MICROBIAL ENZYMES:
Studies on citrase and acylase

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by
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C O N T E N T S

contd.

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List of abbreviations and trivial names

Acylase,	N-Acylamino acid amido-hydrolase
Citrase,	Citrate oxaloacetate-lyase (E.C.4.1.3.6)
DEAE-cellulose,	Diethylaminoethyl-cellulose
EDTA,	Ethylenediaminetetraacetic acid
hr,	hour
MDH,	Malate dehydrogenase (L-malate:NAD oxidoreductase, E.C. 1.1.1.37)
min,	minute
NAD,	Nicotinamide-adenine dinucleotide
$NADH_2$,	Reduced nicotinamide-adenine dinucleotide
NADP,	Nicotinamide-adenine dinucleotide phosphate
$NADPH_2$,	Reduced nicotinamide-adenine dinucleotide phosphate
O.D.	Optical Density
$O.D._{340}$	Optical Density at 340 mμ
$\triangle O.D._{340}$	Change in Optical Density at 340 mμ

Microorganisms have become an essential part of several industrial processes that involve chemical changes, both of degradation and of synthesis. Such transformations are the result of enzymatic activities and the rapid strides made in this field of applied microbiology have become possible only through an understanding of the relevant enzymes which are produced by the organism.

As apart from their industrial significance, several microbial enzymes have found extensive use as biochemical "reagents" in analytical procedures. The special value of enzymes in analysis lies in their ability often to react specifically with an individual compound in a complex mixture without interference from the other components.

In the present investigation two different microbial enzymes, citrase and α-amino acid acylase have been studied.

Citrase, isolated from a bacterial source, has been adapted to the rapid, specific and sensitive assay of citrate in biological materials for which there is at present no quick

and accurate assay procedure.

An α-amino acid acylase has been isolated and purified from a fungal source and has been shown to differ in some of its properties from those reported hitherto in literature for this group of enzymes. Acylases have great commercial value in the preparation of optically active amino acids.

The studies on citrase are described in Part I of this thesis and the studies on acylase in Part II.

PART I.

STUDIES ON CITRASE:

AN ENZYMATIC PROCEDURE FOR

THE ASSAY OF CITRATE IN BIOLOGICAL MATERIALS

INTRODUCTION

Citric acid is extensively distributed in living systems. High concentrations of the acid are present in several plant tissues, particularly in citrus fruits from which it was isolated for the first time in crystalline form by Scheele in 1784. Among other plant sources rich in citrate are cotton leaves, potatoes and pineapples (Thunberg, 1953). In mammals, relatively large concentrations are present in milk and mammary glands (Thunberg, 1953); semen and prostate glands (Mann, 1949) and particularly in bones (Dickens, 1941). Many fungi, such as Aspergillus niger, produce large amounts of citric acid by fermentation and have been used almost exclusively for the commercial production of the acid (Shu and Johnson, 1948).

The importance of citric acid in biological intermediary metabolism has become apparent over the last three decades. As a key compound in the Krebs cycle (Krebs and Johnson, 1937; Krebs and Lowenstein, 1960) citric acid plays an important role in the oxidative metabolism of fats, carbohydrates and amino acids.

Clinical variations in the citrate content of tissues and biological fluids have been associated with particular

abnormalities and disease and has become of significance in diagnostic procedures. Literature on this aspect has been reviewed recently by Nordmann and Nordmann (1961). Some illustrative examples are cited from this review: Low levels of urinary citrate are associated with renal stones and renal tubular acidosis. Alterations in citrate levels of blood and urine have been reported in cases of bone diseases. Urinary citrate output is low in hypothyroidism and in ovarian insufficiency. Citric acid content of plasma is elevated during hepatitis but not in extrahepatic obstruction of the biliary duct. High blood citrate and low urinary citrate are associated with myocardial infarction. Lowering of blood and urinary citrate occurs during acute renal failure. The decrease in urinary citrate is an early symptom during renal failure and has been suggested as a routine test to assess renal function.

In view of the general importance of citric acid in biological metabolism, in clinical chemistry and in industry, particularly of food and pharmaceutics, it is not surprising that several analytical procedures and modifications have been described in the literature for the assay of citrate. The

methods are mainly chemical, some physical and a few enzymatic. The more important of these are considered briefly below:

1. Chemical methods for assay of citrate

a) Pentabromoacetone method: Of the chemical methods the majority involve the oxidation of citric acid to pentabromoacetone (Stahre, 1897) which may be estimated by a variety of gravimetric, titimetric or colorimetric procedures. Since the application of this procedure to biological materials by Pucher, Sherman and Vickery (1936), many variations have been proposed particularly in the conditions of the oxidation and the conversion of the pentabromoacetone to a coloured complex (Natelson, Pincus, Lugovoy, 1948; Hargreaves, Abrahams and Vickery, 1951; Wolcott and Boyer, 1948; Stern, 1957; Weil-Malherbe and Bone, 1949; Taylor, 1953; McArdle, 1955; Ettinger, Goldbaum and Smith, 1952; Beutler and Yeh, 1959; Buffa and Peters, 1950; Cartier and Pin, 1949; Taussky, 1949; McDonald and Waterbury, 1959). The use of ^{82}Br for conversion of citric acid to pentabromoacetone followed by measurement of radioactivity in the derivative has been recommended by Jacobs and Lee (1964).

The oxidation to pentabromoacetone however is non-specific and several substances such as α-keto acids, aldehyde and acetone interfere. These substances have therefore to be eliminated from the test sample by prolonged heating with concentrated sulphuric acid. Further the oxidation conditions have to be carefully controlled and the reaction has to be completed in slow stages. The procedure though sensitive to quantities as low as 10 ug citric acid becomes lengthy, particularly with biological materials, when freedom from interference has to be ensured.

b) Furth-Hermann Reaction:

This colorimetric method first described by Furth and Hermann (1935) depends on the production of a pink colour by the reaction of citric acid with pyridine and acetic anhydride. The method has been modified by Nekhorocheff and Wajzer (1953), Saffran and Denstedt (1948), Marier and Boulet (1958) and Hartford (1962). The procedure though rapid is inaccurate and lacks specificity and fumaric, itaconic, transaconitic, cisaconitic and isocitric acids interfere and the procedure is not to be recommended for assay of biological samples.

c) Berg's reaction: The interaction of ferric chloride and citric acid has been utilised to develop a colorimetric method for citrate (Williams, Muller and Niederl, 1931; Storck, 1954). The method is non-specific and other hydroxy acids interfere.

d) Molybdate method: The bleaching effect of citrate on the colour of reduced molybdate at pH 1.3 has been used by Matulis and Gujon (1964). The method is however not applicable to micro quantities and other hydroxy acids, heavy metals, HPO_4^{-2} and tartrate interfere markedly.

e) Indirect oxidation: An indirect oxidation method was developed by Gordon (1951) in which citrate is oxidised with excess of Br_2, hypochlorite, $KMnO_4$ or ceric sulphate as oxidising agent, the excess being used to oxidise a standard amount of dye. The procedure again is applicable only to relatively pure citrate samples and a wide variety of organic compounds interfere in the assay.

f) Acetone method: Backstrom (1942) oxidised citrate to acetone with $KMnO_4$ and estimated the ketone by treatment with KOH and vanillin to develop colour proportional to the original citrate concentration. This procedure is open to interference from

aldehydes and ketones.

g) Manometric method: Ajl, Wong and Hersey (1952) have described a rapid procedure whereby citric acid is oxidised under controlled conditions with ceric sulphate in Warburg flasks and measured manometrically by the CO_2 produced. Among substances which interfere are α-ketoglutaric acid, pyruvic acid, oxaloacetic acid and malic acid. For application to biological material, citric acid has to be preliminarily isolated by chromatography to remove interfering substances.

2. Physical Methods

In recent times paper, column and thin layer chromatography have been used to separate citrate prior to its determination.

a) Paper chromatography: A general technique of two dimensional chromatography has been described by Nordmann et al. (1954) for the separation of non-volatile organic acids using the systems: ethanol, ammonia and water, and n-propanol, formic acid, eucalyptol and water. Isocitric acid is not separated from citric acid in these chromatograms.

b) Silica gel chromatography: The separation of organic acids on silica gel columns has been described by (Isherwood, 1946; Kinnory, Takeda and Greenberg, 1955). The procedure does not separate citric acid and isocitric acid.

c) Thin layer chromatography: Plates with cellulose powder have been used by Goebell and Klingenberg (1962) for separation of components of the tricarboxylic acid cycle. Two dimension technique is used with the systems: ethanol-ammonia-water and isobutanol-formic acid.

3. Enzymatic Methods

a) Thunberg method: A sensitive method for citric acid determination applicable to 100 ug of the acid was developed by Thunberg as early as 1920. The procedure depends on the bleaching of methylene blue when a crude seed extract of cucumber (Cucumus sativus) is added to citrate in Thunberg tubes in the presence of the dye. The amount of citric acid is evaluated by an elaborate procedure of measuring the period of time required for the complete oxidation by the enzymes of the seed extract in presence as well as absence of test sample. Further as the seed

extract contains a variety of enzymes, the determination is subject to many interfering substances such as malic acid, ethanol, fumaric acid, and a host of other compounds of natural occurrence which make the procedure unsuitable for the assay of biological materials (Natelson, Lugovoy and Pincus, 1947).

b) Determination with aconitase and isocitric dehydrogenase:

A method has been suggested by Siebert (1963) for the determination of citrate by the initial conversion of citrate to isocitrate by the action of the enzyme aconitase;

citrate ⟷ (cis-aconitate) ⟷ isocitrate

and the subsequent oxidation and decarboxylation of isocitrate by the NADP-linked isocitric dehydrogenase:

isocitrate + NADP ——— α-ketoglutarate + CO_2 + $NADPH_2$.

The increase in optical density at 366 mu (or 340 mu) due to the formation of $NADPH_2$ from NADP being a measure of the reaction.

The procedure does not distinguish between citrate and cisaconitate. But the most serious drawback is that the method is applicable only to pure citrate solutions and recovery experiments in which standard citrate solutions are added to tissue extracts result in very low recoveries.

c) Determination of citrate with citrase and oxaloacetate decarboxylase

The estimation of citrate by the use of coupled enzyme reactions initiated by citrase was first developed by Dagley & Dawes (1953). Citrase may be induced in Aerobacter aerogenes (Dagley and Dawes, 1953a; 1955), Streptococcus faecalis (Gillespie and Gunsalus, 1953) and Escherichia coli (Grunberg-Manago and Gunsalus, 1953; Dagley, 1955) and is present constitutively in Streptococcus diacetilactis (Harvey and Collins, 1963). The enzyme catalyzes the cleavage of citrate to oxaloacetate and acetate and requires divalent metal ions, Mg^{2+} being the most effective. The reaction is reversible (Smith, Stamer and Gunsalus, 1956; Harvey and Collins, 1963; Tate and Datta, 1965) and the enzyme is inhibited by the oxaloacetate produced (Dagley and Dawes, 1955; Wheat and Ajl, 1955; Bowen and Rogers, 1963 a and b). A quantitative change of citrate by citrase is obtained only when oxaloacetate is removed from the system. This was achieved by Dagley and Dawes (1956) by the use of a crude extract of citrate from Aerobacter aerogenes which contains a very active oxaloacetate decarboxylase in addition to citrase. By the coupled

action of the two enzymes, the citrate was quantitatively converted to acetate, pyruvate and carbon dioxide; and the pyruvate formed was estimated by the colorimetric procedure of Friedemann and Haugen (1943). The sensitivity of this procedure was greatly increased by Bowen and SivaRaman (1960) and Dagley and SivaRaman (1963) by partially purifying the Aerobacter aerogenes extract by heat denaturation and treatment with alumina gel to remove $NADH_2$ oxidases and estimating the pyruvate formed from citrate by further coupling with the reduction of pyruvate by NAD-linked lactate dehydrogenase. The use of crude or of only partially purified bacterial extracts, however, presents certain problems since contaminating enzymes greatly reduce the specificity of the procedure. Dagley and SivaRaman (1963) had thus observed the interference by glutamate and α-ketoglutarate; and in the application to biological samples many steps were required to eliminate these compounds, such as treatment with a cation exchanger and heating with sulphuric acid.

PRESENT INVESTIGATIONS

It will be evident from the foregoing introduction that although chemical procedures based on the pentabromoacetone derivative are available for the sensitive and specific assay of citrate in biological specimens, these are somewhat lengthy and require several hours of careful manipulation. The development of a rapid and accurate procedure for assay in biological samples of a metabolically important compound such as citrate would be an useful contribution to biochemical analyses.

A method that appeared to have interesting possibilities was one developed in this Laboratory by Sivakaman and Subramanian (1963) for the assay of pure citrate solutions. This rapid and sensitive procedure is based on the use of a purified and stable preparation of citrase to break citrate down to oxaloacetate and acetate, coupled with the reduction of oxaloacetate by $NADH_2$ and

malate dehydrogenase according to the following reactions:

$$\text{Citrate} \rightleftharpoons \text{acetate} + \text{oxaloacetate}$$
$$\text{Oxaloacetate} + NADH_2 \rightleftharpoons \text{malate} + NAD$$

At a pH of 7.4, $NADH_2$ is oxidised rapidly when citrate is the limiting substrate to give a stochiometric assay of citrate, the cleavage of one mole of citrate resulting in the oxidation of one mole $NADH_2$. The assay is carried out spectrophotometrically by measuring the absorption of $NADH_2$ at 340 mu. This sensitive procedure has been used very recently by Ward and Srere (1965) for the measurement of citrase activity.

In the present investigation the application of this rapid and elegant technique to the determination of citrate in a variety of biological samples has been worked out and the accuracy and specificity of the assay procedure and freedom from interference from a wide range of compounds normally present in biological materials have been established.

MATERIALS

The following chemicals and enzyme reagents were used in the assay procedure.

I. Chemicals

1. Citric acid. $1H_2O$, Analytical Reagent obtained from B.D.H., England, was used for the preparation of standard solutions.

2. Reduced nicotinamide-adenine dinucleotide, $NADH_2$ (sodium salt, $Na_2.NADH_2.4H_2O$, Type 1) was obtained from Sigma Chemical Co., U.S.A.

3. Metaphosphoric acid, HPO_3, containing about 80% HPO_3 was a product of Thomas Tyres, England.

4. Magnesium sulphate, $MgSO_4.7H_2O$; Sulphuric acid, H_2SO_4; Sodium hydroxide, NaOH and Potassium dihydrogen phosphate, KH_2PO_4 were Analytical grade reagents obtained from B.D.H.

5. Dipotassium hydrogen phosphate, K_2HPO_4 was a reagent grade manufactured by B.D.H.

II. Enzyme Reagents

1. Citrase: (Citrate oxaloacetate-lyase, E.C. 4.1.3.6)

The enzyme was isolated from Aerobacter aerogenes, type NCTC 418.

The culture was routinely maintained on nutrient broth agar slants.

(a) Growth of cells: A.aerogenes cells were grown without aeration at 30°C in 10 L flat bottomed flasks filled to the neck with the mineral salts-citrate medium of Dagley and Dawes (1953a). The medium was prepared in two parts as follows:

Composition of medium:

(i) Na_3 citrate.$2H_2O$ 90 g

KH_2PO_4 20 g

Water to make to about 8.8 L

N NaOH (about 90 ml) to adjust pH to 7.0

(ii) $(NH_4)_2SO_4$ 10 g

$MgSO_4.7H_2O$ 4 g

Water to make to 1 L

and the two separately sterilised at 15 p.s.i. for 20 min and mixed after cooling. The medium was inoculated with 100 ml of a 24 hr culture of cells grown previously on the citrate medium.

The cells were harvested after 18 hr in a Sharples supercentrifuge. Yield of cell paste from 10 L was about 15 g.

(b) Extraction of cells: Batches of 10 g cell paste were suspended in 50 ml 0.03 M potassium phosphate buffer, pH 7.0 and sonicated at 10 KC/sec for 20 min in a Raytheon sonic

oscillator Model 101 F. The sonicates were centrifuged at 20,000 x g for 30 min at 4°C in a Spinco Rotor 21 of the Model L ultracentrifuge. The clear supernatant containing about 1% protein was adjusted to a protein content of 0.5% with 0.03 M potassium phosphate buffer, pH 7.0 and batches of 150 ml were used for purification of the enzyme.

c) Purification of citrase

The enzyme was purified according to the procedure of Sivakaman (1961). All operations were carried out at 0° to 5°C. The diluted sonic extract (150 ml, 0.5% protein) was treated with streptomycin sulphate to a final concentration of 1.4% of the sulphate and the precipitated nucleic acids removed by centrifuging after 1.5 hr at 20,000 x g for 30 min in a Spinco Rotor 21. The clear supernatant was treated with 5.6 ml of alumina C_γ gel (Willstatter and Kraut, 1923; gel preparation of dry wt. 11 mg/ml). The suspension was cleared by centrifugation at 3000 x g for 15 min in the PR 2 refrigerated centrifuge. The supernatant which contained the activity was treated with another 17 ml of alumina gel (dry wt 11 mg/ml) to absorb the activity. The gel was then collected by centrifugation as in the earlier case. The

gel containing the adsorbed enzyme was washed with 40 ml of 0.01 M potassium phosphate buffer pH 7.0 containing $MgSO_4$ (1.6 mM) and the gel separated by centrifugation. All buffers used in subsequent purification operations contained $MgSO_4$ (1.6 mM) to stabilise the enzyme. The enzyme was then eluted from the washed gel with 40 ml of 0.05 M potassium phosphate buffer, pH 7.0.

The enzyme was precipitated from the eluate (37 ml) by addition of powdered ammonium sulphate to 0.45 saturation (10.4 g). The precipitate which was collected by centrifugation at 20,000 x g for 20 min in a Spinco Rotor 30 was dissolved in 2 ml of 0.01 M potassium phosphate buffer, pH 7.4 and dialysed overnight against 100 ml buffer of the same composition.

The dialysed enzyme was then chromatographed on DEAE-cellulose (Bio Rad, Cellex D, exchange capacity 0.62 meq./g) as follows: The DEAE cellulose (4 g) was pretreated with alkali and acid as recommended by Peterson and Sober (1962) and was then washed first with five lots of 0.5 M potassium phosphate buffer, pH 7.4 (50 ml) and then with five lots of 0.01 M potassium phosphate buffer, pH 7.4 (100 ml); both buffers containing $MgSO_4$ (1.6 mM). The cellulose exchanger was then packed in one

lot to give a 1 x 20 cm column. The dialysed enzyme solution (40 mg protein) was loaded on the column and chromatographed by continuous gradient elution with potassium phosphate buffer, pH 7.4, with gradual increase in concentration over the range 0.01 to 0.3 M. The buffer concentration gradient was obtained by the use of 200 ml of 0.01 M buffer in the mixing chamber and 200 ml of 0.5 M buffer in the separating funnel and flow rates of 15 sec/drop from both the column and the separating funnel (Fig. 1.I). Fractions of 3 ml size were collected on a Technicon Automatic Fraction Collector and the elution of the enzyme detected by sampling a drop from each tube for citrase activity according to the procedure of Dagley and Dawes (1955). The enzyme emerged at about the 20th tube and fractions containing the enzyme were pooled (20 ml) and treated with powdered ammonium sulphate to 0.5 saturation (6.2 g). The precipitate was collected by centrifugation at 20,000 x g for 15 min in a Spinco Rotor 30 and dissolved in 0.01 M potassium phosphate buffer, pH 7.0 (1 ml) and the enzyme fractionally precipitated with saturated ammonium sulphate solution neutralised to pH 7.0, the initial slight precipitate obtained on first addition of ammonium sulphate solution

Fig. 1.1

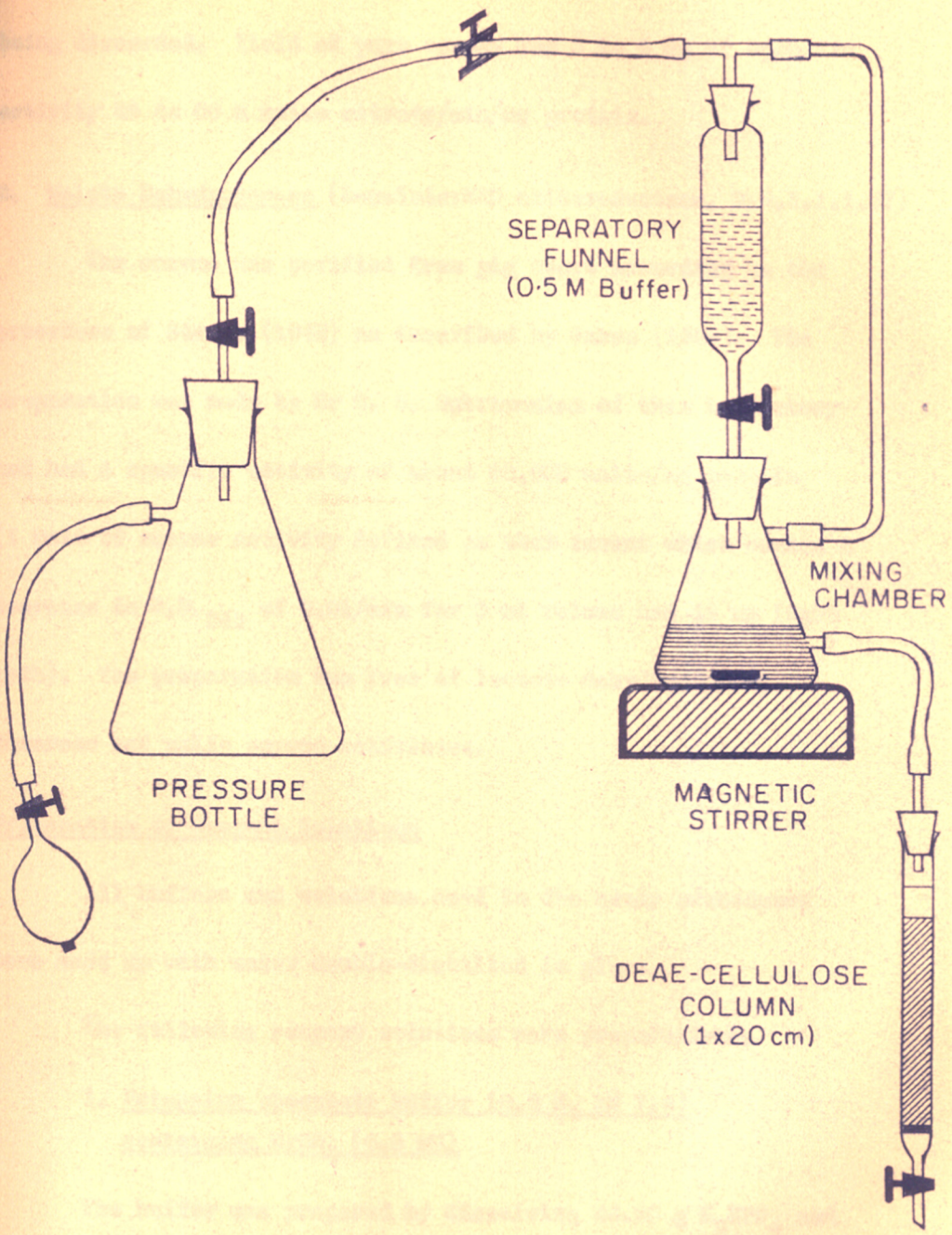

FIG. 1.1 DEVICE FOR CONTINUOUS GRADIENT ELUTION OF DEAE-CELLULOSE COLUMN

being discarded. Yield of pure enzyme was 5 to 6 mg of specific activity 45 to 60 u moles citrate/min/mg protein.

2. Malate Dehydrogenase (L-malate:NAD oxidoreductase, E.C.1.1.1.37)

The enzyme was purified from pig heart according to the procedure of Straub (1942) as described by Ochoa (1955). The preparation was made by Mr S. S. Subramanian of this Laboratory and had a specific activity of about 60,000 units/mg protein (1 unit of enzyme activity defined as that amount which causes a decrease in $O.D._{340}$ of 0.01/min for 3 ml volume and 10 mm light path). The preparation was free of lactate dehydrogenase, fumarase and malic enzyme activities.

Preparation of Reagent Solutions

All buffers and solutions used in the assay procedures were made up with water double-distilled in glass.

The following reagent solutions were preparations.

1. Potassium phosphate buffer (0.3 M, pH 7.4) containing $MgSO_4$ (4.8 mM)

The buffer was prepared by dissolving 43.33 g K_2HPO_4 and 6.98 g KH_2PO_4 in about 700 ml water, adding 120 ml of a 1% w/v aqueous solution of $MgSO_4.7H_2O$ and diluting the mixture to 1L

with water. The buffer was stored at 0°C.

2. Standard Citrate: 21.0 mg citric acid.$1H_2O$ were dissolved in about 50 ml water, treated with 24.2 mg anhydrous sodium bicarbonate ($NaHCO_3$, AR grade) and made up to 100 ml. The solution which is neutral was stored frozen at -20°C.

3. Metaphosphoric acid: Sticks of HPO_3 were dissolved in ice-cold water and adjusted to 2.5 M. The reagent was stored at 0°C and prepared fresh every month.

4. 1.5 N sodium hydroxide solution.

5. Citrase:

Stock solution: The purified enzyme was dissolved (10 mg/ml) in ice cold 0.01 M potassium phosphate buffer, pH 7.4 containing 1.0 mM $MgSO_4$, distributed into 0.5 ml portions and stored at -20°C. The solution was thawed in an ice-bath when required. Frozen solutions are stable and have been used in the assay with no apparent difference up to a period of one year.

Reagent Solution:

Fresh solutions are prepared daily by diluting the stock solution with ice-cold 0.01 M potassium phosphate buffer, pH 7.4,

containing 1.6 mM $MgSO_4$ to a protein concentration of 1.5 mg/ml. Reagent solutions stored frozen and thawed every day have been used in the assay for a period of 1 week without any apparent difference.

6. Malate dehydrogenase (MDH)

Stock suspension: A suspension of the purified enzyme (16 mg) was made with ice cold 70% aqueous ammonium sulphate, $(NH_4)_2SO_4$ solution (2 ml) and stored frozen at -20°C. The preparation is stable for at least a year.

Reagent solution: Fresh solutions are prepared daily by diluting the stock suspension with 0.1 M potassium phosphate buffer, pH 7.4 to a protein concentration of 0.16 mg/ml.

7. Reduced Nicotinamide-adenine dinucleotide (NADH$_2$)

11.5 mg of the $NADH_2$ disodium salt are dissolved in 5 ml water. The solution (2 to 3 mM) is stored frozen and prepared fresh every week.

METHODS & RESULTS

1. Measurements

All pH measurements were made on a Beckman Model G pH meter with glass and calomel electrodes.

Spectrophotometric measurements were made on a Beckman DU Spectrophotometer fitted with a photomultiplier attachment. Measurements were made in 3 ml capacity Pyrex or Corex rectangular glass cells of light path of 10 mm and provided with lids. All enzyme measurements were made at room temperature of 25° to 30°C.

Protein estimations were made by the spectrophotometric method of Warburg and Christian (1941).

2. Chemical assay

In order to check the accuracy of the present enzymatic procedure, all values obtained with biological samples were compared with the average value from determinations in triplicate by the pentabromoacetone method of Natelson, Pincus and Lugovoy (1948) as described by Stern (1957).

The procedure was briefly as follows: Trichloroacetic acid was mixed with the solution to be tested to a final concentration of 5%. The precipitate was removed by centrifugation and an aliquot containing 10 to 100 ug citric acid taken in a glass stoppered tube and brought to 3.0 ml volume with water. 0.1 ml 18N H_2SO_4 and a glass bead were added to the tube and

the tube unstoppered and heated in an air-oven at 120°-140°C till the solution evaporated to about 1 ml. The solution was then cooled and treated with 5 drops M KBr then with 5% $KMnO_4$ (about 10 drops) with shaking over a period of 10 min to maintain a purple colour. The tube was cooled in ice-water and the excess $KMnO_4$ just decolourised with 6% H_2O_2. The final end-point was obtained with alternate addition of 0.5% $KMnO_4$ and 0.6% H_2O_2 followed by 1 drop 0.05% $KMnO_4$ to give a very faint permanent pink colour. 2.5 ml heptane was then added to the tube and stoppered and shaken vigorously for 2 min. The tube was lightly centrifuged for 5 min to separate water and heptane layers. 2.0 ml of the heptane layer was transferred to a clean glass stoppered tube and treated with 4.0 ml 4% thiourea solution containing 2% sodium borate. A tube with heptane and thiourea was included for the reagent blank. The tubes were stoppered and shaken vigorously for 2 min. A sample of the aqueous layer was taken in Beckman cuvettes and read at 430 mu. A reading was also taken at 650 mu and subtracted from that at 430 mu to correct for any cloudiness. A standard citric acid solution equivalent to 40 ug was always carried through the procedure. A linear relationship was obtained between amount and

colour intensity over the range 10 to 100 ug of citric acid. A standard curve is illustrated in Fig. 1.2.

GENERAL ASSAY PROCEDURE

Standard curve for citrate

All solutions except of $NADH_2$ and enzymes were equilibrated to 30° before use. Determinations were carried out in triplicate.

The following solutions are pipetted into a silica (or glass) cuvette.

1.00 ml	0.3 M phosphate buffer containing $MgSO_4$ (solution 1)
0.10 ml	$NADH_2$ (solution 7)
1.78 ml	standard citrate solution containing 0.01 to 0.20 uM citrate
0.10 ml	MDH reagent solution (6)
2.98 ml	

The cuvettes are covered with lids and the contents mixed by inverting twice or thrice and the initial optical density read at 340 mu against water as blank.

0.02 ml citrase reagent solution (5) is pipetted onto a small glass spatula and stirred into the experimental cuvette.

Fig. 1.2

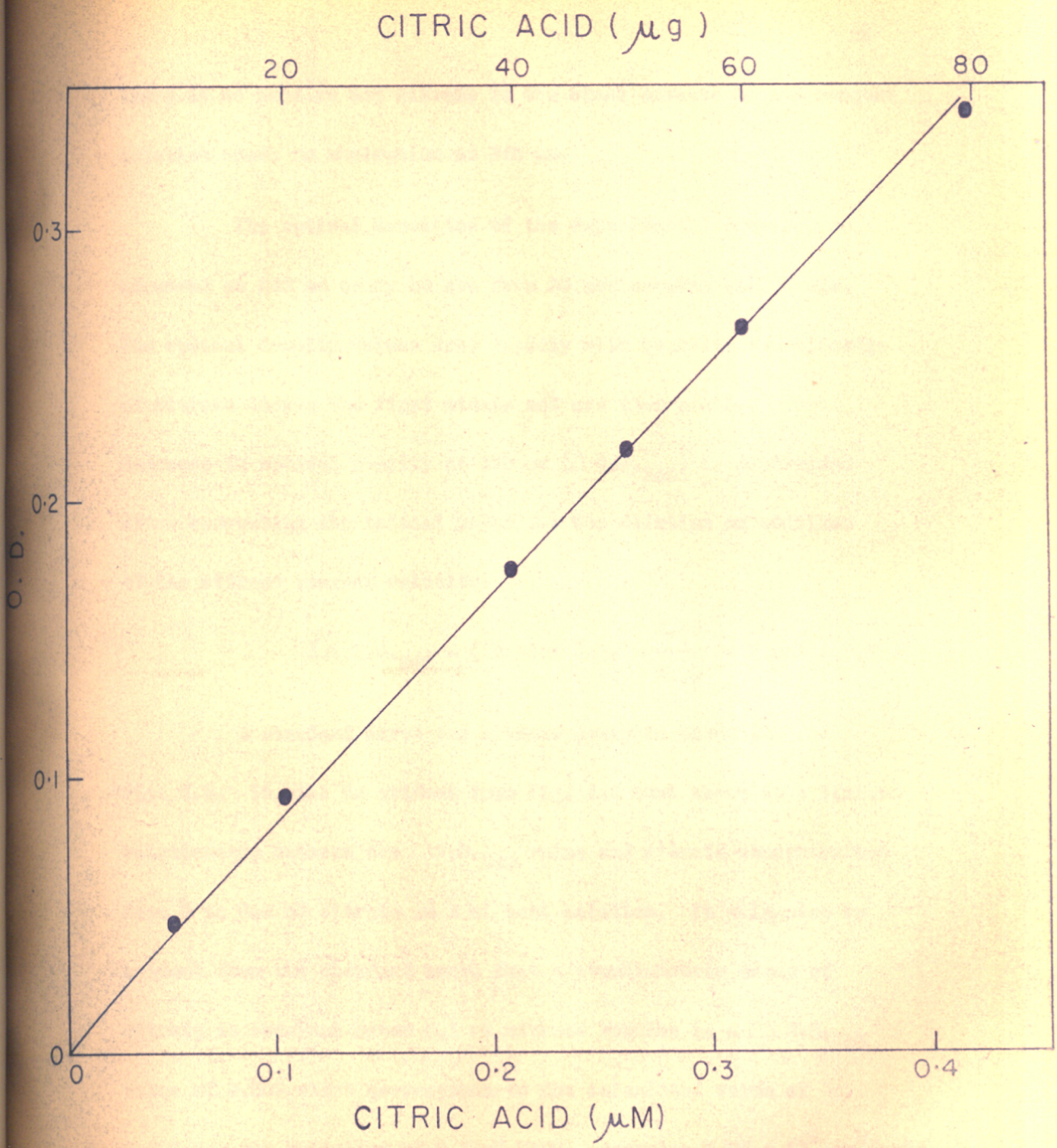

FIG. 1.2 STANDARD CURVE FOR CHEMICAL ASSAY OF CITRIC ACID BY THE PROCEDURE OF NATELSON et al. (1948).

RELATIONSHIP BETWEEN AMOUNT OF CITRIC ACID IN SAMPLE OF STANDARD SOLUTION AND O.D. AT 430 mµ FOR CUVETTE OF 10 mm LIGHT PATH.

CITRIC ACID SOLUTION, 1 ml; 2 ml (OUT OF 2.5 ml) HEPTANE LAYER TREATED WITH 4 ml THIOUREA.

There is no need to add citrase to the blank cuvette as the reagent solution shows no absorption at 340 mμ.

The optical densities of the experimental cuvette are measured at 340 mμ every 15 sec from 30 sec onwards till 3 min. The optical density values drop rapidly with quantitative cleavage of citrate during the first minute and are then stable. The decrease in optical density at 340 mμ ($\triangle O.D._{340}$) is determined after correcting the initial value for the dilution on addition of the citrase reagent solution:

$$\triangle O.D._{340} = \left(\text{Initial } O.D. \times \frac{2.98}{3.00} - \text{Final } O.D.\right)$$

A standard curve for citrate assay is shown in Fig. 1.3. It will be evident from Fig. 1.3 that there is a linear relationship between the $\triangle O.D._{340}$ value and citrate concentration from 0 to 0.2 uM citrate in 3 ml test solution. It will also be evident from the standard curve that a stoichiometric assay of citrate is obtained since 0.1 uM citrate results in a $\triangle O.D._{340}$ value of 0.205 which corresponds to the calculated value of 0.207 for the oxidation of 0.1 uM $NADH_2$, assuming 6.22×10^6 cm^2/mole as the molecular extinction coefficient of $NADH_2$ (Horecker and Kornberg, 1948).

Fig. 1.3

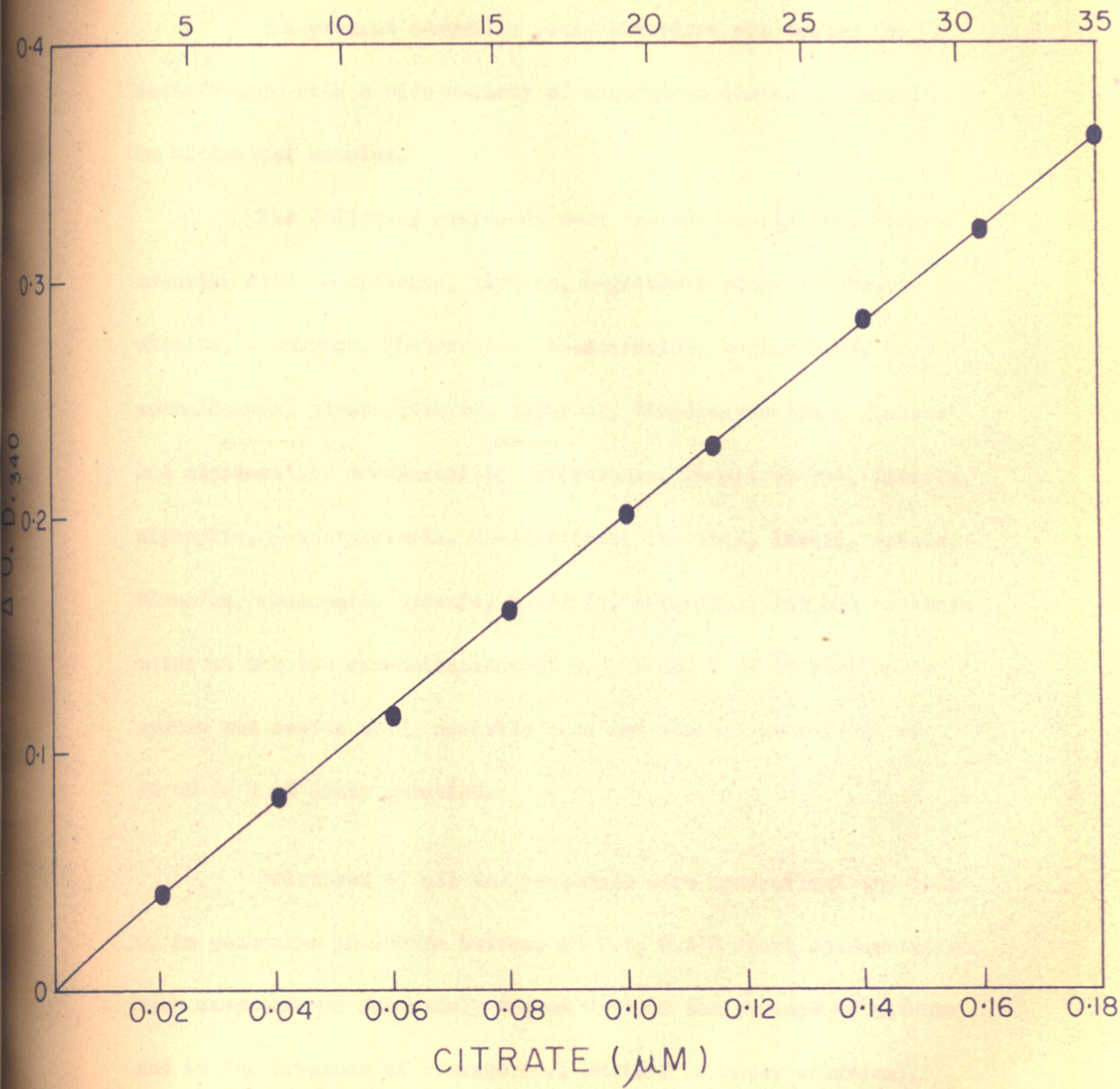

FIG. 1.3 STANDARD CURVE FOR ENZYMATIC ASSAY OF CITRATE WITH CITRASE-MDH-$NADH_2$.

SYSTEM: POTASSIUM PHOSPHATE BUFFER, pH 7.4 CONTAINING $MgSO_4$ (4.8×10^{-3}M), 1 ml; CITRATE SOLUTION, 1.78 ml; MDH REAGENT SOLUTION (15 ug), 0.1 ml; $NADH_2$ SOLUTION, 0.1 ml; CITRASE REAGENT SOLUTION (30 ug), 0.02 ml. CUVETTES OF 10 mm LIGHT PATH.

The present enzymatic assay procedure was tested for interference with a wide variety of substances commonly present in biological samples.

The following compounds were tested: DL-alanine, DL-aspartic acid, L-cysteine, glycine, L-glutamic acid, DL-phenylalanine, DL-serine, DL-tyrosine, L-asparagine, L-glutamine, acetaldehyde, glyceraldehyde, glycerol, dihydroxyacetone, glucose and cisaconitic, transaconitic, citraconic, DL-citramalic, fumaric, glyoxylic, α-ketoglutaric, DL-isocitric, itaconic, lactic, maleic, DL-malic, mesaconic, pyruvic, succinic, tricarballylic and tartaric acids at the two concentrations of 0.3 uM and 1 uM in 3 ml assay system and acetic acid, ascorbic acid and ethanol at a level of 10 uM in 3 ml assay solution.

Solutions of all the compounds were neutralized and made up in potassium phosphate buffer, pH 7.4, 0.1 M final concentration. Each compound was separately tested both in the absence of citrate and in the presence of citrate (0.1 uM in 3 ml assay solution).

No oxidation of $NADH_2$ could be detected when the compounds were tested in the absence of citrate. In the presence of 0.1 uM citrate all experimental cuvettes gave ΔO.D.$_{340}$ value of

0.200 to 0.210, indicating complete absence of interference.

The influence of added oxaloacetic acid in the system was also checked at 0.1 uM level in 3 ml test system. In this case the concentration of $NADH_2$ in the test system was increased by ~~two~~ by pipetting 0.15 ml $NADH_2$ solution 7 and reducing the sample size proportionately. In the absence of citrate 0.1 uM $NADH_2$ was oxidized within a period of 1 min. The test with citrate was carried out by taking the initial $O.D._{340}$ reading after 1 min of addition of MDH reagent solution 6, followed by addition of citrase reagent solution 5 and recording O.D. readings thereafter at 15 sec intervals. Under these conditions $\triangle O.D._{340}$ for citrate showed the expected value of 0.2.

APPLICATION TO BIOLOGICAL MATERIALS

1. Preparation of biological samples for assay

General

The advantage of enzymatic methods of analysis lies mainly in the specificity of an enzyme to react with an individual component of a mixture. This makes any lengthy procedure for the elimination of interfering substances unnecessary. The freedom of the present

procedure from interference from all of a wide range of substances that were tested in these studies makes the procedure directly applicable to most biological samples. In such cases, initial treatment such as heating to stop autolytic changes and to denature enzymes that might cause oxidation of $NADH_2$ may be required. Examples of material that require such treatment only are samples of fungal and bacterial culture media and plant extracts which contain little protein. The presence of large amounts of protein as in serum and bacterial sonicates, however, leads to an opalescence on heating which subsequently interferes to some extent with the spectrophotometric determinations at 340 mμ. Such samples have, therefore, to be treated with a deproteinising agent for an accurate assay. Metaphosphoric acid which was recommended first by Krebs and Eggleston (1944) and later by Dagley and Sivaraman (1963) was used in the deproteination step. The latter authors had already indicated the unsuitability of other agents such as zinc sulphate with NaOH or $Ba(OH)_2$ and trichloracetic acid for enzymic assay of citrate. Excess metaphosphoric acid was converted quantitatively to orthophosphoric acid by heating for 30 min at 100°C since neutralised metaphosphoric acid inhibits citrase (Dagley and Sivaraman, 1963).

2) Biological Samples

Parallel assays were carried out by both the new enzymatic procedure and the pentabromoacetone procedure as described by Stern (1957). The enzymatic assay was carried out as described for standard citrate solutions.

a) Culture Fluids:

The production of citric acid by Aspergillus niger (NCIM 613) grown on Currie's medium and the utilisation of citrate by cells of Aerobacter aerogenes (NCTC 418) grown in a mineral salts-excess citrate medium (Dagley and Dawes, 1953b) were followed by the enzymatic procedure. Samples of the culture medium of A.niger were removed on the 7th day and filtered. Samples of the A.aerogenes grown medium were withdrawn aseptically at periodic intervals and centrifuged for 10 min. at 10,000 x g. Aliquots of the clear filtrate or supernatant were heated for 15 min in test tubes immersed in a boiling water bath, cooled and made up to a suitable volume. Aliquots of the clear treated solutions were neutralised and taken for citrate assay.

b) Leaf tissue of Gossypium herbaceum (cotton)

Cotton leaves are a rich source of citric acid. Samples of the air dried leaf powder were extracted with 100 volumes 0.1 N H_2SO_4 by

mechanical stirring for 20 min at room temperature followed by heating 10 min in a boiling water bath. The extract was cooled, filtered and the residue washed with water. The filtrate and washings were combined and made to volume and aliquots neutralised with N NaOH and again made to volume. Aliquots of the final neutral solution were taken for the assay of citrate.

c) Serum:

Fresh serum obtained from horse and from rabbit were used for assay. Samples varying from 0.5 ml to 5 ml size can be assayed conveniently. In the case of the lower range the final assay has to be carried out in 1 ml capacity microcuvettes with 10 mm light path. Details for the larger volume is described below: 5 ml serum was treated with 1 ml of 2.5 M HPO_3 solution in 7 ml centrifuge tubes while stirring with a thin glass rod. The voluminous precipitate which formed was kept for 5 min and then centrifuged off at 5000 x g for 15 min. 3.0 ml of the clear centrifugate was transferred to a stoppered test tube and heated in a boiling water bath for 30 min. The solution was then cooled, adjusted to pH 7.4 with 1.5 N NaOH and made up to a volume of 10 ml with water. The amount of NaOH required for neutralisation is determined either by titration of an aliquot using bromothymol blue as indicator or by adjusting the pH using a

glass electrode. In the former case where an indicator is used the aliquot is discarded while in the latter this is unnecessary. 1 ml to 1.5 ml aliquots of the final solution were taken for citrate assay.

The results obtained with the various biological samples are summarised in Table 1.1.

It will be evident from these results that there is excellent agreement in all cases between the values obtained by the enzymatic procedure and those obtained by the chemical method of Natelson et al. (1948).

3) Recovery Trials

As a further check on the reliability of the enzymatic procedure for citrate assay in biological samples, recovery trials were performed with biological specimens to which known amounts of citrate had been added. Details of particular examples are given below.

a) Culture fluids and cotton leaf extracts

Samples of culture filtrates and cotton leaf extracts were heated for 15 min, cooled, neutralised as described earlier and made up to a suitable volume. Known amounts of citric acid were then added to aliquots of the materials and samples were assayed as

TABLE 1.1

Estimation of citrate in biological materials by the enzymatic system: citrase–malate dehydrogenase–$NADH_2$. Parallel determinations carried out by the chemical procedure of Natelson et al. (1948) as check.

Material		Citric acid content: Enzymatic assay	Citric acid content: Chemical assay
1. Culture Medium (*A. niger*) 7 days growth		21.80 mg/ml	22.12 mg/ml
2. Culture Medium (*A. aerogenes* grown on citrate–mineral salts medium)	0 hr	47.3 uM/ml	47.0 uM/ml
	2 hr	43.8 uM/ml	44.7 uM/ml
	4 hr	34.0 uM/ml	33.0 uM/ml
	6 hr	18.1 uM/ml	-
	8 hr	0.0	0.0
3. Cotton leaf powder		4.62 g/100 g	4.70 g/100 g
4. Horse serum		31.46 mg/100 ml	32.00 mg/100 ml

described earlier. Control samples without added citrate were also analysed to determine the original citric acid contents.

b) Serum samples

Serum samples were treated with known amounts of citric acid and immediately deproteinised with metaphosphoric acid and analysed as described earlier. Control samples of serum without added citrate were estimated for the original citric acid content.

c) Bacterial crushes

Recovery trials were also performed with samples of sonicates of Aerobacter aerogenes prepared as described earlier under the method for isolation of citrase. Aliquots of the centrifuged sonicate were heated for 15 min in a boiling water bath, cooled and treated with known amounts of citric acid. Aliquots were deproteinised as in the case of serum samples and assayed also under similar conditions. Control samples were analysed and found to contain no citrate.

The results of the recovery experiments are summarised in Table 1.2.

It is evident from Table 1.2 that the recovery of added citrate from a large variety of biological sample is practically quantitative by the enzymatic procedure.

TABLE 1.2

Recovery of added citrate from biological material. Assay by the enzymatic system: citrase-malate dehydrogenase-$NADH_2$.

Biological material	Citrate Added uM	Citrate Recovered uM	Recovery %
1. Culture medium (A.niger) 7 day	0.100	0.100	100
2. Culture medium (A.aerogenes) 8 hr	0.100	0.097	97
3. Cotton leaf extract	0.200	0.202	101
4. Cell sonicate (A.aerogenes)	0.200	0.200	100
5. Rabbit serum	1.000	1.040	104
6. Horse serum	1.000	0.980	98

DISCUSSION

All accurate procedures which have hitherto been applied to citrate in biological samples have the drawback of being lengthy on account of their intrinsic non-specificity and the difficulty in removing compounds that interfere. The most accurate of the chemical procedures, which is the pentabromoacetone method, thus involves several steps for removal of α-keto acids, aldehyde and acetone (Natelson et al. 1948). Amongst the enzymatic procedures, the most accurate and sensitive method described in literature is that of Dagley and SivaRaman (1963) using partially purified extracts of Aerobacter aerogenes. The presence of contaminating enzymes however in this instance produces interference in presence of glutamic acid and α-ketoglutarate and the removal of these involves tedious procedures.

The enzymatic procedure suggested by SivaRaman and Subramanian (1963) in which purified citrase and malate dehydrogenase are used in the spectrophotometric assay of citrate has been shown in the present studies to have none of these disadvantages. The method is sensitive and amounts as low as 0.02 mM or about 4 ug citric acid can be accurately assayed in standard 3 ml capacity spectrophotometric cuvettes of 10 mm light path.

The new enzymatic procedure is also extremely rapid, a direct assay requiring only a minute after mixing of reagents. The procedure is intrinsically free from interference and shows absolute specificity for citrate from among a wide range of compounds that have been tested. Where protein levels are low in biological samples, the only pretreatment required for the specimen is heat denaturation of the enzymes in the sample which might react either with citrate or with $NADH_2$. Where protein levels are high and an opalescence results on heat treatment, deproteinisation with metaphosphoric acid is recommended followed by conversion of excess metaphosphoric acid to orthophosphoric acid by heating for 30 min at 100°C and neutralisation with alkali. In this case the entire assay procedure takes no more than an hour and a half.

The present enzymatic procedure is also accurate and results obtained with several different biological specimens are in complete agreement with parallel determinations carried out by the pentabromoacetone procedure of Natelson et al. (1948). The accuracy of the new procedure has also been established by recovery trials where added citrate has been quantitatively accounted for in different biological samples.

The enzymes used in the present studies have also been shown

to have excellent keeping qualities. Pure citrase stock preparations have been used without any difficulty after several months of storage for as long as a year in the freeze, while repeated thawing at 0°C and freezing at -20°C has also been found to have no apparent deleterious effect on the rapidity and accuracy of citrate assay. Stock samples of malate dehydrogenase also show remarkable keeping qualities and have been used for at least a year without apparent loss of effectiveness in the assay system. In this connection it may be pointed out that the application of enzymes such as the citrate cleaving enzyme (Srere and Lipmann, 1953; Srere, 1959) to citrate assay was also considered but was not pursued as the enzyme is unstable and loses as much as 40% of its activity on storage overnight at -20°C.

SUMMARY

A new procedure has been worked out for the enzymatic assay of citrate in biological materials based on the coupled action of the two enzymes citrase and malate dehydrogenase in presence of $NADH_2$. Under the conditions described the breakdown of 1 mole citrate results in the oxidation of exactly 1 mole $NADH_2$. The estimation is carried out spectrophotometrically by measuring the decrease in the optical density at 340 mμ due to the oxidation of $NADH_2$.

1. The procedure is highly sensitive and amounts as low as

1 to 4 ug citric acid can be accurately assayed.

2. The method is rapid and the enzymatic reaction is complete within a minute.

3. The enzymatic procedure shows freedom from interference from the following compounds that were tested: alanine, aspartic acid, cysteine, glycine, glutamic acid, phenylalanine, serine, tyrosine, asparagine, glutamine, acetaldehyde, glyceraldehyde, glycerol, dihydroxyacetone, glucose, cisaconitic, transaconitic, citraconic, citramalic, fumaric, glyoxylic, α-ketoglutaric, isocitric, itaconic, lactic, maleic, malic, mesaconic, pyruvic, succinic, tricarballylic and tartaric acids at levels of 1 uM in 3 ml test system and ethanol, acetic acid and ascorbic acid at a level of 10 uM in 3 ml. In the presence of oxaloacetic acid, citrate can be estimated by the use of an adequate $NADH_2$ level and addition of citrase after the initial reduction of oxaloacetate is complete.

4. The specificity of the analytical procedure renders all pretreatment for removal of interfering substances unnecessary in the case of samples of low protein content such as culture fluids and leaf extracts. In such cases the entire assay can be completed within 30 minutes. Where biological samples are rich in protein, such as serum and bacterial crushes, removal of protein with metaphosphoric

acid is recommended followed by removal of precipitated protein and heating for 30 min to convert excess of the metaphosphoric acid to orthophosphoric acid. In these instances the assay takes about an hour and a half.

5. The accuracy of the new assay procedure has been checked both by parallel chemical determinations of citrate in a variety of biological specimens as well as by recovery trials.

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PART II.

STUDIES ON α-AMINO ACID ACYLASE: PURIFICATION AND PROPERTIES OF AN ACYLASE FROM *Aspergillus flavus-oryzae* NRRL 5 3 6

INTRODUCTION

The group of enzymes classed as α-amino acid acylases or N-acylamino acid amido-hydrolases are peptidases capable of hydrolysing the peptide bond of N-acylated amino acid derivatives. The first report of such an enzyme was made by Schmiedeberg (1881), who observed the hydrolysis of hippuric acid (benzoylglycine) by aqueous extracts of animal tissues and called the enzyme "histozyme". The nomenclature was later changed to "hippuricase" (Clementi, 1923) and the more general notation "aminoacylase" (Smorodinzev, 1923). Greenstein and his co-workers who initiated extensive studies on this class of enzymes in more recent years, adopted the nomenclature "amino acid acylase" which has now been further modified to the systematic name of N-acylamino acid amido-hydrolases.

1. TYPES AND OCCURRENCE

At least five different enzymes have been well characterised in this group:

i) Acylase I (N-acylamino acid amido-hydrolase, E.C. 3.5.1.14) which is the most extensively studied in the group has a wide range of specificity and hydrolyses the N-acyl-derivatives of several L-α-amino acids with the exception of aspartic acid and the aromatic acids, particularly tryptophan (Greenstein and Winitz, 1961; Bruns and Schulze, 1962). Acylase I occurs in mammalian tissues such as liver, kidney, pancreas and hepatoma (Abderhalden and Abderhalden, 1938;

Price and Greenstein, 1948). The richest source reported for the enzyme is hog kidney, where it occurs particularly in the cytoplasm with decreasing levels in the order; microsomal, nuclear and mitochondrial fractions (Hanson, Hermann and Blech, 1959).

ii) Acylase II, which is present in mammalian renal tissue, is more specific in its activity than acylase I and hydrolyses only N-acylated derivatives of L-aspartic acid (Birnbaum, Levintov, Kingsley and Greenstein, 1952).

iii) An enzyme, ϵ-lysine acylase, specific for ϵ-acylated-L-lysine derivatives has been isolated from rat, hog and chick kidneys (Paik, Bloch-Frankenthal, Birnbaum, Winitz and Greenstein, 1957; Paik and Benoitin, 1963) and from bacterial sources like a strain of Pseudomonas (Wada, 1959) and the soil organism, Achromobacter pestifer (Chibata, 1964). The renal enzyme differs from the bacterial in its substrate specificity. The enzymes do not act readily on α-substituted derivatives of L-lysine.

iv) Microbial and plant acylase III, has been isolated from several fungi (Michi and Nonaka, 1954a, b; Borkar, 1957; Michi and Tsuda, 1957); yeast (Chibata and Ishikawa, 1958), soil Pseudomonas (Kameda, Toyoura and Kimura, 1958) and plants (Chibata and Tosa, 1959; Ozaki and Wetter, 1960). The enzyme has a wide range of specificity acting on the N-acylated derivatives of several L-α-amino acids and differs from renal acylase I particularly in the susceptibility of acylated aromatic amino acids to hydrolysis by the mould and plant enzyme and the marked resistance of these to the renal

enzyme. The possibility of the existence of a renal acylase III capable of acting on acylated aromatic amino acids has been suggested by Greenstein and his group (Greenstein and Winitz, 1961; Rao, Birnbaum, Kingsley and Greenstein, 1952).

v) An interesting enzyme isolated from soil bacteria has been shown to be specific towards N-acylated derivatives of D-amino acids (Kameda, Toyoura and Kimura, 1958).

Among the less well authenticated acylases is a mammalian kidney microsomal enzyme claimed to be specific for long-chain N-acyl derivatives of glycine and L-amino acids like palmitoyl-glycine and myristoyl-L-phenylalanine (Hazama, Kitagawa and Yamamura, 1963).

2. PURIFICATION & PROPERTIES OF ACYLASES

Some of the important investigations on acylases reported in the literature are summarized briefly below:

(i) RENAL ACYLASE I

Partial purification of the enzyme to the extent of a 35-fold concentration from hog kidney was obtained by Birnbaum, Levintow, Kingsley and Greenstein (1952) by a procedure involving isoelectric precipitation and ammonium sulphate and acetone fractionations. Chi and Orekhovich (1959) reported a 100-fold concentration from the same source by starch gel electrophoresis. Renal acylase I was isolated in pure form by Bruns and Schulze (1962) and the final

preparation after 300 to 350-fold concentration was shown to be homogeneous on the basis of ultracentrifugal, electrophoretic and immunoelectrophoretic behaviour. The purification procedure involved isoelectric precipitation, ammonium sulphate and ethanol fractionations and chromatography on DEAE-cellulose. The enzyme has a molecular weight of 76,500 and an activity of 20,000 moles acetylmethionine/mole protein/min at 37°C.

(a) OPTICAL SPECIFICITY

Smorodinzev (1923) first made the important observation that crude enzyme extracts from animal tissues, like liver and kidney, has marked optical specificity and hydrolysed N-benzoyl-L-aminobutyric acid but not N-benzoyl-D-aminobutyric acid.

The application of this property of optical specificity to the resolution of racemic α-amino acids has been extensively investigated by Greenstein and his co-workers (Greenstein, 1954; Greenstein and Winitz, 1961). The basis of this enzymatic procedure is the remarkably high optical specificity of renal acylase I towards most acylated, particularly acetylated, L-amino acid derivatives, the ratio of the rates of hydrolysis of the acetyl-L-amino acid to that of the acetyl-D-amino acid being 40,000:1 or greater when the N-acetylated racemate is the substrate (Birnbaum, Levintov, Kingsley and Greenstein, 1952). By the application of this procedure of asymmetric hydrolysis, Greenstein and his coworkers have resolved nearly all known α-amino acids. The general procedure involves the initial hydrolysis of the N-acylated DL-amino acid to give the L-amino acid,

acyl acid and N-acyl-D-amino acid. The L-amino acid is then separated from the acyl-D-amino acid either by differential solubility or by the use of ion exchange resins. The D-amino acid is then obtained from the acyl-D-amino acid by acid hydrolysis. The isomers separated by such procedures have been shown by the use of optically specific amino acid oxidases or decarboxylases to be remarkably pure, usually containing not more than 1 part in 1000 of the antipode (Meister, Levintov, Kingsley and Greenstein, 1951).

An interesting extension of this procedure for the isolation of optical isomers of amino acids is covered under a recent Patent (Fr. 1964, 1,374,707) wherein a mold acylase preparation is copolymerised with N-carboxy-γ-methyl-glutamic anhydride and the resulting copolymer is packed in a column and the acylated racemate resolved by percolating through the column.

(b) CHEMICAL STRUCTURE AND SUBSTRATE SUSCEPTIBILITY

The original observation that renal acylases are α-amino acid acylases was also made by Smorodinzev (1923) who showed that animal tissue extracts hydrolysed N-benzoyl-α-amino acids and not β-amino acids derivatives, like benzoyl-β-alanine and benzoyl-β-amino butyric acid. Later workers have shown that partially purified renal acylase I acts on diacylated derivatives of α-γ-diamino butyric acid, ornithine and lysine to yield the corresponding ω-acyl amino acids (Fu, Rao, Birnbaum and Greenstein, 1952).

A free α-carboxyl group has been shown to be essential for the enzymatic action of acylase I and acyl-amino acid amides

and esters are resistant to hydrolysis under conditions where the free acid is rapidly hydrolysed (Fu, Birnbaum and Greenstein,1954).

The influence of the nature of the amino acid residue and of the acyl group on susceptibility to the action of acylase I has also been studied extensively by Greenstein and his group using relatively crude enzyme preparations and more recently by Bruns and Schultze (1962) with the pure enzyme. The earlier data have been reviewed by Greenstein and Winitz (1961). Among the amino acid derivatives tested those of methionine were the most susceptible. Those involving the aliphatic amino acids were in general preferred substrates. In the straight-chain aliphatic homologues of glycine, susceptibility increased with chain length to a maximum of n-valine, the rates decreasing thereafter with the longer chain derivatives. The branching of the amino acid chain depressed the rate and tert-leucine with a completely substituted β-carbon was resistant to renal and pancreatic acylases. Allostereoisomers of threonine and isoleucine differed in their susceptibility from that of the corresponding normal form. Derivatives involving aspartic acid, proline, hydroxyproline and tryptophan were resistant to the action of acylase I.

In studies on sensitivity of acylase I to the nature of the acyl group, halogenacyl derivatives such as chloroacetyl have been shown to be hydrolysed faster than the corresponding acetyl derivative, while benzoyl derivatives are acted on at rates slower than the corresponding acetyl, propionyl or formyl derivative. In

the monohalogen series, rates decreased in the order of decreasing electronegativity of the halogen atom, fluoroacetyl derivatives being the most susceptible and iodoacetyl the least. When the N-acyl group is trifluoroacetyl, the hydrolysis rates were generally the fastest, but the high degree of optical specificity of the enzyme was then considerably lowered and the trifluoroacetyl D-amino acid derivatives were also split at measurable rates (Fones and Lee,1954). A similar lowering effect on optical specificity was observed with chloropropionyl derivatives (Fu, Birnbaum and Greenstein, 1954).

Acylase I also acts on dipeptides such as glycyl and alanyl amino acids, in the case of the latter, L-alanyl derivatives being split faster than the D-alanyl (Rao, Birnbaum, Kingsley and Greenstein, 1952). The hydrolysis of glycylglycine by acylase I has been shown to differ from that of the same substrate by glycylglycine dipeptidase (Smith,1951) in that Co^{2+} is required for the action of the dipeptidase while the metal inhibits hydrolysis by acylase I (Bruns and Schulze, 1962).

Pure acylase I has been shown to have no cathepsin-like activity (Bruns and Schulze, 1962) unlike less pure preparations (Chi and Orekhovich, 1958).

(e) INHIBITORS AND METAL REQUIREMENT

Pure acylase I has been shown to be strongly inhibited by -SH group-specific reagents (Bruns and Schulze, 1962). The pure enzyme has also been shown to require no metal for its activity

particularly towards the more susceptible derivatives, the presence of Co^{2+} actually acting as an inhibitor in these instances. The presence of 1×10^{-3}M Co^{2+} has been shown, however, to enhance hydrolysis of the less susceptible amino acid derivatives such as benzoylglycine (Bruns and Schulze, 1962) and the acyl D-amino acid derivatives (Marshall, Birnbaum and Greenstein, 1956). Partially purified acylase I was shown to contain no cobalt but the metal could be incorporated by treating the enzyme preparation with Co^{2+} followed by dialysis and lyophilisation. The Co^{2+} modified preparation has then been shown to hydrolyse all substrates at a rate higher than that of the native enzyme and the cobalt-treated acylase was unaffected by ethylene diamine tetraacetic acid (EDTA) (Marshall, Birnbaum and Greenstein, 1956).

ii) ACYLASE II

The enzyme has been separated from acylase I and partially purified by Birnbaum, Levintov, Kingsley and Greenstein (1952) using hog kidney as the source. The enzyme is specifically an L-aspartic acid acylase and hydrolyses asymmetrically, N-acylated derivatives of L-aspartic acid such as N-chloroacetyl-L-aspartic acid. Dipeptides of L-aspartic acid, like glycyl-L-aspartic acid, are hydrolysed at slow rates. N-acylated-L-asparagine is not susceptible to acylase II but is readily hydrolysed by acylase I.

Co^{2+} + Mn^{2+} have no effect on the activity of acylase II (Rao, Birnbaum, Kingsley and Greenstein, 1952).

iii) ∈ -LYSINE ACYLASE

Paik, Block-Frankenthal, Birnbaum, Winitz and Greenstein (1957) reported an acylase system in rat kidney which hydrolysed ∈-acylated-L-lysine derivatives stereospecifically. The enzyme was concentrated 160-fold by Paik and Benoitin (1963) from hog kidneys. EDTA and metals had no effect on this renal enzyme. Aliphatic ∈-N acyl derivatives were readily hydrolysed while aromatic derivatives were unaffected. In marked contrast, the α-N-acyl-derivatives of lysine were hydrolysed very slowly while α-N-aryl derivatives were acted on at appreciable rates. ∈-N-Glycyl-L-lysine was resistant but the ∈-N-acetyl group of lysine was still hydrolysed when the α-amino or the carboxyl or both were in peptide linkages.

∈-Lysine acylases have been shown to be present in a strain of Pseudomonas (Wade, 1959), in Aspergillus oryzae (Chibata, Ishikawa and Tosa, 1960) and in Achromobacter pestifer EA (Chibata, Ishikawa and Tosa, 1962). The fungal enzyme, unlike the renal and bacterial, has no optical specificity. The bacterial enzyme has been purified from toluene-treated cells of A.pestifer by fractionation with ammonium sulfate, precipitation with Rivanol (6:9-diamino-2-ethoxy-acridine lactate), acetone and ammonium sulphate fractionation and electrophoresis on cellulose columns. The bacterial enzyme required no metal and was inhibited by -SH group-specific reagents and has been shown to differ from the renal preparation in its substrate specificity (Chibata, Tosa and Ishikawa, 1964). ∈-N-Benzoyl-L-lysine was thus hydrolysed at a rate comparable to the acetyl

derivative while the aryl derivative was resistant to hydrolysis by the renal acylase. Also unlike the renal preparation, α- ε-N- diacyl-L-lysine derivatives and compounds having no free -COOH group were resistant to the action of the bacterial enzyme.

iv) MICROBIAL AND PLANT ACYLASES (ACYLASE III)

Investigations on microbial acylases have been carried out mainly by Japanese workers over the last decade, particularly after Greenstein and his colleagues had established the usefulness of this group of enzymes in the preparation of optically pure amino acids.

The earlier investigations were restricted mainly to a study of the enzymatic hydrolysis of hippuric acid. Thus Dox (1910) showed the presence of a hippuric acid hydrolysing enzyme in several cheese moulds like Penicillium camemberti and P.chrysogenum and Grassman and Basu (1931) reported such an activity in bran cultures of Aspergillus oryzae.

Neuberg and Linhardt (1924) observed that the acylase from takadiastase, a commercial enzyme product from mouldy bran (A.oryzae) asymmetrically hydrolysed benzoyl-DL-alanine leaving benzoyl-D-alanine unreacted in the digest. The procedure was applied to the resolution of alanine by Hoppert (1925) and two-and-a-half decades later by Neuberg and Mandl (1950) to the resolution of methionine, tryptophan, phenylalanine, isoleucine, aspartic acid, lysine and tyrosine. The

takadiastase preparations used in these studies had only low levels of activity and prolonged digestion often over several days were required with massive amounts of the enzyme preparation, even as much as the substrate by weight.

Ellis and Walker (1942) investigated the rates of hydrolysis of hippuric acid and various isomeric ring substituted derivatives of hippuric acid by the acylase of takadiastase. The nature of the substituents was found to have little effect on rates of hydrolysis but the position of the substituent was found to be important.

Michi and Nonaka (1954a) systematically studied several fungal strains for acylase activity towards N-acylated-DL-glutamic acid. Extracts of mouldy bran were found to contain acylase activity, the highest levels being associated with Aspergillus tamarii, A.oryzae, Penicillium vinaceous, P. oxalicum and P.corymbiferum. Michi and Nonaka (1954b) concentrated the activity about five-fold by ammonium sulphate and acetone fractionations and used the concentrates for the resolution of glutamic acid and valine. Borkar (1957) obtained about 400-fold concentration of activity of extracts of wheat bran cultures of Aspergillus flavus-oryzae by a procedure involving acetone and ammonium sulphate fractionations, adsorption on calcium phosphate gel and refractionation with ammonium sulphate. The partially purified preparation, most active at pH 6.3 to 6.4, was shown to hydrolyse N-acylated tryptophan and phenylalanine derivatives more rapidly than the corresponding alanine derivatives in marked contrast to renal acylase I which does not act on tryptophan derivatives

and hydrolyses phenylalanine derivatives at about a thirtieth the rate of the corresponding alanine ones (Bruns and Schulze, 1962). The mould preparation was shown to be active towards chloroacetyl, acetyl and formyl derivatives of several amino acids, the formyl derivatives being most susceptible and acetyl the least. The susceptibility of the N-acyl derivatives tested increased in the following order: aspartic acid, histidine, glutamic acid, alanine, tryptophan, methionine, phenylalanine. The enzyme was not inhibited by -SH-group-specific reagents but was completely inactive in the presence of EDTA, o-phenanthroline and α, α'-dipyridyl. The enzyme was completely inactive after treatment with EDTA followed by dialysis and was reactivated by Fe^{2+} and Zn^{2+} while Co^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+} were without effect. The mould preparation was also used for the resolution of tryptophan. Michi and Tsuda (1957) and Michi (1958) obtained essentially similar data on substrate susceptibility with an enzyme concentration from Penicillium vinaceous cultivated on wheat bran-calcium carbonate. These authors obtained the concentrate by a procedure involving ammonium sulphate fractionation, precipitation with Rivanol (6:9-diamino-2-ethoxyacridine lactate) and refractionation with ammonium sulphate. The preparation was used for the resolution of tryptophan, phenylalanine and methionine. The preparation was also readily active towards α-N-acetyl lysine, a substrate which is only slowly acted on by renal acylase. This mould acylase concentrate was reported to be activated by Co^{2+}.

The enzyme from takadiastase has been concentrated 1000-fold by Inouye and Akabori (1960) using a procedure involving starch

column electrophoresis. These authors reported an activation by Co^{2+} for the substrates acetylphenylalanine and acetylvaline, while other divalent metals inhibited hydrolysis. The optimum pH was 8.6 for low levels of Co^{2+}. The enzyme lost half its activity in the presence of 3.6×10^{-4} M p-chloromercuribenzoate. More recently, Doi and Hata (1963a) have reported a 1500-fold concentration in activity of taka-diastase acylase by a procedure involving precipitation with calcium chloride, elution and fractionation with ammonium sulphate, chromatography on DEAE-sephadex and acetone fractionation. The purified enzyme was found to run as a single component in free electrophoresis at pH 7.0 but the sedimentation pattern in the ultracentrifuge indicated the lack of homogeneity. These authors have also reported that the hydrolytic rate of acetylleucine for the purified enzyme was enhanced by Co^{2+} while that of chloroacetylphenylalanine was lowered in presence of Co^{2+}. The enzyme could not be rendered metal free by treatment with EDTA followed by fractionation on a sephadex column. However the metal free protein could be obtained by treatment with o-phenanthroline followed by dialysis. The metal-free enzyme was inactive but was reactivated by Co^{2+} and Zn^{2+}, Co^{2+} being more effective than Zn^{2+} in hydrolysis of acetylleucine but less effective than Zn^{2+} in hydrolysis of chloroacetylphenylalanine (Doi and Hata, 1963b).

An acylase has been isolated from brewers yeast by Chibata and Ishikawa (1958) and concentrated 10-fold by successive liquefaction with chloroform and precipitation with ammonium sulphate. The preparation

rapidly hydrolysed phenylalanine and tyrosine derivatives but acted on tryptophan derivatives at relatively lower rates than the mould enzymes.

An acylase has been concentrated 150-fold from rape seeds by Ozaki and Wetter (1961) by ammonium sulphate and calcium phosphate gel fractionation. The enzyme preparation was stereospecific for the L-isomer and hydrolysed acetylphenylalanine, acetyltyrosine and acetylmethionine rapidly but acetyltryptophan and acetylalanine more slowly. Co^{2+} was required for activation of this seed enzyme.

Low levels of acylase activity have been reported in Escherichia coli (Vogel and Bonner,1956; Chibata, Kisumi and Yamada,1958) but no studies seem to have been carried out on the substrate requirements of the enzyme from this source.

v) D-AMINO ACID ACYLASE

The simultaneous presence of L- and D-amino acid acylases capable of hydrolysing benzoyl derivatives of phenylalanine, tyrosine and alanine have been reported by Kamada, Toyoura and Kimura (1958) in a strain of Pseudomonas. The activity towards the L-isomer was separated from that towards the D-form by fractionation with protamine sulphate. The D-acylase was concentrated 15-fold during the separation procedure and was used for the resolution of phenylalanine. Some strains of Pseudomonas have also been isolated with single optical specificity towards the L-isomer (Kamada, Toyoura, Kimura and Matsui, 1958).

PRESENT INVESTIGATION

In earlier work carried out at this Laboratory by Borkar (1957), an acylase had been concentrated 400-fold from wheat bran cultures of Aspergillus flavus-oryzae NRRL 536. This enzyme was shown to differ from renal acylase I in its substrate and metal requirements. The main distinguishing feature in substrate susceptibility is the preference of the mould enzyme for aromatic amino acid derivatives while the aliphatic and not the aromatic derivatives are the preferred substrates for renal acylase I (Greenstein and Winitz, 1961). Thus acylated tryptophan derivatives were hydrolysed by the mould preparation faster than corresponding alanine derivatives while the former are resistant to hydrolysis by acylase I and the latter are rapidly hydrolysed (Bruns and Schulze, 1962). Further, the partially purified mould preparation was rendered completely inactive after EDTA treatment followed by dialysis and required Zn^{2+} or Fe^{2+} and not Co^{2+} for reactivation (Borkar, 1957) while renal acylase I is not inhibited by EDTA (Marshall, Birnbaum and Greenstein, 1956) and does not require metal ions for its action on susceptible substrates (Rao, Birnbaum, Kingsley and Greenstein, 1952).

Several groups working in Japan have concentrated mould acylases, particularly from Penicillium vinaceous and takadiastase and shown that these also behave like the enzyme from A. flavus-oryzae in hydrolysing the N-acylated aromatic amino acids with greater ease than the corresponding N-acylated aliphatic amino acids (Michi, 1958; Chibata and Ishikawa, 1958).

Although the substrate susceptibilities of the A.flavus-oryzae and other mould acylases appear to follow a common pattern, the two seem to differ in their metal activation behaviour. While takadiastase acylase which has been rendered metal-free is reactivated by either Co^{2+} or Zn^{2+} (Doi and Hata, 1963b), partially purified A.flavus-oryzae acylase which has been treated with EDTA and dialysed is activated by Zn^{2+} or Fe^{2+} and not at all by Co^{2+} (Borkar, 1957).

In view of the distinctive properties of the A.flavus-oryzae acylase as compared to other microbial acylases as well as to renal acylase I, it was decided to resume the earlier work of Borkar (1957), particularly in regard to the purification and properties of the enzyme. In the earlier work, the enzyme concentrated 400-fold had been shown electrophoretically to contain at least 3 components in about equal proportions. A further 3 to 4-fold concentration of acylase activity was therefore considered desirable before studying the properties of the enzyme preparation.

In the results reported here, a 1500- to 2000-fold purification of A.flavus-oryzae enzyme is reported both from wheat bran cultures and surface growth on a liquid, semi-synthetic medium. The influence of metal ions, pH, substrate structure and some inhibitors on the activity of the purified enzyme is also described.

EXPERIMENTAL

METHODS AND MATERIALS

1. Growth of organism

(a) Organism: *Aspergillus flavus-oryzae* NRRL 536 was used as the source of the enzyme. Among several fungi screened for activity this organism had been found to be the best acylase producer by Borkar (1957).

(b) Maintenance of stock culture: The mould was grown on potato-dextrose agar slants prepared as follows:

200 g peeled and cut potatoes were steamed with 700 ml distilled water for 1 hr and the extract strained through muslin. 20 g dextrose and 30 g shredded agar were added to the extract and the volume was made to 1 L with distilled water. The medium was steamed for about 1 hr till the agar dissolved completely. About 10 ml lots of the medium were dispensed in pyrex test tubes (6" x 1") and autoclaved at 15 p.s.i. for 20 min and slants prepared in the usual manner.

The slants were stored at 5°C.

(c) Inoculum: Active spores were obtained by growing the organism on malt agar slants prepared as follows:

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

were obtained as described above.

The malt agar slants were inoculated with a loop of spores from 5 days-old stock cultures on potato-dextrose agar and were grown for 10 days at 37°C. Two successive subcultures were made on malt agar slants to obtain active spores. Spore suspensions for inoculation were obtained by adding 5 to 10 ml sterile water and teasing the spores off each slant with a nichrome inoculating needle.

(d) Surface cultures on corn steep liquor-dextrose medium:

The organism was found to grow well on a simple corn steep liquor medium of the following composition:

Corn steep liquor (50% solids)	-	30 ml
Dextrose	-	40 g
Water	-	1 L

pH adjusted to 6.4 with N NaOH.

For determining the optimum period of growth for acylase production, 250 ml medium were dispensed in 1 L pyrex conical flasks and autoclaved at 15 p.s.i. for 30 min. The medium was then inoculated with spores from one malt agar slant and grown at 30°C under stationary conditions.

For enzyme preparation, 1 L lots of medium were dispensed in 4 L pyrex Fernbach culture flasks and steam sterilised at 15 p.s.i. for 30 min. Spores from four malt agar slants were used for inoculating each flask and grown under stationary conditions at 30°C for 8 days.

(e) Wheat bran cultures: Mouldy bran was prepared according to the procedure described by Borkar (1957).

Wheat bran was ground in a Wiley mill and passed through a 50-mesh sieve. 450 g fresh powdered bran was mixed with 550 ml water and sterilised in a covered enamelled basin by autoclaving for 2 hr at 20 p.s.i. The quantity of water used was just sufficient to moisten the bran. The bran was then spread under sterile conditions in a sterilized shallow enamel tray (18" x 15" x 2") to give a uniform layer of about ½" to 1" thickness.

Spores from 8 malt agar slants were used for each tray containing 1 kg moist bran. The bran was inoculated uniformly and the trays covered with sterilized filter paper sheets and left 72 hr at 28°C in a sterile chamber in which the humidity was maintained with shallow trays containing water. A thick greenish-yellow carpet of mouldy bran was obtained at the end of the period of growth.

2. Purification of acylase

(a) Extraction of acylase activity

(i) Surface cultures on liquid medium: The pale straw yellow coloured mycelium was harvested and washed once with 3 volumes distilled water on a Buchner funnel. The mat was then extracted at 0° to 5°C with 2 volumes saturated, ice cold toluene-water by grinding in a mortar and pestle with 1.5 parts cooled, acid-washed sand. The extract was squeezed through muslin and clarified by centrifugation at 2000 x g for 45 min at 0°C. 100 g

mat usually yielded about 200 to 250 ml brown coloured, clear extract containing 0.5% protein.

(ii) Mouldy bran: Extraction was carried out as described by Borkar (1957). 1 kg mouldy bran was broken up and taken in an ice-cold porcelain ball mill jar of about 10 L capacity. 1.5 L of saturated ice-cold toluene water was added to the bran culture and the contents ground with porcelain balls (1/3 volume of jar) for 1 hr in the mill. After the end of this period the mouldy bran was obtained as a uniform paste at a temperature of about 15°C. The pasty mass was squeezed through muslin and the extract filtered overnight at 0°C. Approximately 1.1 L filtrate was obtained from each tray of 1 kg of bran.

(b) Acetone precipitation

The acetone used was refluxed over anhydrous K_2CO_3 and distilled.

(c) Tricalcium phosphate - cellulose column chromatography

Tricalcium phosphate adsorbent was prepared according to the method of Swingle and Tiselius (1951) by the slow addition of orthophosphoric acid to a chilled solution of a calcium complex of sucrose (MacIntire, Palmer and Marshall, 1945). The details are given below.

To 225 g pure sucrose in 1 L water were added 37.5 g of CaO prepared by calcining 75 g of precipitated $CaCO_3$ at 1000°C for 3 hr. The suspension was agitated periodically for several hours until most of the CaO had dissolved. The solution was clarified

by centrifuging at 1000 x g for 30 min. Concentrated (85%, sp.gr. 1.71) orthphosphoric acid (about 9 ml) was added dropwise with stirring over the course of an hr to 400 ml of the calcium-sucrose solution chilled to about 5°C until the pH was 9.5. The stirring was continued for 4 hr. The resulting precipitate of tricalcium phosphate was washed thoroughly with water and was stored as a suspension (dry weight 30 mg/ml).

Tricalcium phosphate-cellulose column for chromatography was prepared according to the procedure of Massey (1960). 20 g Whatman cellulose powder (ashless, standard grade) were suspended in 200 ml water and mixed with 100 ml of the 3% tricalcium phosphate suspension. The mixture was evacuated in a stoppered Buchner flask at the water pump for 15 min to remove occluded air. The material was packed in a glass tube with a coarse sintered disc at the base over which a 2 mm thickness of cellulose powder had been layered previously. The dimensions of the column were 25 x 2.2 cm. The rate of flow was about 15 ml/hr. Chromatography was carried out at 0° to 5°C and 3 to 4 ml fractions were collected on a Technicon Automatic fraction collector.

(d) DEAE-cellulose chromatography

The DEAE-cellulose column was prepared as described earlier under Purification of Citrase in Part I (page 19), except that buffers used contained no $MgSO_4$.

Chromatography was carried out at 0° to 5°C and 3 to 4 ml fractions were collected on a Technicon fraction collector.

(e) Determination of acylase activity

The purification of acylase was followed using N-acetyl-DL-tryptophan as substrate. The test system consisted of 2 ml neutralised 0.1 M N-acetyl-DL-tryptophan; 0.6 ml 0.1 M phosphate buffer pH 6.4, enzyme and water to make to 3 ml total volume. The reaction was carried out for 15 min at 37°C. The reaction was stopped by the addition of 1 ml glacial acetic acid and the amount of hydrolysis of the acyl amino acid was determined by the Van Slyke nitrous acid procedure (Van Slyke, 1929). The quantity of enzyme was always adjusted to give no more than 5% hydrolysis of the acetyl racemate in 20 min. Enzyme and substrate blanks were routinely run under identical conditions.

In following the purification of the enzyme, parallel determinations were carried out using sodium β-glycerophosphate buffer, pH 6.4, in place of orthophosphate together with separate supplements of the following metal salts: $ZnSO_4$ (3×10^{-4}M), $FeSO_4$ (3×10^{-3}M), $CoCl_2$ (3×10^{-3}M) to give final metal ion concentrations as indicated in parenthesis.

In the chromatographic separation of acylase on columns of tricalcium phosphate-cellulose and DEAE-cellulose, the following rapid test was carried out to qualitatively identify activity in the effluent. A drop of column effluent from each fraction was added to the test system of 0.2 ml of neutralised 0.1 M N-acetyl-DL-tryptophan solution and 0.1 ml of 0.1 M potassium phosphate buffer, pH 6.4 and the mixture left for 20 min at 37°C. A

spot (5 to 10 ul) from each test sample was then placed on a sheet of filter paper within pencilled circles and dried in a current of hot air. The sheet was then sprayed with 0.1 w/v % solution of ninhydrin in n-butanol and heated for 8 min at 80°C. The presence of activity was immediately apparent by the appearance of a purple spot on the filter paper sheet due to the liberated amino acid, while the inactive fractions showed no colour at all. A spot of each fraction was also checked directly by placing on filter paper and developing with ninhydrin to rule out the presence of ninhydrin-reacting substances in the column effluents.

In studies on the properties of the purified enzyme, the extent of hydrolysis of the acyl derivative was determined by estimating the liberated amino acid by a modification of the ninhydrin method of Moore and Stein (1948,1954). The test system comprised of 1.0 ml of 0.1 M neutralised N-acyl derivative of the racemate (or 0.05 M of the acyl derivative of the optically active form); 0.3 ml 0.1 M veronal buffer, pH 8.0; 0.1 ml $ZnSO_4$ solution (6×10^{-3}M) at 37°C. 0.1 ml enzyme was added to the test system, mixed and 0.2 ml sample withdrawn for assay within 15 sec of adding the enzyme followed by further samples of 0.2 ml after intervals of 5, 10, 15 min or any other stated interval from the time of withdrawing the first sample. The reaction was arrested by adding the 0.2 ml samples to 1.8 ml ninhydrin reagent made up as follows:

Ninhydrin reagent: 400 mg ninhydrin (B.D.H., A.R.) were dissolved just before use in 20 ml of methyl cellosolve(ethylene glycol-monomethyl ether, E. Merck) and treated with stirring with

10 ml of 4 M sodium acetate buffer, pH 5.5, containing $SnCl_2$. The acetate buffer containing $SnCl_2$ was prepared by dissolving 200 mg $SnCl_2.2H_2O$ (Reanal, A.R.) in 125 ml 4 M acetate buffer, pH 5.5. The methyl cellosolve solution after treatment with the acetate buffer was further diluted with 10 ml 4 M sodium acetate buffer, pH 5.5 and 32 ml 0.2 M sodium citrate buffer, pH 5.0. The presence of citrate prevented turbidity during colour development with test samples.

1.8 ml lots of the ninhydrin reagent were pipetted into test tubes (19 x 1 cm) provided with aluminium caps. After the addition of 0.2 ml test samples the tubes were capped and left in a vigorously boiling water bath for 30 min. The tubes were then cooled quickly in running tap water and diluted with 2 ml of 50:50 ethanol-water. The tubes were read at 570 mμ in 10 mm light path glass cuvettes in an Unicam SP 600 spectrophotometer. Standard curves were obtained with pure amino acid samples and linear relationships between amino acid concentration and O.D. 570 were obtained in all cases under the conditions of assay. The colour yields for the different amino acids calculated on a molar basis with reference to leucine were in agreement with the values given by Moore and Stein (1948).

Protein measurements were made with the Folin phenol reagent according to the procedure of Lowry, Rosebrough, Farr and Randall (1951) as described by Layne (1957). Crystalline bovine plasma albumin (Armour & Co.) was used as the working standard and

colorimetric measurements were made on an Unicam SP 600 spectrophotometer at 500 mu (or 750 mu for the lower range of values). Enzyme solutions were diluted usually to contain 25 to 250 ug protein/ml.

Protein measurements were also made by the spectrophotometric procedure of Warburg and Christian (1941). Values obtained by this procedure were in good agreement with those obtained by the use of the phenol reagent.

The total activity of fractions have been expressed as moles substrate hydrolysed per hr at 37°C and specific activity as umoles substrate hydrolysed initially per hr per mg protein nitrogen. The units of expressing activity were similar to those used by Greenstein and coworkers and most others in the field.

3. Properties of purified acylase

(a) Substrates

Acetyl-DL-tryptophan which was routinely used for following the purification procedure and in the study of most of the enzyme properties was prepared by the method described by de Vigneaud and Sealock (1932). 6.8 g recrystallised DL-tryptophan were dissolved in 10 ml water with addition of 16.75 ml of 2N NaOH and treated with 8.0 ml of 2N NaOH and 8 ml of acetic anhydride added in eight portions during an interval of 16 min. The reaction mixture was kept in an ice bath during the treatment and the flask was vigorously

shaken after each addition of acetic anhydride. After the flask was allowed to stand at room temperature for 20 min, 35.8 ml of 6N H_2SO_4 were added and the solution cooled in an ice-salt bath. The white crystalline compound which precipitated was filtered and then freed from any traces of tryptophan by washing with 25 ml of 0.2 N HCl and finally with water. The derivative was recrystallised from water. Yield 5.4 g, m.p. 204°C.

Acetyl-L-tryptophan, acetyl-D-tryptophan, formyl-DL-tryptophan, chloroacetyl-DL-tryptophan, formyl-DL-alanine, acetyl-DL-alanine, chloroacetyl-DL-alanine, formyl-DL-phenylalanine, acetyl-DL-phenylalanine, acetyl-DL-methionine and chloroacetyl-DL-aspartic acid were preparations made by Dr. P. S. Borkar at this Laboratory. All substrates were recrystallised before use.

RESULTS

1. OPTIMUM PERIOD OF GROWTH FOR ACYLASE PRODUCTION

The activity extracted from mycelia at various stages of growth is shown in Table 2.1.

TABLE 2.1

250 ml corn steep liquor-dextrose medium at 30°C

Age of culture (days)	Mycelium		
	Wet wt. g	Total activity (uM/hr)	Specific activity (uM/hr/mg N)
3	21	250	10
5	23	300	—
7	29	550	14
8	30	700	14
10	36	700	13

It will be evident from Table 2.1 that the highest yields are obtained on about the 8th day. Mycelium was therefore harvested at this stage of growth for working up the acylase activity.

2. PURIFICATION OF A.flavus-oryzae ACYLASE

Extracts of wheat bran cultures and mycelium harvested from surface cultures on corn steep liquor-dextrose medium were

worked up separately. The following were the steps in the fractionation procedure:

(i) Acetone fractionation:

(a) Wheat bran cultures: This step was similar to that used by Borkar (1957). Batches of 900 ml extract were worked up at a time. 900 ml of crude extract at 0°C were stirred in a 2 L beaker and treated with 100 ml ice-cold sodium acetate buffer, 1 M, pH 5.6. The beaker containing the buffered extract was then chilled in a bath of ice-salt mixture and addition of acetone was started when the temperature of the buffered extract was -2°C. The acetone was added from a separating funnel the tip of which was drawn out to a capillary and extended nearly to the bottom of the beaker. 500 ml acetone were added over a period of 1 to 2 hr with continuous stirring. The mixture was then stirred for another hr and the resulting precipitate separated by centrifugation in a refrigerated PR-2 centrifuge at -10°C and 2000 x g for 15 min. The clear supernatant was poured into a 2 L beaker cooled in ice-salt mixture and the precipitate extracted with water. The deep brown extract was centrifuged and dialysed overnight against water at 0° to remove residual acetone. This fraction was found to have little activity towards acetyl tryptophan and was discarded.

The supernatant from the initial treatment with acetone was treated at -10°C with a further volume of 200 ml acetone over a period of 1 hr. The mixture was allowed to stand for an hr at -10°C and the precipitate separated as in the earlier case. The

precipitate was extracted twice with 20 ml water each time at 0°C and the clear brown solution dialysed against water and stored frozen at -20°C.

(b) Mycelium grown on corn steep liquor-dextrose medium

Conditions for acetone fractionation of extracts of mycelium from surface cultures were different from those required for the wheat bran extract. 450 ml batches of mycelial extract were treated with 50 ml M sodium acetate buffer, pH 5.6 and treated with 325 ml acetone under conditions similar to those used for the bran extract. The precipitate was removed by centrifugation and the supernatant treated with a further lot of 250 ml acetone. Both precipitates were separately taken up in water and dialysed overnight against distilled water at 0°C to remove acetone. The first acetone fraction had a low specific activity and was discarded. The second fraction containing about half the original activity was worked up further for isolation of acylase.

The acetone fractions were stable for several months on storage at -20°C.

ii) Tricalcium phosphate-cellulose column chromatography

From this stage onwards, bran and mycelial isolates were treated under identical conditions. The dialysed extract of the second acetone fraction was loaded on the calcium phosphate-cellulose column (25 x 2.2 cm) and washed overnight with 150 ml 0.01 M potassium phosphate buffer, pH 7.4. No activity was extracted from the

column by washing with this buffer. The buffer was then changed and the column washed with 0.05 M potassium phosphate buffer, pH 7.4. The activity was now eluted from the column in a volume of about 40 ml commencing usually with about 80 ml after change of buffer.

The fractions containing the activity were pooled (40 ml) and treated at 0°C with ammonium sulphate (19.2 g) to 0.75 saturation. The precipitate containing the activity was separated by centrifugation in a Spinco rotor 21 at 25,000 x g for 30 min at 0°C. The clear supernatant was poured off and the white precipitate extracted with 5 ml water and the clear extract dialysed overnight against 1 L water at 0°C. The fraction showed the same activity towards acetyl tryptophan both in the absence of metal and in presence of $ZnSO_4$ (3×10^{-4}M).

iii) DEAE-cellulose chromatography

The dialysed fraction from the calcium phosphate-cellulose column chromatography was loaded on the DEAE-cellulose column (20 x 1 cm) and chromatographed by continuous gradient elution with potassium phosphate buffer (pH 7.4) with gradual increase in buffer strength over the range 0.01 - 0.3 M (see Fig. 1.1). The activity was eluted from the column in a volume of about 25 ml of a buffer strength corresponding to about 0.1 M concentration.

The fractions containing the activity were pooled (25 ml) and treated at 0°C with ammonium sulphate (12 g) to 0.75 saturation. The precipitate was separated in a Spinco rotor 30 at 25,000 x g

for 30 min at 0°C. The clear supernatant was decanted off and the white precipitate extracted with 2 ml water and the colourless, water-clear extract dialysed overnight against water at 0°C with several changes.

This fraction when tested without added metal showed an activity towards acetyl tryptophan of 1,204 uM/hr/mg N in the case of bran cultures and 2,961 uM/hr/mg N in the case of the surface growth. In the presence of Zn^{2+} (3×10^{-4}M) however the specific activity increased to 14,260 uM/hr/mg N at pH 6.4 and 22,300 uM/hr/mg N at pH 8.0 in the case of bran cultures and 10,280 uM/hr/mg N at pH 6.4 and 21,000 uM/hr/mg N at pH 8.0 in the case of the mycelium. In the presence of Fe^{2+} (3×10^{-3}M) activation effect was less marked, the values at pH 6.4 being 8,332 uM/hr/mg N for the bran culture concentrate and 7,313 for mycelium. Co^{2+} (3×10^{-3}M) had no effect on the hydrolytic rates.

The results of typical experiments with bran cultures and the mycelium from corn steep liquor-dextrose medium are summarised separately in Tables 2.2 and 2.3.

No check on homogeneity could be carried out by ultra-centrifugation or by electrophoresis in free solution as the material was in inadequate amounts.

TABLE 2.2

Purification of *A.flavus-oryzae* acylase from mouldy bran

Test substrate: N-acetyl-DL-tryptophan.

Fractions (i), (ii), (iii) and (iv) assayed without metal supplement at pH 6.4. Fraction (v) assayed with Zn^{2+} (final concentration 3×10^{-4}M) and at pH 8.0.

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (uM/hr/mg N)	Total activity (mM / hr)	% Recovery of initial activity
i) Extract	1000	30.5	13	63.4	100
ii) First acetone (0 to 0.5 v/v)	40	4.7	51.7	1.56	2.5
iii) Second acetone (0.5 to 0.7 v/v)	40	23.7	199	30.2	47.6
iv) $Ca_3(PO_4)_2$ gel-cellulose	10	1.88	1678	5.05	8.0
v) DEAE-cellulose	3	0.33	22,300	3.53	5.6

TABLE 2.3

Purification of A.flavus-oryzae acylase from mycelium grown on corn steep liquor-dextrose medium

Test substrate: N-acetyl-DL-tryptophan.

Fractions, (i), (ii), (iii), (iv) assayed without metal supplement at pH 6.4. Fraction (v) assayed with Zn^{2+} (final concentration 3×10^{-4}M) and at pH 8.0.

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (uM/hr/mg N)	Total activity (mM / hr)	% Recovery of initial activity
i) Extract	500	5.0	14.0	5.60	100
ii) First Acetone (0 to 0.65 v/v)	20	7.9	43.9	1.11	19.8
iii) Second Acetone (0.65 to 1.15 v/v)	30	3.4	250	4.11	73.3
iv) $Ca_3(PO_4)_2$ gel-cellulose	8	1.0	2569	3.29	58.8
v) DEAE-cellulose	3	0.12	21,000	1.21	21.6

3. PROPERTIES OF PURIFIED ACYLASE

The properties reported here are those of the purified preparation from wheat bran cultures.

i) Substrate hydrolysis: The graph of the time plotted against per cent hydrolysis of N-acetyl-DL-tryptophan by the purified A.flavus-oryzae enzyme is shown in Fig. 2.1. It will be evident from Fig. 2.1 that the hydrolysis catalysed by the purified enzyme follows zero-order kinetics upto at least 5% splitting of the total racemate derivative under the conditions of assay.

The curve of enzyme concentrations versus per cent hydrolysis of N-acetyl-DL-tryptophan is shown in Fig. 2.2. It can be seen from Fig. 2.2 that the rate of hydrolysis of the substrate is also proportional to enzyme concentration upto at least 5% splitting of the total racemate.

ii) Effect of pH: The effect of pH on the activity of the purified acylase towards N-acetyl-DL-tryptophan is shown in Fig. 2.3. The determinations were made in the presence of Zn^{2+} (final concentration, 4×10^{-4}M). It is seen from the plot that the optimum pH for hydrolysis of acetyltryptophan is 8.0 and the rate of hydrolysis at this pH value is 22,000 uM/hr/mg N. This pH value is markedly higher than the optimum pH of about 6.4 reported by Borkar (1957) for the action of the cruder preparation of A.flavus-oryzae (4500 uM/hr/mg N). However, the cruder fractions require no metal supplement for activation and the purified

Fig. 2.1

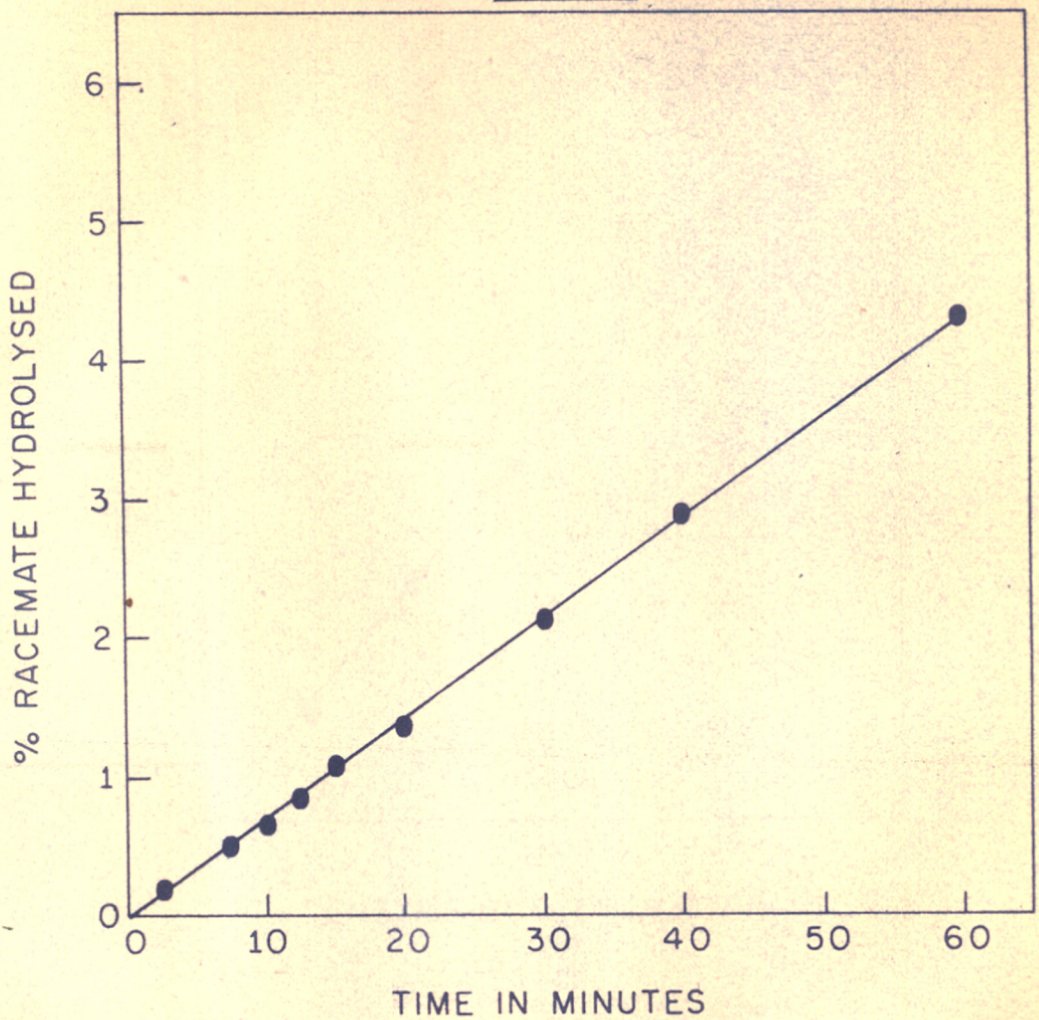

FIG. 2.1 PLOT OF TIME VERSUS ACTIVITY OF PURIFIED A. FLAVUS–ORYZAE ACYLASE TOWARDS ACETYL-DL-TRYPTOPHAN.

SYSTEM: 0.1 M SUBSTRATE SOLUTION, 10 ml; VERONAL BUFFER (0.1 M, pH 8.0) 3 ml; $ZnSO_4$ SOLUTION (6×10^{-3}M) 1 ml; ENZYME SOLUTION (5 ug) 1 ml.

TEMPERATURE, 37°C.

SUBSTRATE BLANKS WITHOUT ENZYME SHOWED NO HYDROLYSIS.

Fig. 2.2

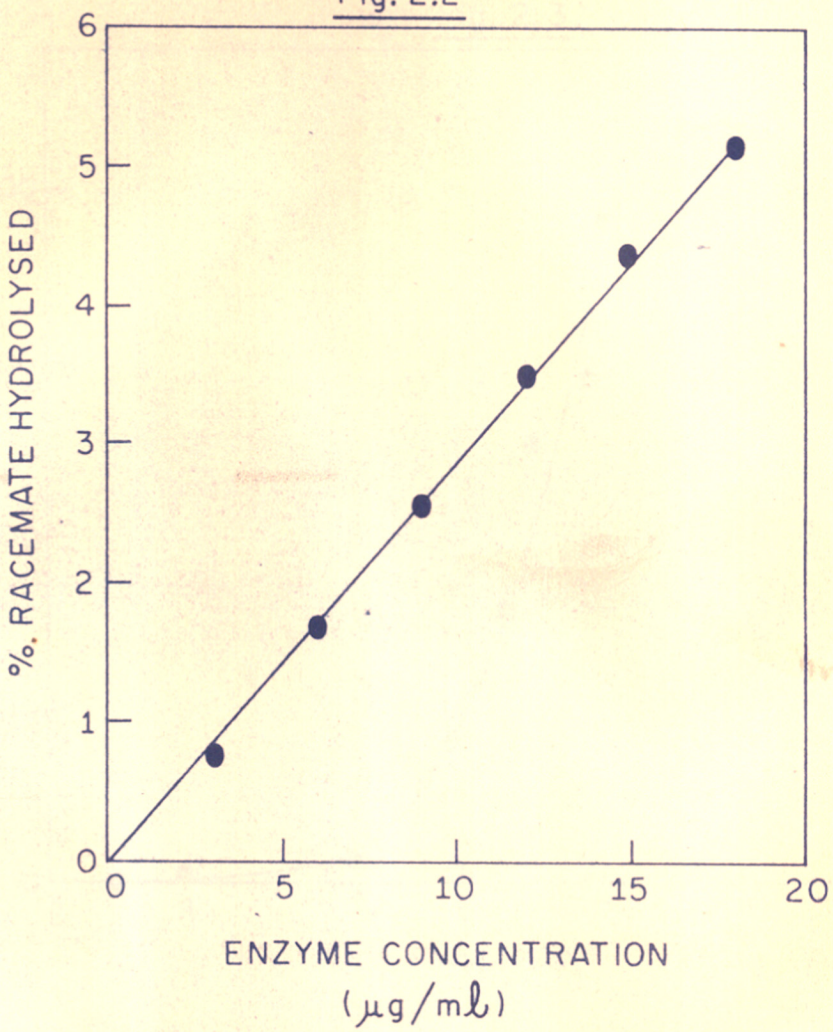

FIG. 2.2 EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF PURIFIED A.FLAVUS-ORYZAE ACYLASE TOWARDS ACETYL-DL-TRYPTOPHAN.

SYSTEM; 0.1 M SUBSTRATE SOLUTION, 1 ml; VERONAL BUFFER (0.1 M, pH 8.0) 0.3 ml; $ZnSO_4$ SOLUTION (6×10^{-3}M), 0.1 ml; ENZYME SOLUTION (5 to 10 ug), 0.1 ml.

5 min at 37°C.

Fig. 2.3

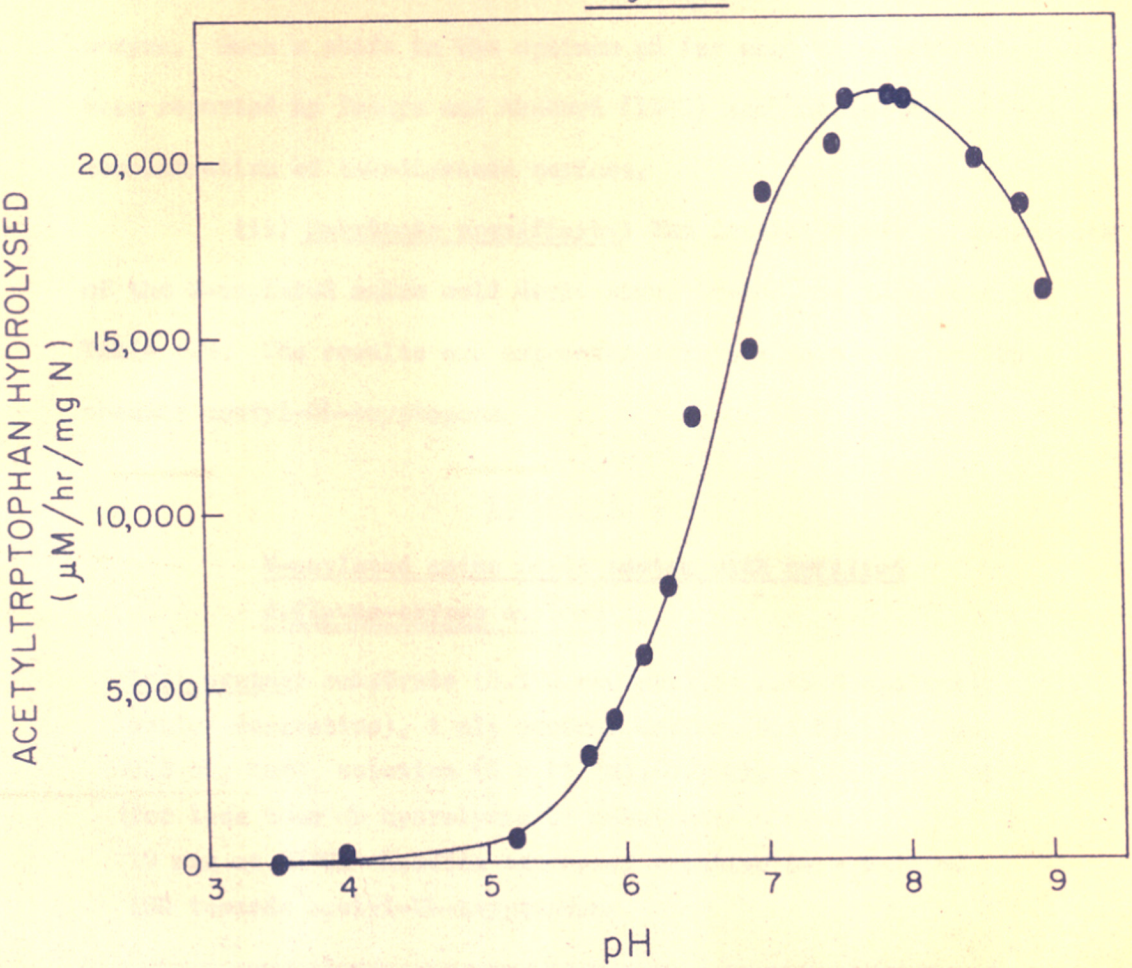

FIG. 2.3 EFFECT OF pH ON ACTIVITY OF PURIFIED A.FLAVUS–ORYZAE ACYLASE.

BUFFERS USED: CITRATE (pH 3.5 to 5.8); POTASSIUM PHOSPHATE (pH 6.0 to 6.8); VERONAL (pH 7.0 to 9.0).

SYSTEM: 0.1 M ACETYLTRYPTOPHAN SOLUTION, 1 ml; BUFFER (0.1 M), 0.3 ml; $ZnSO_4$ SOLUTION (6×10^{-3}M), 0.1 ml; ENZYME (5 to 15 ug) SOLUTION, 0.1 ml.

TEMPERATURE 37°C.

SUBSTRATE BLANKS WITHOUT ENZYME SHOWED NO HYDROLYSIS.

preparation probably corresponds to a metal-free or metal-deficient enzyme. Such a shift in the optimum pH for enzymatic action has also been reported by Inouye and Akabori (1960) and Doi and Hata (1963a) on concentration of takadiastase acylase.

iii) Substrate specificity: The initial rates of hydrolysis of the N-acylated amino acid derivatives tested are described in Table 2.4. The results are expressed relative to a rate of 100 towards acetyl-DL-tryptophan.

TABLE 2.4

N-acylated amino acids tested with purified A.flavus-oryzae acylase

Test system: substrate (0.1 M racemate or 0.05 M optically active derivative), 1 ml; veronal buffer (0.1 M), pH 8.0, 0.3 ml; $ZnSO_4$ solution (6×10^{-3} M), 0.1 ml; enzyme, 0.1 ml (for less than 5% hydrolysis of substrate/10 min). 10 min at 37°C. Results expressed relative to a rate of 100 towards acetyl-DL-tryptophan.

Amino acid moiety	Relative rates of hydrolysis		
	Formyl	Acetyl	Chloroacetyl
L-Tryptophan	-	100	-
D-Tryptophan	-	0	-
DL-Tryptophan	81	100	107
DL-Alanine	163	79	105
DL-Phenylalanine	668	144	-
DL-Methionine	-	120	-
DL-Aspartic acid	-	-	3

Substrates showed no hydrolysis without enzyme.

The results clearly indicate that the acylase is optically specific for the acyl derivative of the L-amino acid. Thus while N-acetyl-L-tryptophan is hydrolysed rapidly, no hydrolysis of N-acetyl-D-tryptophan could be observed. Further the presence of acetyl-D-tryptophan in the racemate appears to have no effect on the hydrolysis rate of the susceptible L-amino acid derivative.

The results show that the different substrates are hydrolysed at widely varying rates by the purified enzyme, derivatives of phenylalanine being the most susceptible particularly the formyl derivative. Acetyl and chloroacetyl derivatives of tryptophan are hydrolysed faster than the corresponding alanine derivatives but formyl alanine is hydrolysed twice as fast as formyl tryptophan. Among the acetyl derivatives tested, that of methionine is hydrolysed at a rate intermediate between that of phenylalanine and tryptophan.

A marked influence of the nature of the acyl group of hydrolytic rates is evident for derivatives of alanine and phenylalanine, the formyl derivative of alanine being hydrolysed twice as fast as the acetyl and 1½ times as fast as the chloroacetyl derivatives, while the formyl derivative of phenylalanine is hydrolysed four times as fast as the acetyl derivative.

It must be pointed out that the hydrolysis of the various substrates was carried out at pH 8.0 and in presence of

Zn^{2+} (final concentration 4×10^{-4}M) which is optimum for acetyl tryptophan but may not be so for the other substrates.

The difference between the mould acylase and pancreatic carboxypeptidase is also evident from Table 2.4. While acetyl tryptophan and chloroacetyl tryptophan are hydrolysed at about similar rates by the acylase, the acetyl derivative is hydrolysed 12 times slower than the chloroacetyl by carboxypeptidase (Gilbert, Price and Greenstein, 1949).

iv) Inhibitors: The effect of some inhibitors on the activity of the purified enzyme towards N-acetyl-DL-tryptophan is described in Table 2.5.

TABLE 2.5

Effect of inhibitors on activity of purified acylase towards acetyl-DL-tryptophan

The composition of the assay system was the same as in Table 2.4 except that inhibitors were added at the indicated levels and that Zn^{2+} was omitted when o-phenanthroline was included in the medium.

Reagent	Final concentration	Inhibition %
o-Phenanthroline	1.3×10^{-2}M	100
p-Chloromercuribenzoate	1×10^{-3}M	0
Iodoacetate	5×10^{-3}M	40

The enzyme is completely inhibited by the metal binding agent, o-phenanthroline.

p-Chloromercuribenzoate has no effect on the enzyme activity at the level tested while iodoacetate causes a 40% inhibition under the conditions of assay.

v) Stability: The stability of the purified enzyme to storage in the frozen state at -20°C was low and roughly 2/3 of the initial activity was lost on storage for 3 to 4 months.

DISCUSSION

Purification: The present procedure for purification of acylase from extracts of A.flavus-oryzae which involves the following steps: acetone fraction, tricalcium phosphate-cellulose column chromatography and DEAE-cellulose chromatography yields a concentrate which hydrolyses N-acetyl-L-tryptophan at a rate of about 20,000 uM/hr/mg N. The corresponding specific activity obtained in earlier work carried out by Berkar (1957) was 4500 uM/hr/mg N. The highest activity reported hitherto in literature for mould acylases is by Doi and Hata (1963a) for a takadiastase acylase preparation and the value reported for their preparation is 28,100 uM/hr/mg N towards acetyl methionine which compares with the corresponding value of 27,000 uM/hr/mg N for the present preparation. The preparation described by Doi and Hata (1963a) is reported to be homogeneous electrophoretically at pH 7.0 while the ultracentrifugal sedimentation pattern indicates

the presence of fast moving components with a main peak. Renal acylase I which has been obtained in a homogeneous state by Bruns and Schulze (1962) does not hydrolyse acetyltryptophan but hydrolyses acetylmethionine at a rate of 98,000 uM/hr/mg N. In its substrate specificity, the purified A.flavus-oryzae acylase resembles the cruder preparations described by Borkar (1957) in its main requirements. Aromatic amino acids are thus hydrolysed rapidly by both preparations, phenylalanine derivatives being the most susceptible particularly the formyl derivative. Tryptophan derivatives are hydrolysed rapidly by both preparations, but while Borkar (1957) has reported faster rates for formyl, acetyl and chloroacetyl tryptophan derivatives as compared with the corresponding alanine derivatives, this is so for the present preparation only with regard to acetyl and chloroacetyl derivatives, the formyl derivative of alanine being hydrolysed twice as fast as the tryptophan derivative in the present case. Chloroacetyl aspartic acid is resistant to both enzyme preparations and acetyl methionine is easily susceptible to both. The susceptibility of acylated aromatic amino acid derivatives towards the mould acylase is in agreement with the results reported by other workers as indicated in the introductory chapter. In marked contrast to mould acylase, renal acylase I does not act on tryptophan derivatives (Bruns and Schulze, 1962).

In its metal requirements the purified A.flavus-oryzae acylase is unique. The purified preparation is apparently rendered

metal-deficient and requires Zn^{2+} for activation. Fe^{2+} has a slightly lower activation effect, while Co^{2+} has no effect at all. The cruder acylase preparation obtained by Berkar (1957) from the same mould is not activated any further by Zn^{2+}, Fe^{2+} or Co^{2+} supplements but the metal-free enzyme obtained by EDTA-treatment followed by dialysis is reactivated completely by Zn^{2+} at a final concentration of about 1.7×10^{-4}M and Fe^{2+} at a corresponding concentration of 1.7×10^{-3}M while Co^{2+} is without effect. The concentrate obtained by Doi and Hata (1963a) from takadiastase requires no metal supplements for its action on chloroacetyl-L-phenylalanine except when rendered metal-free by treatment with o-phenanthroline followed by dialysis. The metal-free enzyme is reactivated by Zn^{2+} or Co^{2+} for its action on chloroacetyl phenylalanine. The hydrolysis of acetyl-L-leucine by the native enzyme however, is reported to be strongly activated by Co^{2+} but inhibited by Zn^{2+}. It is interesting to note that renal acylase I is not inhibited by metal binding reagents like EDTA, unlike the mould enzyme and does not require metal for its action on susceptible substrates (Bruns and Schulze, 1962). The renal enzyme is affected however by SH group-specific reagents like p-chloromercuribenzoate, unlike purified A.flavus-oryzae acylase. p-Chloromercuribenzoate has been reported by Inouye and Akabori (1960) to inhibit a takadiastase acylase concentrate to the extent of 48% at a concentration of 3.6×10^{-4}M.

SUMMARY

1. An acylase of Aspergillus flavus-oryzae NRRL 536 was concentrated 1500 to 2000-fold from mycelium grown on liquid corn steep liquor-dextrose medium under stationary conditions as well as from wheat bran cultures by a procedure involving extraction of the enzyme, fractionation with acetone, chromatography on tricalcium phosphate-cellulose and DEAE-cellulose columns.

2. The purified enzyme required added Zn^{2+} for its activity. Fe^{2+} also activated the purified enzyme but less so than Zn^{2+}. Co^{2+} was without any effect. The differences in metal requirement of the enzyme and that of other mould acylases are discussed.

3. The purified enzyme had an optimum pH of 8.0 for its action on acetyltryptophan in the presence of Zn^{2+}.

4. The purified enzyme showed optical specificity towards N-acetyl-L-tryptophan, N-acetyl-D-tryptophan being resistant to hydrolysis. The enzyme hydrolysed acetyl-L-tryptophan at a rate of 22,000 uM/hr/mg N at pH 8.0 in presence of Zn^{2+}.

5. The enzyme acted rapidly on aromatic amino acid derivatives, like other microbial acylases and unlike renal acylase I. The differences in substrate specificity of the mould preparation from renal acylase I and pancreatic carboxypeptidase are discussed.

6. The mould enzyme was completely inhibited by o-phenanthroline unlike renal acylase I. p-Chloromercuribenzoate had no effect on the mould preparation unlike the renal enzyme. These properties are in agreement with the results reported earlier for a 400-fold concentrate of the enzyme from the same source and distinguish the enzyme from renal acylase I and some of the other mould acylases.

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Statement I

The following new facts, which contribute to new knowledge on the subject were discovered:

1) A new procedure has been standardised for the enzymatic assay of citrate in biological materials, based on the coupled action of the two enzymes citrase and malate dehydrogenase in presence of reduced nicotinamide-adenine dinucleotide ($NADH_2$). Under the conditions described the breakdown of 1 mole citrate results in the oxidation of exactly 1 mole $NADH_2$. The estimation is carried out spectrophotometrically by measuring the decrease in the optical density at 340 mu due to the oxidation of $NADH_2$.

2) The procedure is sensitive and amounts as low as 1 to 4 ug citric acid can be assayed accurately.

3) The new enzymatic procedure is specific for citrate and the presence of a large variety of compounds have been shown to cause no interference in the assay.

4) The specificity of the new assay procedure makes any lengthy pretreatment of biological samples unnecessary. The procedure is therefore rapid and the assay step is completed within a minute and a complete assay of biological samples such as culture fluids and plant extracts can be carried out within 30 minutes while serum and bacterial sonicates take an hour longer.

5) The accuracy of the new assay procedure has been established both by parallel chemical determination and recovery trials in a wide range of biological specimens.

6) The new assay procedure is the only one available now for the rapid, specific and accurate assay of citrate in biological samples.

7) a new procedure for the concentration of an acylase from Aspergillus flavus-oryzae NRRL 536 grown both on wheat bran and on a liquid corn steep liquor-dextrose medium has been worked out. The enzyme which is concentrated 1500- to 2000-fold has a specific activity of 22,000 uM/hr/mg N towards acetyl-L-tryptophan and is as active as the purest mould acylase preparation reported hitherto in literature and obtained from takadiastase.

8) Like renal acylase I and mould acylase III from other sources, the purified enzyme shows optical specificity towards the L-amino acid derivative.

9) The purified enzyme acts rapidly on aromatic amino acid derivatives like other microbial acylases and unlike renal acylase I.

10) The purified enzyme requires Zn^{2+} or Fe^{2+} for its activity, the former being more effective than the latter, while Co^{2+} is without effect. The enzyme shows differences in its metal requirements from others of its class reported hitherto in literature.

(H. M. Sayagaver)
Signature of the Candidate

(M. R. Raghavendra Rao)
Signature of the Guiding Teacher

Statement II

All the findings reported in the thesis are derived from my own original work carried out in the National Chemical Laboratory, Poona under the guidance of Dr. M. R. Raghavendra Rao.

(H. M. Sayagaver)
Signature of the Candidate

(M. R. Raghavendra Rao)
Signature of the Guiding Teacher