenol reagent method of Lowry, Rosebrough, Farr and Randall (1951) and with values calculated from total - N (micro-Kjeldahl).

Calcium Phosphate gel

The gel was prepared by the method of Keilin and Hartee (1951) and was aged for 6 months or longer before use.

RESULTS

PURIFICATION OF MOULD ACYLASE

The following were the results obtained in a typical experiment :

Extraction :

The bran cultures of A. flavus-oryzae NRRL 536 were grown in trays as described earlier in Part I and extraction with tolune-water was also carried out by the same procedure. Three trays, each with an initial charge of 1 Kg. of moist wheat bran, were worked up at a time. 20 to 25 litres of extract from 21 trays of mould bran were worked up finally in every batch, the lots being pooled after acetone fractionation. The pH of the clear reddish brown crude extract was 5.6 (Fraction A). The specific activities of Fraction A were 8.3 μmoles/hr./mg.N and 12.9 μmoles/hr./mg.N against N-acetyl-DL-alanine and N-formyl-DL-tryptophane, respectively; and the total activities 1013 mM/hr. and 1575 mM/hr., respectively, for the two substrates.

Acetone fractionation :

Batches of 4.5 litres or less were worked up at

a time. 4.5 litres of crude extract were stirred in a 10-litre beaker and treated with 500 ml. of pH 5.6 acetate buffer (1M) at 0°C. The beaker containing the buffered extract was chilled in a bath of ice-salt mixture. Addition of acetone was started only when the temperature of the buffered extract was -2°C. and the temperature was allowed to drop to -10°C. during the course of the addition. 2.5 litres of acetone were added from a separatory funnel the tip of which was drawn out to a capillary and extended nearly to the bottom of the beaker. The addition was made over a period of 4 hours with continuous stirring. The resulting precipitate was allowed to settle for two hours at -10°C. As much as possible of the supernatant solution was then siphoned off into a 10-litre beaker at -10°C. and the residual liquid was centrifuged in a refrigerated centrifuge at -10°C. and 2500 r.p.m. for 15 minutes. The clear supernatant obtained on centrifugation was then added to the rest of the supernatant at -10°C. The precipitate was extracted thrice with water at 0°C. and the extracts separated by centrifugation. The deep reddish brown extracts were combined and dialyzed for 2 hours against water at 0°C. with frequent changes to remove traces of acetone. (Fraction B.1). This fraction was found to have no Acylase I or Acylase II activity and was discarded.

The supernatant from the initial treatment with acetone was treated at -10°C. with a further volume of 1 litre

of acetone. The addition of the acetone was made over a period of 1½ hours. The mixture was allowed to stand for 1½ hours at -10°C. and the precipitate separated as in the case of the earlier fraction. The precipitate was extracted with 200 ml. water at 0°C. and the extracts from the various batches were dialyzed and pooled again (Fraction B.2). The extract was clear and reddish brown in colour and contained most of the initial enzyme activity. The fraction was stable for months on storage at -10°C.

Ammonium sulphate fractionation :

Ammonium sulphate fractionation and all further operations were carried out at 0°C.

Fraction B.2 (1050 ml.) from the total pooled batches corresponding to 25 litres initial extract was adjusted to pH 6.0 with molar acetate buffer (120 ml.) and the buffered solution brought to 0.65 saturation with solid ammonium sulphate (532 g.) and left aside for 2 hours. No precipitate was obtained till 0.5 saturation with ammonium sulphate. The precipitate which separated at 0.65 saturation was removed by centrifugation for 30 minutes at 3500 r.p.m. The precipitate which contained most of the activity was dissolved in ice water and the solution was dialyzed overnight against water for 12 hours with frequent changes to remove

salts and was stored frozen at $-20^{\circ}C$, (Fraction C.1). The supernatant from the initial treatment was brought to 0.75 saturation with solid ammonium sulphate (82 g.). The resulting precipitate was centrifuged and the supernatant which was inactive was discarded. The sediment was dissolved in 50 ml. water. The solution was dialyzed against water and stored frozen (Fraction C.2). The pH of the dialyzed solutions (Fractions C.1 and C.2) were 6.0.

Calcium phosphate gel adsorption :

Treatment with gel was always tried out first on aliquots of the active preparation. The quantity of gel required in the various steps varied from batch to batch and the results reported below represent the amounts required for one batch.

Fraction C.1 (300 ml.) was stirred and treated with calcium phosphate gel (80 ml., solid content 32.5 mg./ml.) previously cooled to $0^{\circ}C$. The gel was added in six portions and the treated solution stirred for 15 minutes and centrifuged. The gel residue was discarded. The supernatant contained the activity. The 1st gel treatment removed most of the colour from the enzyme solution and the supernatant had a pale amber colour. (Fraction D.1).

Fraction D.1 was treated further with calcium

phosphate gel (80 ml., solid content 32.5 mg./ml.) the activity was now completely adsorbed by the gel. The gel was centrifuged off and the supernatant discarded. The gel was then extracted thrice with 0.3 saturated ammonium sulphate solution. During elution the gel was mixed in a Potter-Elvehjem homogenizer by hand for a few minutes to break up lumps. The extracts were pooled after centrifugation. The clear, pale amber-coloured eluate was brought to 0.65 saturation by the addition of solid ammonium sulphate to precipitate the active fraction. The precipitate was then centrifuged and dissolved in ice water (Fraction D.2) and dialyzed against water overnight with frequent changes to remove ammonium sulphate. The supernatant was brought to 0.75 saturation with solid ammonium sulphate. The resulting precipitate was centrifuged and the sediment dissolved in ice water (Fraction D.3).

<u>Refractionation with ammonium sulphate</u> :

Fraction D.2 (76 ml.) was lyophilized. A pale yellow powder was obtained without loss in activity. The powder was dissolved in 10 ml. 0.1 M phosphate buffer (pH 7.4) and precipitated with 3.85 g. solid ammonium sulphate (Final concentration of ammonium sulphate, 0.55 saturation). The

solution was allowed to stand 30 minutes and centrifuged for 20 minutes (18000 x g.). The supernatant was discarded and the precipitate dissolved in ice water and the solution dialyzed overnight against water. The dialyzed solution (Fraction E) was stored frozen. The fraction was stable to storage at -20°C. The specific activities of Fraction E were 4390 μM/hr./mg.N and 5980 μM/hr./mg. against N-acetyl-DL-alanine and N-formyl-DL-tryptophane respectively; and the total activities 76 mM/hr. and 105 mM/hr. respectively for the two substrates. The final solution had a pale yellow tinge and was water clear. No further purification was obtained by repetition of gel adsorption or ammonium sulphate fractionation.

The results of a typical experiment are summarized in Tables IV and V for the two substrates N-acetyl-DL-alanine and N-formyl-DL-tryptophane respectively. Reproducible results were obtained with five different batches of the mould bran extract. The final fraction had no amylase activity.

Electrophoresis :

Electrophoresis of the final fraction at pH 7.4 in 0.1 ionic strength phosphate buffer indicated that the fraction was grossly heterogeneous. Electrophoresis was carried out in a Perkin-Elmer Model 38 Tiselius electrophoresis apparatus. Plate I shows the pattern obtained.

TABLE - IV

Purification of mould acylase of A. Flavus-oryzae.

21 Kg. mouldy bran.

Test substrate : N-acetyl-DL-alanine

Test system : 2 ml. $\frac{M}{10}$ substrate, 0.6 ml. $\frac{M}{15}$ phosphate buffer (pH 6.3) and 0.4 ml. diluted enzyme. Readings taken after 20 minutes at 37°C.

	Fraction	Volume ml.	Protein mg./ml.	Specific activity (μM/hr/mg.N)	Total activity (mM/hr)	Recovery of initial activity (per cent)
A	Extract	25,000	30.5	8.3	1013	-
B.1	Acetone fraction 0%-50% v/v	4,000	30.0	0	0	0
B.2	Acetone fraction 50%-70% v/v	1,050	25.9	205	892	88
C.1	Ammonium sulphate 0.0-0.65 saturation	300	14.4	515	357	35.2
C.2	Ammonium sulphate 0.65-0.75 saturation	51	4.7	180	69.2	6.83
D.1	1st calcium phosphate gel supernatant	300	11.4	699	383	38
D.2	Eluate from 2nd calcium phosphate gel precipitated by ammonium sulphate 0.3-0.65 saturation	76	7.0	1842	158	15.6
D.3	Supernatant from D.2 precipitated by ammonium sulphate 0.65-0.75 saturation	15	6.3	178	27	2.7
E	Reprecipitation of D.2 at pH 7.4 and 0.55 saturation ammonium sulphate	17	6.4	4390	76.4	7.54

TABLE - V

Purification of mould acylase of A. flavus-oryzae.
21 Kg. mouldy bran.

Test substrate : N-formyl-DL-tryptophane

Test system : 2 ml. $\frac{M}{10}$ substrate, 0.6 ml. $\frac{M}{15}$ phosphate buffer (pH 6.3) and 0.4 ml. diluted enzyme. Readings taken after 20 minutes at 37°C.

	Fraction	Volume ml.	Protein mg./ml.	Specific activity (μM/hr/mg.N)	Total activity (mM/hr)	Recovery of initial activity per cent
A	Extract	25,000	30.5	12.9	1575	-
B.1	Acetone fraction 0%-50% v/v	4,000	30.0	0	0	0
B.2	Acetone fraction 50%-70% v/v	1,050	25.9	312.5	1360	86.4
C.1	Ammonium sulphate 0.0-0.65 saturation	300	14.4	774	560	35.6
C.2	Ammonium sulphate 0.65-0.75 saturation	51	4.7	252	96.8	6.15
D.1	1st calcium phosphate gel supernatant	300	11.4	985	539	34.2
D.2	Eluate from 2nd calcium phosphate gel precipitated by ammonium sulphate 0.3-0.65 saturation	76	7.0	2763	236	15.0
D.3	Supernatant from D.2 precipitated by ammonium sulphate 0.65-0.75 saturation	15	6.3	267	40.5	2.6
E	Reprecipitation of D.2 at pH 7.4 and 0.55 saturation ammonium sulphate	17	6.4	5980	105	6.6

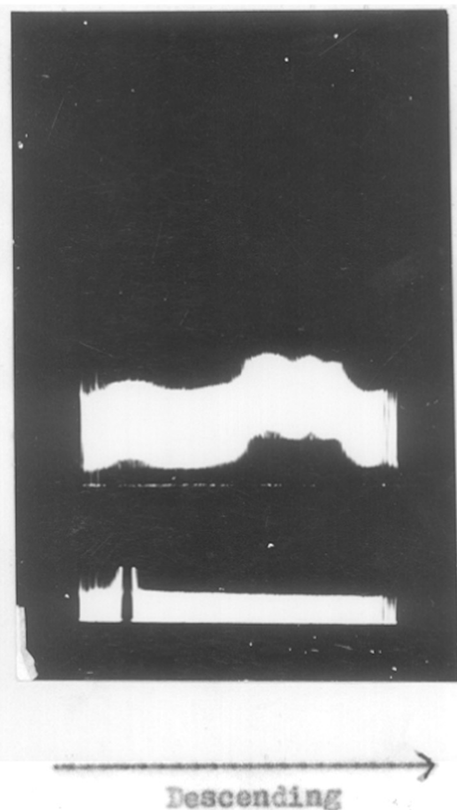

Plate : 1 :- Electrophoresis diagram ~~of diagram~~ of purified acylase of A. flavus-oryzae (Fraction E) in phosphate buffer, pH 7.4, ionic strength 0.1,

Properties and Kinetics

Substrate hydrolysis :

The hydrolysis catalyzed by the mould enzyme followed zero-order kinetics upto at least 10 per cent splitting of the total racemate derivative, under the conditions of assay. The graphs of time plotted against per cent hydrolysis of racemate for the purified enzyme, Fraction E, and the substrates N-acetyl-DL-alanine and N-formyl-DL-tryptophane are shown in Fig. I. The rates of hydrolysis of the substrates were also proportional to enzyme concentration upto 10 per cent splitting of the total racemic derivative. The curves of enzyme concentration versus per cent hydrolysis of the racemate for the final Fraction E and the two substrates N-acetyl-DL-alanine and N-formyl-DL-tryptophane are shown in Fig. II.

Specific activities were determined for the various substrates at values obtained at about 5 per cent splitting of the racemate.

Substrate specificity :

The initial rates of hydrolysis of N-acylated-DL-amino acids are given in Table VI. The results show that the substrates are hydrolyzed at widely varying rates by the mould

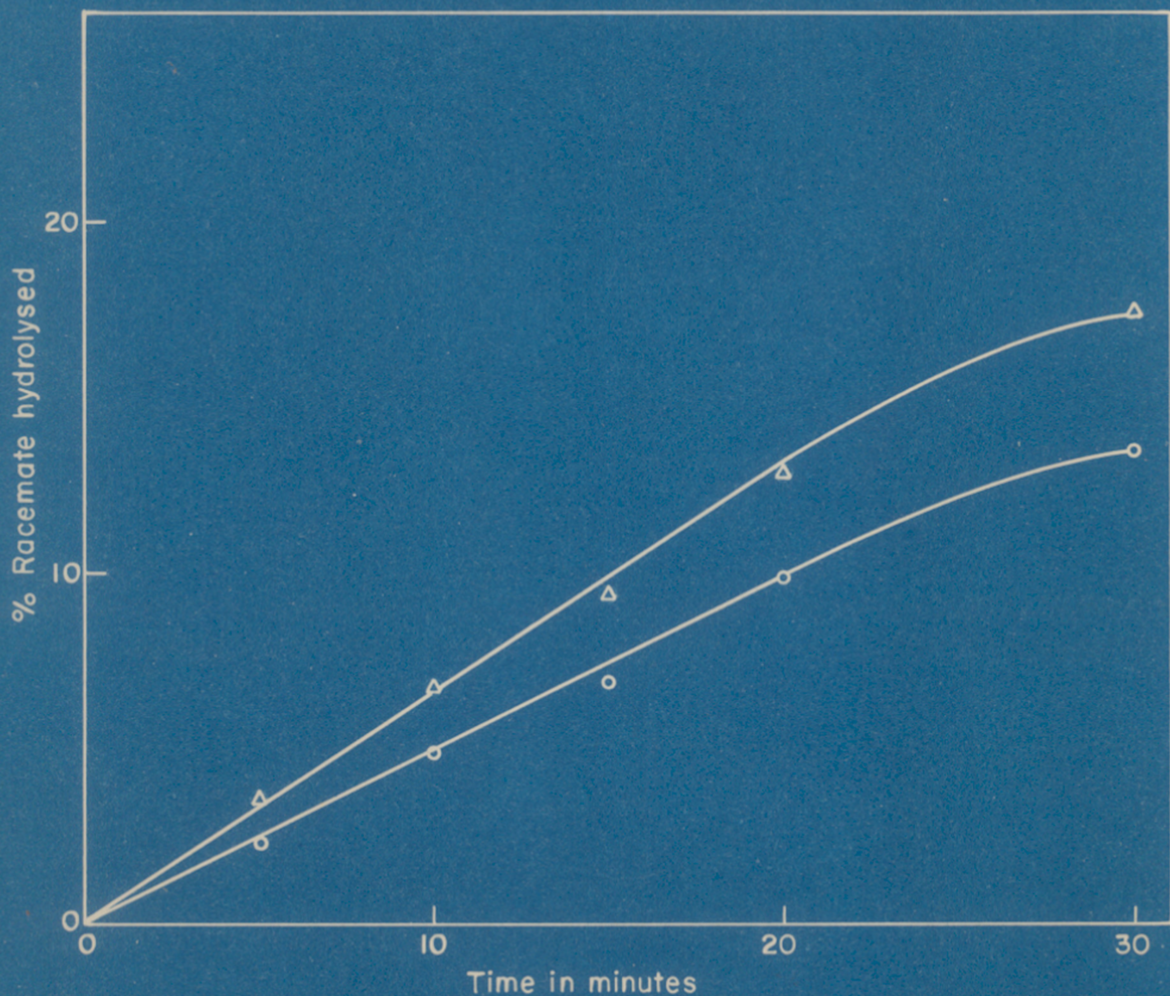

Fig. I : Plot of time versus activity of purified mould acylase Fraction E. The test solution contained 2 ml. 0.1 M neutralized substrate, 0.6 ml. $\frac{M}{15}$ phosphate buffer (pH 6.3) and 0.4 ml. enzyme solution containing 156 μg. protein.

O—O N-Acetyl-DL-alanine.

△—△ N-Formyl-DL-tryptophane.

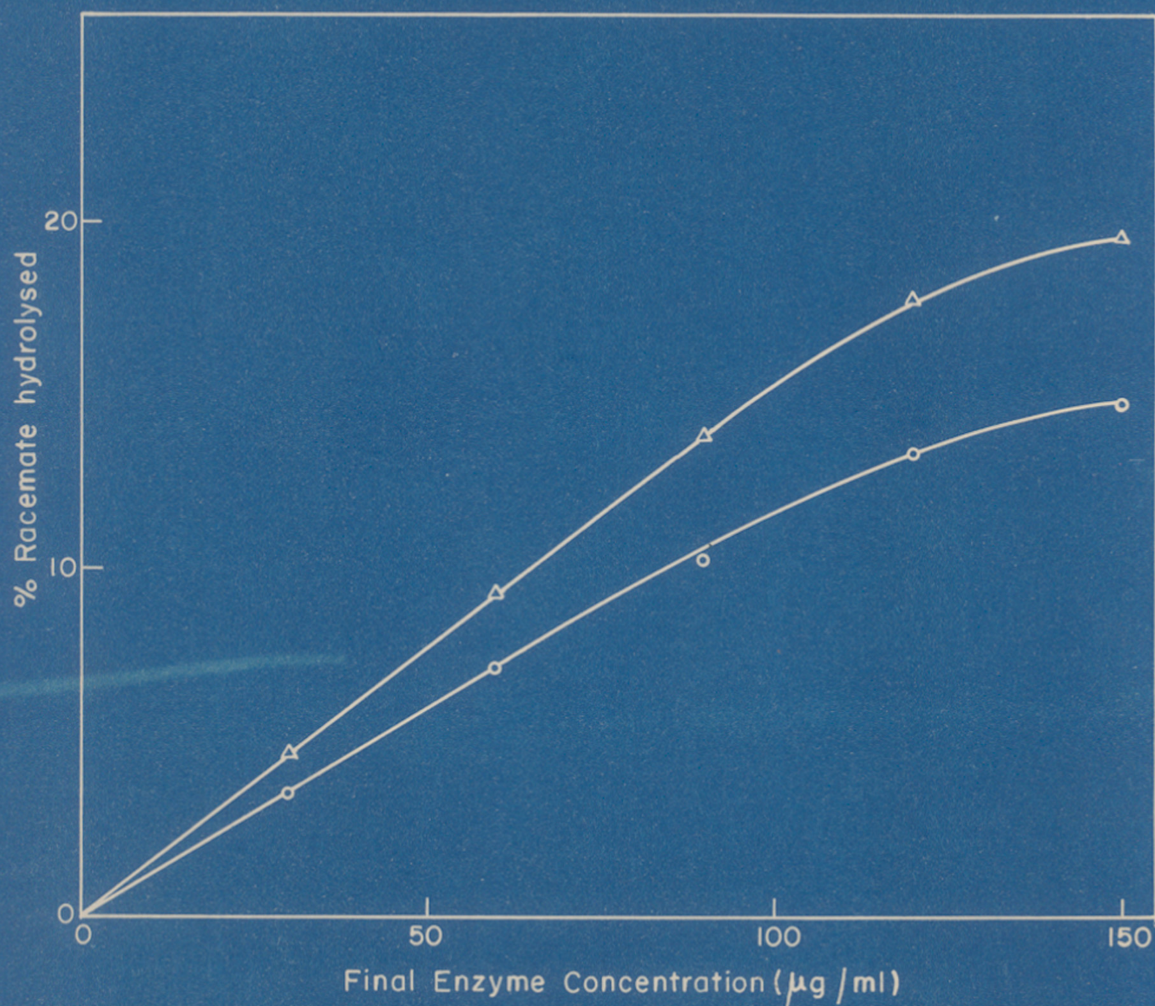

Fig. II : Effect of enzyme concentration on activity of purified mould acylase Fraction E. The test solution contained 2 ml. 0.1 M neutralized substrate, 0.6 ml. $\frac{M}{15}$ phosphate buffer (pH 6.3) and 0.4 ml. enzyme solution. Temp. 37°C. Time 20 minutes.

O—O N-Acetyl-DL-alanine.

△—△ N-Formyl-DL-tryptophane.

enzyme concentrate, derivatives of phenylalanine being the most susceptible. The rates decrease in the following order, phenylalanine, methionine, tryptophane, alanine, glutamic acid, histidine, aspartic acid.

TABLE - VI

N-acylated racemic amino acids tested with mould enzyme concentrate: Fraction E.

Test system as usual

Amino acid	Specific activity (μ M/hr./mg.N)		
	Formyl	Chloroacetyl	Acetyl
Phenylalanine	25,000	13,000	7,000
Methionine	10,300	8,000	5,700
Tryptophane	6,300	5,400	4,500
Alanine	4,200	3,900	3,600
Glutamic acid	3,000	2,150	1,400
Aspartic acid	1,200	560	370
Histidine	2,000	---	800

The mould enzyme is less sensitive to variation of the acyl group in the N-acylated amino acids studied. Among the formyl, chloroacetyl and acetyl derivatives studied, the formyl derivatives were the most susceptible in every case and acetyl the least.

Heat denaturation :

The concentrates of the mould enzyme were stable to storage at -20°C. and showed no loss in activity after three months. The concentrates were also completely stable to dialysis for 24 hours at 0°C. against water. The effect of temperature on the stability of the final concentrate, Fraction E, of the mould enzyme in phosphate buffer at pH 6.3 is shown in Table VII.

TABLE - VII

Heat denaturation of Fraction E of mould acylase on preheating in 0.04 M phosphate buffer at pH 6.3 for 30 minutes at a concentration of 1 mg. enzyme per ml. buffer solution.

Test system as usual.

Temperature	Specific activity towards N-acetyl-DL-alanine. (μM/hr./mg.N)	Loss in activity (per - cent)	Specific activity towards N-Formyl DL-tryptophane (μM/hr./mg.N)	Loss in activity (per cent)
Control	3600		6300	
40°C	3600	0	6300	0
50°C	3540	1.7	6200	1.6
60°C	2200	38.9	3950	37.4
70°C	0	100	0	100

It will be evident from the table that heat denaturation of the mould enzyme affects the hydrolysis rates of alanine and tryptophane derivatives to the same extent.

Temperature velocity constant :

The effect of temperature on the enzyme activity of Fraction E towards N-acetyl-DL-alanine and N-formyl-DL-tryptophane is shown in Fig. III. The temperature velocity constant μ, (or the Arrhenius constant, E) obtained in the usual manner (Lardy, 1949) was 12,500 Cal. for the alanine derivative and 12,700 Cal. for the tryptophane derivative, which correspond to a value of about 2.0 for the temperature coefficient, Q_{10}, for both hydrolyses.

Effect of pH :

Table VIII summarizes the data on the effect of pH on the activities of Fraction E towards N-formyl-DL-tryptophane and N-acetyl-DL-alanine. The results are also shown graphically in Fig. IV. The optimal pH for hydrolysis of both substrates was 6.3 to 6.4. The same value was obtained for the crude extract of mould enzyme.

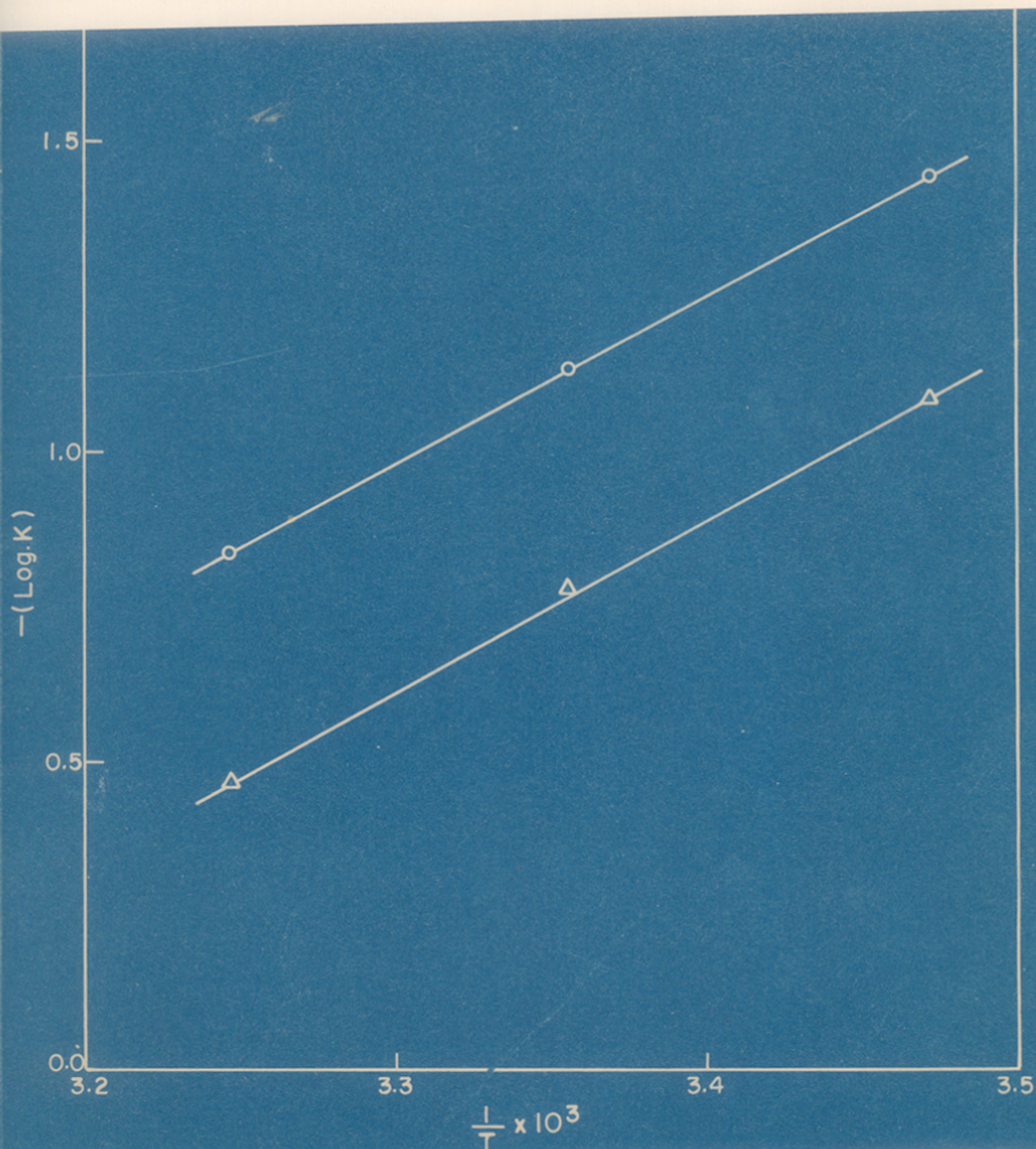

Fig. III : Effect of temperature on the enzyme activity of mould acylase concentrate Fraction E.

O—O N-Acetyl-DL-alanine.

△—△ N-Formyl-DL-tryptophane.

TABLE - VIII

Effect of pH on activity of mould acylase (Fraction B)

Test system : 2 ml. $\frac{N}{10}$ neutralized substrate, 0.6 ml. 0.1M buffer as indicated below and 0.4 ml. enzyme solution.

pH	Buffer	Specific activity towards N-acetyl-DL-alanine (μM/hr./mg.N)	Specific activity towards N-formyl-DL-tryptophane (μM/hr./mg.N)
3.45	0.1M Acetate	120	120
3.88	"	150	270
4.52	"	260	420
5.05	"	2250	3900
5.29	0.1M Phosphate	2700	4530
5.68	"	3300	5320
6.29	"	3600	6300
6.40	"	3600	6300
6.61	"	3600	6240
6.82	"	3460	6120
7.08	"	3000	5700
7.30	"	2630	4980
7.42	"	2100	4200
8.14	0.1M Borate	1290	2880
8.55	"	900	2100
9.20	"	750	1440
9.51	"	750	1380

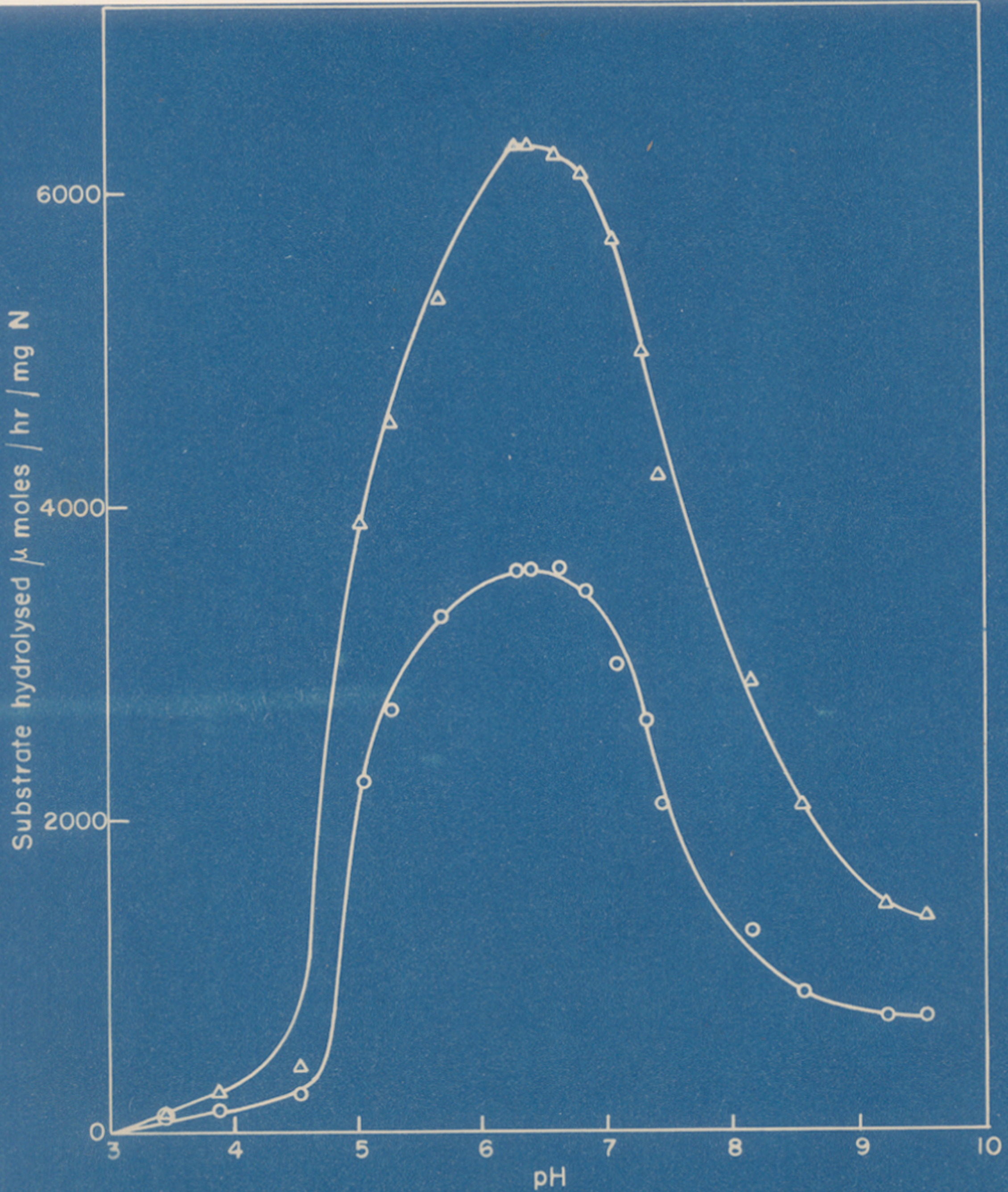

Fig. IV : Effect of pH on activity of purified mould acylase Fraction E.

O—O N-Acetyl-DL-alanine.

△—△ N-Formyl-DL-tryptophane.

Effect of substrate concentration :

The effect of substrate concentration on enzyme activity of Fraction E towards N-acetyl-DL-alanine and N-formyl-DL-tryptophane is shown in Fig. V. The usual constants (Michealis and Menten, 1913; Briggs and Haldane, 1925) were obtained through the use of the Lineweaver-Burk relation (Lineweaver and Burk, 1934) $\frac{[S]}{v} = \frac{[S]}{V} + \frac{Ks}{V}$ where $[S]$ is the substrate concentration, v the rate of hydrolysis and V the maximum activity when the enzyme is saturated with respect to the substrate. The value of Ks was 2.27×10^{-2}M/litre for N-acetyl-DL-alanine and 2.17×10^{-2}M/litre for N-formyl-DL-tryptophane.

Effect of inhibitors :

The effect of inhibitors on the activity of mould acylase Fraction E towards N-acetyl-DL-alanine and N-formyl-DL-tryptophane is described in Table IX. The inhibitors were tried at the indicated final concentrations (Table IX).

Mould acylase was found to be inhibited by metal binding agents such as ethylenediaminetetraacetic acid (EDTA), o-phenanthroline, $\alpha\alpha'$-dipyridyl, cyanide, fluoride and azide, the inhibitory effect being 100 per cent for EDTA, o-phenanthroline and $\alpha\alpha'$-dipyridyl and about 50 per cent for cyanide, fluoride and azide at the levels tested.

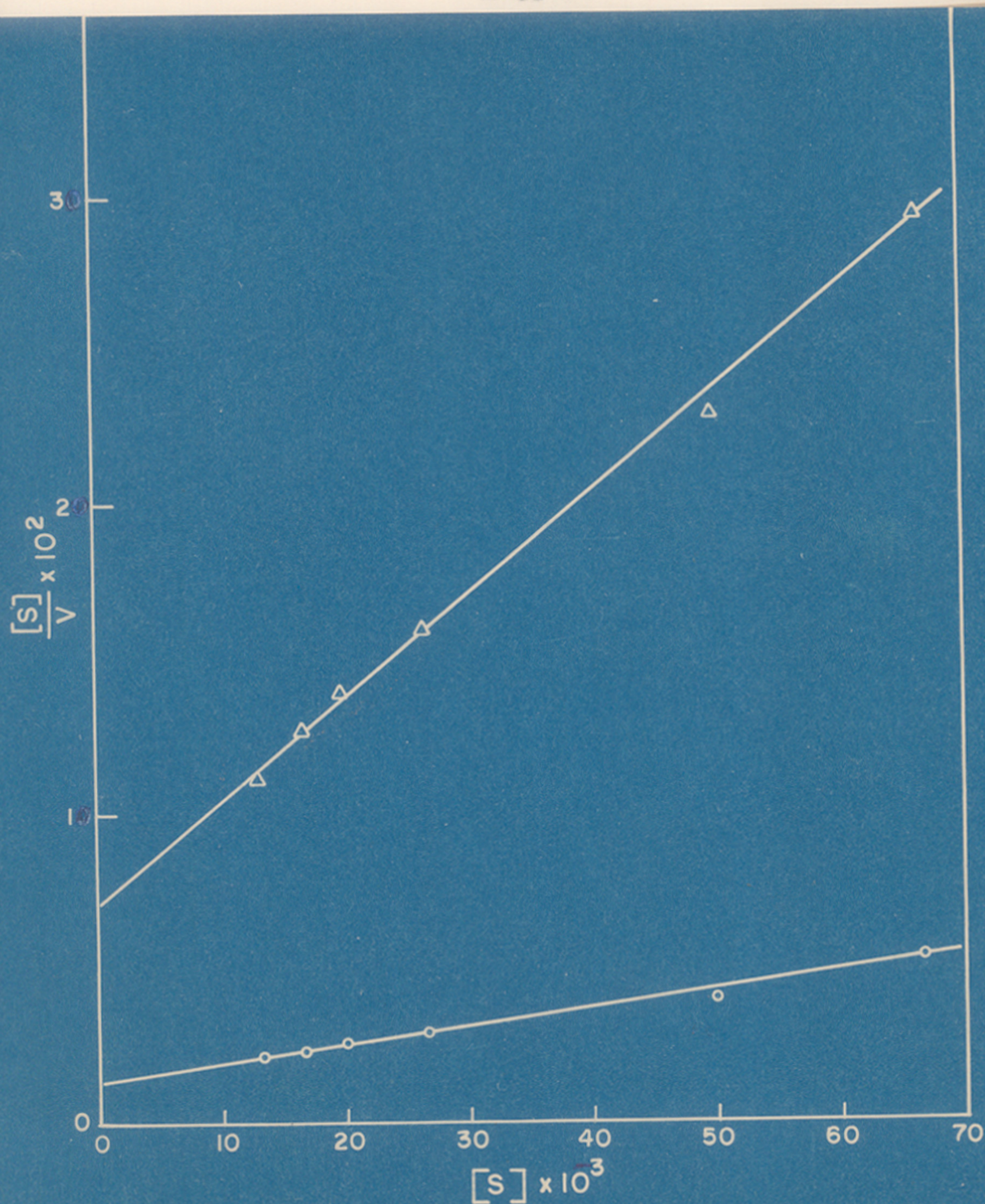

Fig. V : Effect of substrate concentration [S] on enzyme activity of mould acylase Fraction E. V is expressed as μM. substrate hydrolyzed in 20 min. at 37°C.

O—O N-Acetyl-DL-alanine.

Δ—Δ N-Formyl-DL-tryptophane.

TABLE - IX

Effect of inhibitors on activity of mould acylase Fraction E.

The composition of the assay system was the same as in the routine test except for inhibitors added at the indicated levels.

Reagent	Final concentration	μM of substrate hydrolyzed per hr. per mg.N		Inhibition (per cent)
		N-acetyl-DL-alanine	N-formyl-DL-tryptophane	
Control	-	3600	6000	-
o-Phenanthroline	1.3×10^{-2}M	--	--	100
αα'-Dipyridyl	1.5×10^{-2}M	--	--	100
Sodium cyanide	1.3×10^{-3}M	2000	3200	50
EDTA	1.3×10^{-2}M	--	--	100
Sodium fluoride	3×10^{-3}M	2200	3500	40
Sodium azide	3×10^{-3}M	2200	3600	40
Pyrophosphate	2×10^{-3}M	1900	3100	50
Iodoacetate	5×10^{-3}M	3500	5900	nil
p-Chloromercuri-benzoate	1×10^{-3}M	3600	6000	nil

Iodoacetate and p-chloromercuribenzoate had no effect on enzyme activity at the levels tested.

Inhibition by EDTA :

The enzyme solution (3 mg./ml.) was inactivated completely by treatment with EDTA (final concentration 1.2×10^{-2}M) and dialysis for 16 hours against water at 0° with frequent changes.

Metal activation :

Mould acylase Fraction E was fully active towards N-acetyl-DL-alanine and N-formyl-DL-tryptophane in the absence of added metals, addition of cobaltous, zinc, manganous, ferrous, magnesium, calcium and ferric ions having no effect on the activity of the enzyme (Table X).

The effect of metals on the EDTA-treated and dialyzed enzyme is shown in Table XI. Acylase activity towards N-acetyl-DL-alanine and N-formyl-DL-tryptophane was almost completely restored by Zn^{++} and Fe^{++}, while Co^{++}, Mn^{++}, Mg^{++}, Ca^{++} and Fe^{+++} were without effect. The influence of Zn^{++} and Fe^{++} concentration on the specific activity of the EDTA - treated and dialyzed enzyme is shown in Fig. VI and Fig. VII

TABLE - X

Effect of metal ions on hydrolytic rates with mould acylase Fraction E.

Test system : 0.1M Na β-glycerophosphate buffer (pH, 6.3) used in place of phosphate in the routine test system.

Test System	Final concentration	μM of substrate hydrolyzed per hour per mg.N	
		N-acetyl-DL-alanine	N-formyl-DL-tryptophane
Enzyme Control	-	3800	5600
" $CoCl_2$	$1.3 \times 10^{-3}M$	3800	5600
" $ZnSO_4$	$1.7 \times 10^{-3}M$	3800	5600
" $MnSO_4$	$1.0 \times 10^{-3}M$	3800	5600
" $FeSO_4$	$1.0 \times 10^{-3}M$	3800	5600
" $MgSO_4$	$1.17 \times 10^{-3}M$	3800	5600
" $FeCl_3$	$1.17 \times 10^{-3}M$	3800	5600
" $CaCl_2$	$1.3 \times 10^{-3}M$	3800	5600

TABLE - XI

Effect of metal ions on activity of EDTA-treated and dialyzed mould acylase Fraction E.

Test system : 0.1 M Na β-glycerophosphate (pH 6.3) in place of phosphate in the routine test system.

Systems	Concen. of salts	μM. of substrate hydrolyzed per hour per mg.N		Activation (per cent)
		N-Acetyl-DL-alanine	N-Formyl-DL-tryptophane	
Untreated enzyme	-	3600	5600	-
EDTA Treated	-	0	0	-
+ $CoCl_2$	1.7×10^{-3}M	0	0	-
+ $ZnSO_4$	"	3550	5500	98
+ $MnSO_4$	"	0	0	-
+ $FeSO_4$	"	3400	5300	94
+ $MgSO_4$	"	0	0	-
+ $FeCl_3$	"	0	0	-
+ $CaCl_2$	"	0	0	-

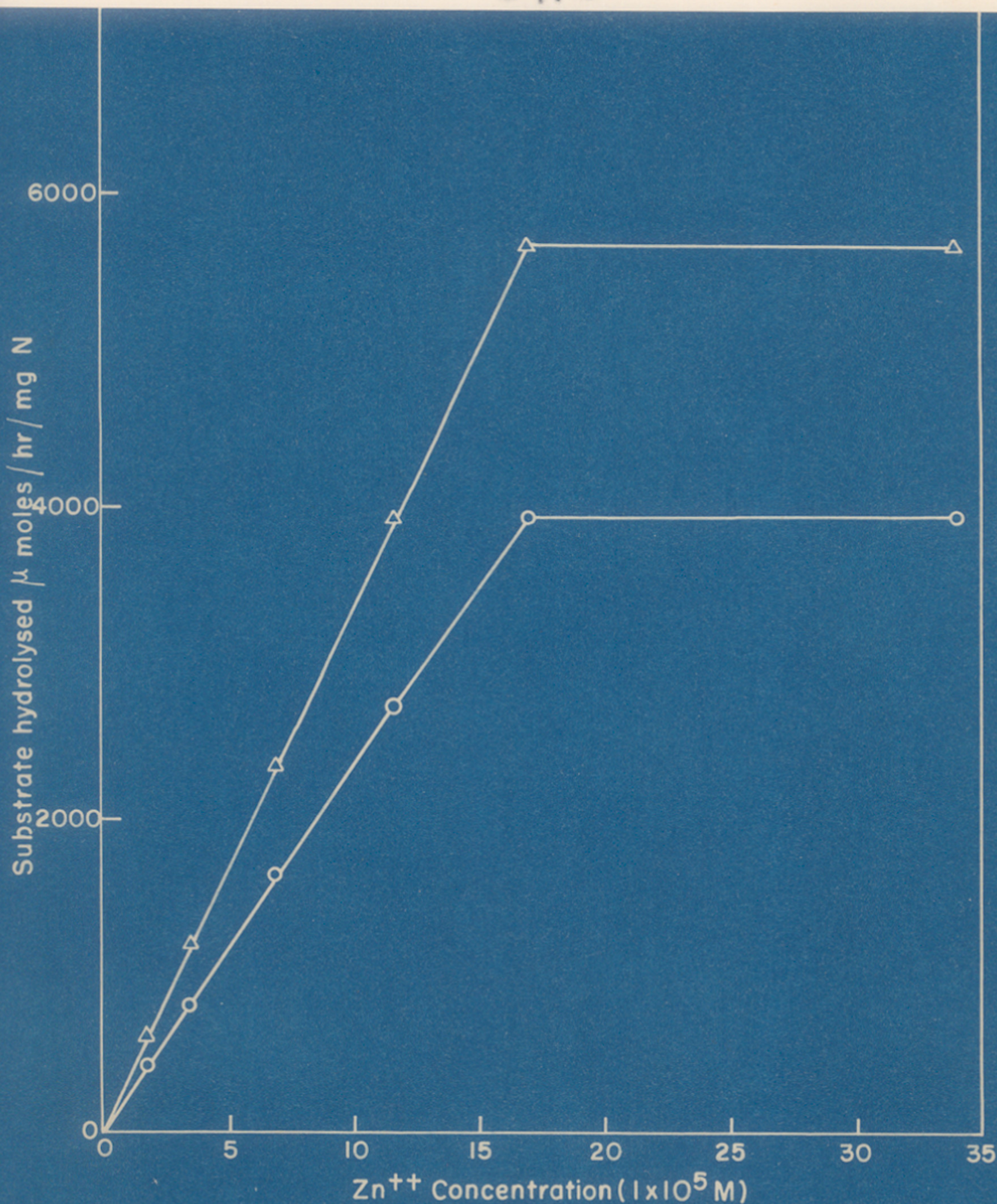

Fig. VI : Effect of Zn^{++} concentration on the specific activity of EDTA-treated and dialyzed mould acylase Fraction E.

O—O N-Acetyl-DL-alanine.

Δ—Δ N-Formyl-DL-tryptophane.

0.1M Na β-glycerophosphate buffer (pH 6.3) in place of phosphate buffer in the routine test system.

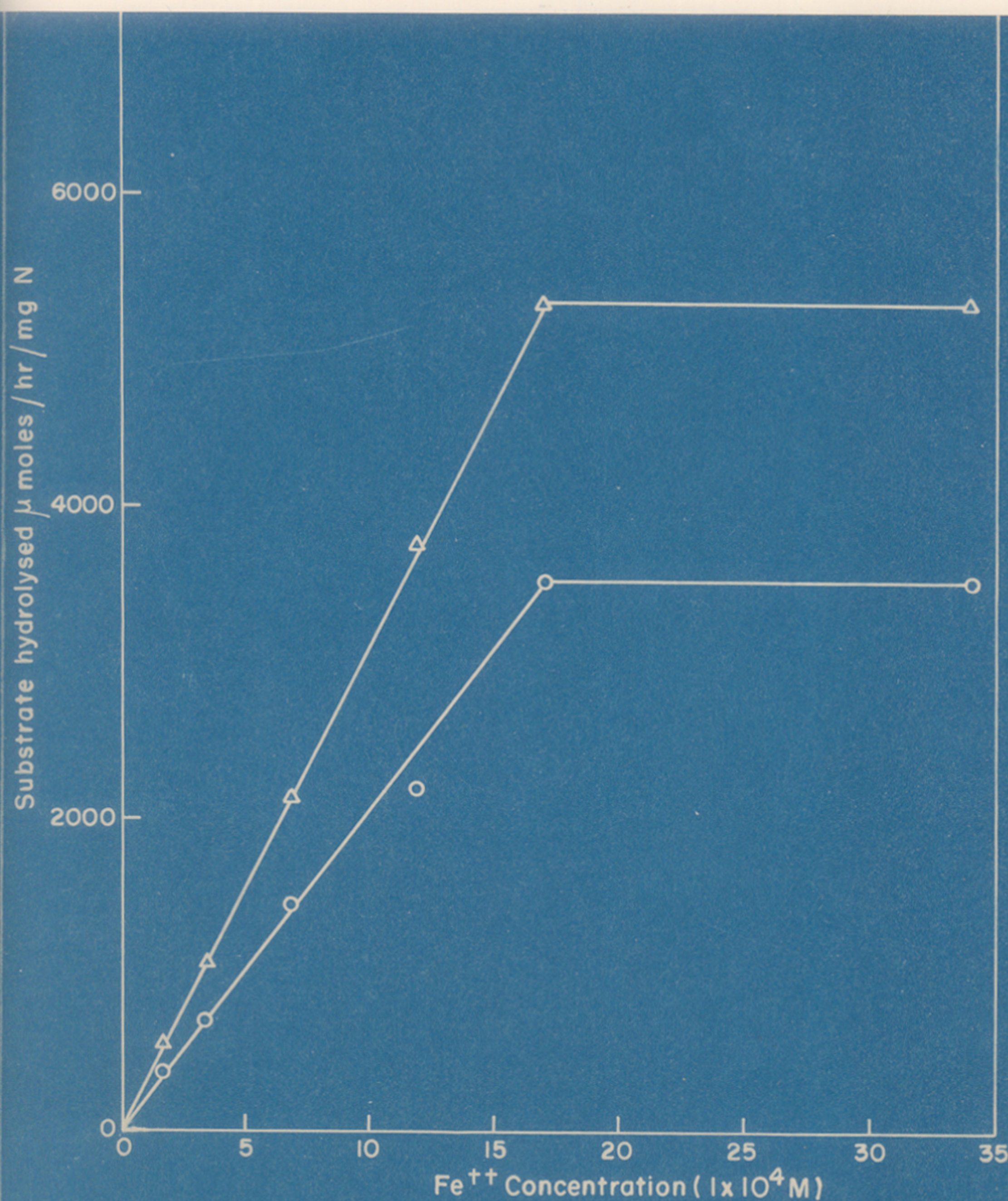

Fig. VII : Effect of Fe^{++} concentration on the specific activity of EDTA-treated and dialyzed mould acylase Fraction E.

O——O N-Acetyl-DL-alanine.

Δ——Δ N-Formyl-DL-tryptophane.

0.1M Na β-glycerophosphate buffer (pH 6.3) in place of phosphate buffer in the routine test system.

respectively. The optimal concentration of Zn^{++} and Fe^{++} for restoring activity were 1.7×10^{-4}M and 1.7×10^{-3}M respectively. The influence of these optimal concentrations of Zn^{++} and Fe^{+++} on the activity of the EDTA-treated and dialyzed enzyme towards formyl, acetyl and chloroacetyl derivatives of DL-alanine and DL-tryptophane are summarized in Table XII.

TABLE - XII

Effect of Zn^{++} and Fe^{+++} on hydrolytic rates for the EDTA - treated and dialyzed mould acylase Fraction E. Test system : 0.1M Na β-glycerophosphate (pH, 6.3) in place of phosphate in the routine system.

Derivative	μM. of substrate hydrolyzed per hour per mg.N		
	EDTA-treated and dialyzed enzyme	Treated enzyme + $FeSO_4$ (final Conc. (1.7×10^{-3}M).	Treated enzyme+ $ZnSO_4$ (final Conc.) 1.7×10^{-4}M
N-Formyl-DL-Alanine	0	3600	3800
N-Acetyl-DL-Alanine	0	3000	3700
N-Chloroacetyl-DL-Alanine	0	3400	3500
N-Formyl-DL-tryptophane	0	6000	6200
N-Acetyl-DL-tryptophane	0	4000	4300
N-Chloroacetyl-DL-tryptophane	0	5000	5100

DISCUSSION

Purification :

The relative increase in activity of Fraction E of mould enzyme concentrate over the crude extract for some of the substrates tested is summarized in Table XIII.

TABLE - XIII

Ratio of activity of mould enzyme Fraction E to activity of crude extract.

Test system as usual.

Rates in μM/hr./mg.N

Derivative	Crude Extract	Concentrate Fraction E	Ratio Fraction E / Crude
N-Formyl-DL-phenylalanine	68	25000	370
N-Acetyl-DL-phenylalanine	20	7000	350
N-Formyl-DL-methionine	33	10300	310
N-Acetyl-DL-methionine	15	5700	390
N-Formyl-DL-tryptophane	15	6300	420
N-Chloroacetyl-DL-tryptophane	11	5400	490
N-Acetyl-DL-tryptophane	10	4500	450
N-Formyl-DL-alanine	10	4200	420
N-Chloroacetyl-DL-alanine	9	3900	430
N-Acetyl-DL-alanine	6	3600	600
N-Formyl-DL-histidine	15	2000	130
N-Acetyl-DL-histidine	9	800	90
N-Chloroacetyl-DL-aspartic acid	5.5	560	100

Towards derivatives of phenylalanine, methionine, tryptophane and alanine the purification on an average is about 400-fold while towards derivatives of histidine and aspartic acid the increase in activity is about 100-fold. Birnbaum, Levintow, Kingsley and Greenstein (1952) reported that their purified renal acylase I was inactive towards N-acylated aspartic acid derivatives. It is not possible to state whether in the case of the mould the same enzyme is involved in the hydrolysis of derivatives of aspartic acid and derivatives of the other amino acids or whether the fractionation procedure failed to separate the different activities.

Substrate specificity :

Mould acylase from bran cultures of *A. flavus-oryzae* shows interesting points of difference from the renal enzyme with regard to substrate susceptibility. In the case of the mould enzyme, both the crude extract and the highly active fraction E hydrolyzed N-formyl-DL-tryptophane at a faster rate than N-formyl-DL-alanine. The ratios of the rate of hydrolysis of the tryptophane derivative to that of N-acetyl-DL-alanine at the various steps in the purification procedure are shown in Table XIV. The constant ratio of about 1.5 throughout the several fractions obtained from the crude extract suggests that the same enzyme is involved in the hydrolysis of both the derivatives. Attempts to separate

TABLE - XIV

Ratio of specific activities of mould acylase of *A. flavus-oryzae* towards the two substrates N-formyl-DL-tryptophane and N-acetyl-DL-alanine at various steps of purification.

	Fraction	Specific activity N-acetyl-DL-alanine (μM/hr./mg.N)	Specific activity N-formyl-DL-tryptophane (μM/hr./mg.N)	Ratio N-formyl-DL-tryptophane / N-Acetyl-DL-alanine
A.	Extract	8.3	12.9	1.6
B.2	Acetone fraction 50%-70% v/v	205	312.5	1.5
C.1	Ammonium sulphate 0.0-0.65 saturation	515	774	1.5
D.1	1st Calcium phosphate gel supernatant	699	985	1.4
D.2	Eluate from 2nd Calcium phosphate gel precipitated by Ammonium sulphate 0.3-0.65 saturation	1842	2763	1.5
E	Reprecipitation of D.2 at pH 7.4 and 0.55 saturation ammonium sulphate	4390	5980	1.4

the activities towards N-acylated derivatives of tryptophane and alanine by heat denaturation were also unsuccessful (Table VII). Further, the effect of pH on the hydrolytic rates of both substrates were identical (Table VIII).

Results obtained with renal acylase I indicate that the N-acylated derivatives of tryptophane and tyrosine may be substrates for a specific acylase distinct from the enzyme that hydrolyzes the N-acylated aliphatic amino acids, since the increase in specific activity of renal acylase I preparation over the crude homogenate towards the substituted aromatic amino acids was about one-eighth to one-tenth of the increase in specific activity towards the derivatives of the aliphatic amino acids (Rao, Birnbaum, Kingsley and Greenstein, 1952).

In contrast to the renal enzyme which acts at markedly slow rates on the substituted aromatic amino acids, phenylalanine, tyrosine and particularly tryptophane (Price, Gilbert and Greenstein, 1949) the mould enzyme readily hydrolyzed the N-acylated derivatives of phenylalanine and tryptophane, in fact at rates greater than with the corresponding alanine derivatives. The rates of hydrolysis of chloroacetyl and acetyl derivatives of tryptophane are 5400 and 4500 μM/hr./mg.N respectively, in the case of the mould enzyme concentrate; the corresponding rates with renal acylase I

being 12 and 5 μM/hr./mg.N, respectively. The slow rates of hydrolysis with the renal enzyme concentrate have precluded its use for resolution of tryptophane. Acylase preparations which can readily hydrolyze tryptophane derivatives have not been described hitherto. The rapid hydrolysis of N-acylated tryptophane derivatives by the mould enzyme concentrates suggest interesting possibilities for the use of the enzyme from this source for the preparation of the enantiomorphs of tryptophane. The application of the mould enzyme concentrate for this purpose was therefore investigated and the results are described in Part III of this thesis. The rates of hydrolysis of the different substrates for the mould enzyme, renal acylase I and pancreatic carboxypeptidase are shown in Table XV for comparison.

The mould enzyme also shows difference with regard to the influence of the acyl group on susceptibility of the substrate to hydrolysis. Of the formyl, acetyl and chloroacetyl derivatives tested, the formyl derivative in every case was the most susceptible to hydrolysis by the mould enzyme and acetyl the least (Table VI), while the chloroacetyl was the most susceptible in the case of the renal enzyme and formyl the least (Fodor, Price and Greenstein, 1950).

These interesting points of difference in substrate pattern distinguish acylases of mould and animal tissues. The only difference in this respect which was

TABLE - XV

Comparison of substrate specificity of the mould enzyme, Renal acylase I and pancreatic carboxypeptidase.

Rates of hydrolyzes of derivatives of phenylalanine, tryptophane and alanine in μM/hr./mg.N.

Substrate	Rate with Mould enzyme Fraction E	Rate with Renal acylase I	Rate with cryst. pancreatic carboxy-peptidase.
N-Formyl-DL-phenylalanine	25,000	14 (2)	80 (2)
N-Chloroacetyl-DL-phenylalanine	13,000	460 (1)	2750 (1)
N-Acetyl-DL-phenylalanine	7,000	138 (1)	20 (3)
N-Formyl-DL-tryptophane	6,300	—	—
N-Chloroacetyl-DL-tryptophane	5,400	12 (1)	—
N-Acetyl-DL-tryptophane	4,500	5 (1)	—
N-Formyl-DL-alanine	4,200	300 (2)	—
N-Chloroacetyl-DL-alanine	3,900	11600 (1)	—
N-Acetyl-DL-alanine	3,600	3200 (1)	—

(1) Birnbaum, Levintow, Kingsley and Greenstein, (1952).
(2) Fones and Lee (1953).
(3) Calculated from ratio given in Reference (1).

reported earlier appears to be the finding of Fones and Lee (1953) that in the case of ring-substituted derivatives of hippuric acid, the nature of the substituent affects the hydrolysis rate of the renal enzyme markedly while for the acylase ("hippuricase") of takadiastase this factor has little influence on the hydrolytic rate (Ellis and Walker, 1942).

Both renal acylase I and mould acylase, however, differ from pancreatic carboxypeptidase with regard to the influence of the acyl group on the hydrolytic rates of N-acylated amino acids. While the two acylases are comparatively insensitive to variation of the acyl group, the rates of hydrolysis with pancreas carboxypeptidase are markedly affected by the structure of this radical. Thus the ratio of the rates of hydrolysis of N-chloroacetylphenylalanine to N-acetylphenylalanine by kidney and mould acylases is 2 to 4 : 1, the corresponding ratio with crystalline carboxypeptidase of pancreas being 150 : 1. (Table XV).

<u>Inhibition by metal binding agents
and metal activation :</u>

Ethylenediaminetetraacetic acid (EDTA) is known to have no effect on the activity of renal acylase I (Marshall,

Birnbaum and Greenstein, 1955; 1956). In contrast the mould enzyme concentrate, Fraction E, is completely inhibited by EDTA and other metal binding agents, such as o-phenanthroline and αα'-dipyridyl (Table IX). The mould enzyme is completely inactive when dialyzed after EDTA-treatment, the activity being restored almost completely by Zn^{++} and Fe^{++}, while Co^{++}, Mn^{++}, Mg^{++}, Ca^{++} and Fe^{+++} are without effect (Table XI). Addition of any of these metal ions to the untreated mould enzyme has no effect on the hydrolytic rates of N-acetyl-DL-alanine and N-formyl-DL-tryptophane. Renal acylase I, on the other hand, hydrolyzes the less susceptible derivatives such as N-acylated tryptophane, at an accelerated rate in the presence of Co^{++}, while the hydrolytic rates of the more susceptible derivatives, such as N-acylated methionine, are inhibited by the presence of Co^{++} (Rao, Birnbaum, Kingsley and Greenstein, 1952).

Acylases from the two sources thus show differences also in their metal ion requirements.

S U M M A R Y

(1) The acylase of *A. flavus-oryzae* was partially purified by acetone and ammonium sulphate fractionations and adsorption on calcium phosphate gel.

(2) The specific activity of the final concentrate

of mould acylase (Fraction E) was about 400 times that of the initial extract when assayed against N-acylated phenylalanine, tryptophane, methionine and alanine derivatives and about 100 times that of the initial extract when assayed against N-acylated histidine and aspartic acid.

(3) The purified mould acylase was electrophoretically heterogeneous.

(4) The properties of the mould enzyme and the kinetics of the reaction are described.

(5) The mould enzyme differed from animal tissue acylase I in its behaviour towards N-acylated derivatives of the substituted aromatic amino acids, phenylalanine and tryptophane. While the renal acylase hydrolyzes the derivatives of phenylalanine and tryptophane at markedly lower rates than those of the aliphatic amino acids such as alanine, the mould enzyme readily hydrolyzed the N-acylated derivatives of phenylalanine and tryptophane more rapidly than those of the corresponding alanine derivatives at rates markedly higher than those reported for acyl-tryptophane hydrolysis for any other purified acylase, hitherto.

(6) The mould enzyme differed from renal acylase also in the effect of the acyl group on the hydrolytic rates of N-acylated amino acids. While among formyl, acetyl and chloroacetyl derivatives of amino acids, the chloroacetyl is the most susceptible and formyl the least in the case of

the renal enzyme, formyl derivatives are the most susceptible and acetyl the least in the case of mould acylase.

(7) The differences in substrate specificity of the renal and mould acylases and pancreatic carboxypeptidase are discussed.

(8) The mould enzyme, unlike renal acylase was inhibited by metal binding agents such as ethylene diamine-tetraacetic acid (EDTA), o-phenanthroline, αα'-dipyridyl, cyanide,azide and fluoride.

(9) The EDTA-treated enzyme remained inactive after dialysis. The inhibition was reversed by bivalent zinc and iron but not by Co^{++}, Mn^{++}, Mg^{++}, Ca^{++} and Fe^{+++}.

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E X P E R I M E N T A L

P A R T - III

PREPARATION OF THE OPTICAL ISOMERS OF DL-TRYPTOPHANE BY USE OF MOULD ACYLASE CONCENTRATE

EXPERIMENTAL

PART - III

PREPARATION OF THE OPTICAL ISOMERS OF DL-TRYPTOPHANE BY USE OF MOULD ACYLASE CONCENTRATE.

Acylases have been extensively employed in the resolution of racemic α-amino acids on account of their high degree of optical specificity and the simplicity and ease in manipulation of the resolution procedure. Active enzyme preparations that rapidly hydrolyze the susceptible substrates have been obtained hitherto only from animal tissues such as hog kidney (Birnbaum, Levintow, Kingsley and Greenstein, 1952). Enzyme preparations from these sources however fail when applied to the resolution of DL-tryptophane, since such enzyme concentrates act extremely slowly (Price, Gilbert and Greenstein, 1949) and incompletely (Rao, Birnbaum, Kingsley and Greenstein, 1952) on the N-acylated derivatives of L-tryptophane. In contrast mould enzyme concentrates isolated from bran cultures of *Aspergillus flavus-oryzae* NRRL 536 have been shown in the present investigation to hydrolyze N-acylated derivatives of tryptophane at rates as high as 6000 μM/hr./mg.N. The application of mould enzyme concentrates to the resolution of tryptophane was therefore investigated.

Racemic tryptophane was readily resolved into its optical isomers by subjecting N-acetyl-DL-tryptophane to asymmetric hydrolysis by a purified mould acylase preparation.

EXPERIMENTAL

Methods and Materials

N-acetyl-DL-tryptophane :

N-acetyl-DL-tryptophane was synthesized according to the procedure of du Vigneaud and Sealock (1932) as described already in Part II.

Enzyme preparation :

Mould acylase concentrate Fraction D.2 obtained from bran cultures of *A. flavus-oryzae*, NRRL 536 as described earlier in Part II of this thesis (Table V) was employed in the resolution procedure. The fraction had a specific activity of about 2000 μM/hr./mg.N towards N-acetyl-DL-tryptophane and a protein concentration of 7 mg./ml. of solution.

R E S U L T S

Resolution Procedure

Enzymic digestion :

N-acetyl-DL-tryptophane (15 g.) was suspended

in 400 ml. distilled water and brought into solution at about pH 7 by cautious addition of N NaOH (60 ml.). The solution was brought to pH 6.3 by adding 130 ml. $\frac{M}{15}$ phosphate buffer of that pH. The solution was diluted to 600 ml. corresponding to a substrate concentration of approximately 0.1M and the diluted solution warmed to 37°C. To this solution was added enough enzyme solution to hydrolyze the L-isomer in 2 hours at 37°C. (Fraction D.2, 50 mg. in 7 ml.), the digest was layered with toluene and the digestion was carried at 37°C. for 16 hours. Fresh enzyme (Fraction D.2, 50 mg. in 7 ml.) was then added and the digestion was allowed to stand for another 8 hours. Determination of liberated amino acid by formol titration in samples of the digestion mixture at the end of this period indicated complete hydrolysis of the susceptible form.

<u>Isolation of L-tryptophane</u> :

The digestion mixture was cooled to 0°C. and acidified with glacial acetic acid to pH 5. L-tryptophane which separated out overnight at 0°C. was filtered by suction and washed with ice water, alcohol, acetone and finally with ether. The mother liquor from the digestion, on concentration in vacuo to a volume of about 200 ml., yielded another crop of the L-amino acid.

The L-amino acid (5 g.) was purified by extraction with hot acetone to remove any traces of N-acetyl-D-tryptophane

and crystallized from hot water. Yield, 4.5 g. or 73 per cent of the theoretical. $[\alpha]_D^{25}$ (in 1 per cent aqueous solution), -32.5.

Isolation of acetyl-D-tryptophane :

The mother liquor from the enzyme digest was concentrated further to a volume of about 50 ml. and the pH adjusted to 2.0 with 4N hydrochloric acid and the N-acetyl-D-tryptophane was extracted several times with ethyl acetate. The ethyl acetate extracts were combined, dried over anhydrous sodium sulphate and filtered. The ethyl acetate was removed in vacuo. The syrupy residue crystallized on cooling to 0°C. and was recrystallized from hot water. Yield, 6.8 g. or 90 per cent of the theoretical. $[\alpha]_D^{25}$ (in 1 per cent absolute methanol), -26.1.

Isolation of D-tryptophane :

N-Acetyl-D-tryptophane was hydrolyzed according to the procedure described by Shabica and Tishler (1949). N-Acetyl-D-tryptophane (6.8 g.) was suspended in 68 ml. of 2N hydrochloric acid, refluxed for 2½ hours and the D-isomer isolated from the hydrolysate according to the method of Shabica and Tishler (1949). Yield, 3.5 g. or 59 per cent the theoretical. $[\alpha]_D^{25}$ (in 1 per cent aqueous solution), + 32.2.

DISCUSSION

The D- and L-isomers of tryptophane were optically pure as determined by their optical rotations. The specific rotations of the isomers obtained by the present procedure were -32.5 for the L-isomer and +32.2 for the D-isomer. Optically pure L- and D-tryptophane were isolated by Gilbert, Price and Greenstein (1949) from chloroacetyl-DL-tryptophane using pancreatic carboxypeptidase for asymmetric cleavage. These authors reported a value $[\alpha]_D^{25}$ of -32.3 for the L-isomer and +32.5 for the D-isomer. The isomers containing less than 1 part in 1000 of the enantiomorph, as measured by optically specific amino acid oxidases (Meister, Levintow, Kingsley and Greenstein, 1951). Optically specific amino acid oxidases were not used in the present study for determining purity of the isomers although these enzymatic methods would have provided a more sensitive criterion of optical purity.

The mould enzyme concentrate thus readily resolves racemic tryptophane into its optical isomers. Crude commercially available enzyme preparations, mostly of mould origin, were used by Neuberg and Mandl (1950) for resolution of N-acetyl-DL-tryptophane. The enzyme preparations employed by these authors were evidently of very low activity as the enzyme used was half the weight of the racemate and the period of digestion was 11 days. Active acylase preparations

suitable for resolution of tryptophane have not been reported, hitherto.

SUMMARY

(1) Racemic tryptophane was readily resolved into its optical isomers by subjecting N-acetyl-DL-tryptophane to asymmetric hydrolysis by a purified mould acylase preparation.

(2) The L- and D- isomers were optically pure as determined by their optical rotations.

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G E N E R A L   S U M M A R Y

(1) Among 15 fungal strains screened for acylase activity towards N-formyl-DL-tryptophane and N-acetyl-DL-alanine, Aspergillus flavus-oryzae NRRL 536 was found to be the best enzyme-producer.

(2) No acylase activity was obtained when the mould was grown in synthetic media.

(3) The yield of enzyme was highest from 62-hour semi-solid mould bran preparations, while yields from liquid media containing bran extract were low, both in stationary and shake cultures. No measurable activity was observed in the culture fluid from the liquid media, all the activity being present in the mycelium.

(4) The acylase of A. flavus-oryzae was partially purified by acetone and ammonium sulphate fractionations and adsorption on calcium phosphate gel.

(5) The specific activity of the final concentrate of mould acylase (Fraction E) was about 400 times that of the initial extract when assayed against N-acylated phenylalanine, tryptophane, methionine and alanine derivatives and about 100 times that of the initial extract when assayed against N-acylated histidine and aspartic acid.

(6) The purified mould acylase was electrophoretically heterogeneous.

(7) The properties of the mould enzyme and the kinetics of the reaction are described.

(8) The mould enzyme differed from animal tissue acylase I in its behaviour towards N-acylated derivatives of the substituted aromatic amino acids, phenylalanine and tryptophane. While the renal acylase hydrolyzes the derivatives of phenylalanine and tryptophane at markedly lower rates than those of the aliphatic amino acids such as alanine, the mould enzyme readily hydrolyzed the N-acylated derivatives of phenylalanine and tryptophane more rapidly than those of the corresponding alanine derivatives at rates markedly higher than those reported for acyl-tryptophane hydrolysis for any other purified acylase, hitherto.

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(10) The differences in substrate specificity of the renal and mould acylases and pancreatic carboxypeptidase are discussed.

(11) The mould enzyme, unlike renal acylase was inhibited by metal binding agents such as ethylene diamine-tetraacetic acid (EDTA), o-phenanthroline, αα′-dipyridyl, cyanide, azide and fluoride.

(12) The EDTA-treated enzyme remained inactive after dialysis. The inhibition was reversed by bivalent zinc and iron but not by Co^{++}, Mn^{++}, Mg^{++}, Ca^{++} and Fe^{+++}.

(13) Racemic tryptophane was readily resolved into its optical isomers by subjecting N-acetyl-DL-tryptophane to asymmetric hydrolysis by a purified mould acylase preparation.

(14) The L- and D- isomers were optically pure as determined by their optical rotations.

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