A STUDY OF THE NICOTINAMIDE ADENINE DINUCLEOTIDE
SPECIFIC ISOCITRIC DEHYDROGENASE OF
ASPERGILLUS NIGER AND ASPERGILLUS FLAVUS

by

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In partial fulfilment
of the requirements for the degree
Master of Science
1964
To My Mother
ACKNOWLEDGEMENTS

For many valuable criticisms and suggestions throughout the course of this investigation, and in the preparation of this thesis, the writer expresses her sincere thanks to Dr. B. D. Sanwal, Department of Microbiology, University of Manitoba, and to Mr. Chester Stachow.
Abbreviations

The following abbreviations are used: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; NAD and NADP, nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate, respectively; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione, reduced; AMP, adenosine 5'-monophosphoric acid; IDH, isocitric dehydrogenase; pCMBA, p-chloromercuribenzoate; Tris, tris (Hydroxymethyl) - aminomethane; DEAE-cellulose, diethylaminoethyl-cellulose.
ABSTRACT

This investigation was carried out with a strain of *Aspergillus niger* which excretes citrate into the medium and with a strain of *Aspergillus flavus* which does not. A kinetic study of the NAD-specific isocitric dehydrogenases was attempted to explain this difference at a molecular or biochemical level. Both isocitric dehydrogenases are found to be extremely labile and require the presence of both adenosine 5'-monophosphoric acid and reduced glutathione for stability. The enzymes were partially purified by ammonium sulfate precipitation. Attempts to purify them further by alcohol, alumina Cγ, and column chromatography failed due to instability of the enzymes. Kinetic studies suggest that both are possible regulatory enzymes with an 'allosteric' site operative at pH 7.5 and not at pH 6.5. Accumulation of citrate in *A. niger* may possibly be due to the loss of sensitivity of the 'allosteric' site for citrate.
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INTRODUCTION

It is well known that Aspergilli generally excrete citric acid in the medium. This characteristic is dependent on a given strain. For instance, most strains of *Aspergillus niger* excrete citric acid while strains of *Aspergillus flavus* very rarely do so. No satisfactory explanation has yet been given for this difference in terms of a molecular or biochemical mechanism. According to one set of ideas (Lewis and Weinhouse, 1951) citrate excretion is considered to be due to a response to unfavorable cultural conditions (overflow shunt mechanism of Foster, 1949), while according to another hypothesis (Ramakrishnan and Martin, 1955; Ramakrishnan, Steel, and Lentz, 1955) citrate is considered to accumulate due to a decreased formation of aconitase and isocitric dehydrogenase during the lag phase of growth.

Our attention was drawn to this problem because of two reports in the literature which allowed for an entirely different plausible explanation in molecular terms of citrate excretion in Aspergilli. Firstly, it has been reported (James et al, 1956) that high citric acid yielding strains of
Aspergillus niger can be obtained by ultra violet irradiation, which suggests that citric acid production may be under genetic control of a regulatory nature (see Historical). Secondly, it has been found recently that in Neurospora crassa, the NAD-specific isocitric dehydrogenase is a regulatory protein, specifically activated by citrate. This enzyme has been found to have a distinct allosteric site (Sanwal et al, 1963). These two observations could be combined into a hypothesis which reasonably explained most of the facts about citrate accumulation. If we assumed that the NAD-specific isocitric dehydrogenase of Aspergillus was also an allosteric protein and require citrate for activation, a loss by mutation of the distinct allosteric site of the enzyme would result in an impaired functioning of the TCA cycle, and under such conditions citrate may conceivably pile up. Thus, A. niger which secretes citric acid should have on this hypothesis a defective isocitric dehydrogenase while A. flavus, which is a non-citric acid accumulator, should have an allosteric enzyme activated by citrate.

To test this hypothesis, results of which were inconclusive, a kinetic study of the isocitric dehydrogenases of A. niger and A. flavus was made. This thesis embodies the results of this study.
Recent research has supplied information on the relationship of the tricarboxylic acid cycle to the formation of citric acid by *Aspergillus niger*. The hypothesis that citric acid is formed through the condensation of a 2-carbon and a 4-carbon compound *in vivo* has been generally accepted. Research carried out with isotopic techniques has given support to this concept. The 2-carbon compound concerned is presumed to be acetate. The 4-carbon compound, a dicarboxylic acid, may be formed by the condensation of two 2-carbon compounds or one 1-carbon and one 3-carbon compound. Using labeled carbon dioxide, Foster and Carson (1950) showed that the carbon may be incorporated into citric acid, in part, at least, through the Wood-Werkman reaction. They also demonstrated the condensation of two 2-carbon compounds to form \( \text{C}_4 \)-dicarboxylic acids by an isotopic-dilution method. Martin, Wilson, and Burris (1950), Martin and Wilson (1951), and Mosbach, Phares, and Carson (1952) have confirmed the role of 1-carbon compounds in the synthesis of citric acid by *Aspergillus niger*. 
Shu, Funk, and Neish (1954) used a medium containing glucose-1-C\textsuperscript{14} as the only source of carbon to study the mechanism of citric acid formation by \textit{A. niger}. As a result of mathematical analysis of their data, the following conclusions were arrived at: (1) 37 to 40 per cent of the citric acid formed was produced from recycled C\textsubscript{4}-dicarboxylic acid; (2) 40 per cent of the dicarboxylic acid was formed by a C\textsubscript{2}, C\textsubscript{2} condensation and the remaining 60 per cent through a C\textsubscript{1}, C\textsubscript{3} condensation; and (3) 78 per cent of the glucose was dissimilated according to the scheme of Embden and Meyerhof and the rest through a mechanism which involved carboxyl-labeled pyruvic acid.

Ramakrishnan and Martin (1955), Ramakrishnan (1954) and Martin (1954) showed that all the enzymes of the Krebs citric acid cycle could be demonstrated in cell-free extracts of \textit{A. niger} when grown in a noncitrate-accumulating medium. Their noncitrate-accumulating medium contained 3\% malt extract, 0.5\% yeast extract, and 1\% glucose. Ramakrishnan, Steel, and Lentz (1955) obtained similar results on a citric acid accumulating medium. In this medium, carbon source was beet sugar molasses. They prepared the mashes by diluting the molasses with water to about 12 per cent sugar, adjusting the pH to 6.0 with
hydrochloric acid, sterilizing, adding 0.5 to 1.0 g ferrocyanide per liter to the hot mash, cooling, and adding 0.1 g to 1.0 g of dibasic potassium phosphate trihydrate per liter. Martin and Waters (1952) reported that the phosphate tended to reduce the toxicity of the ferrocyanide and thus exerted a favorable influence on citric acid production. In general, sugar concentration of 14 to 20% were found to be optimum for citric acid production on the basis of sugar utilized. Low yields were obtained with low sugar concentrations.

Ramakrishnan, Steel and Lentz, showed that after 46 hr., when citric acid began to accumulate, the specific activity of the condensing enzyme increased, whereas the TPN-specific isocitric dehydrogenase activity disappeared. At the time of maximum citric acid accumulation (after 96 hr.), the specific activity of the condensing enzyme had increased almost tenfold, and no TPN-specific isocitrate dehydrogenase or aconitase activities were detected. From their observations, these workers suggested an interruption of the Kreb's tricarboxylic acid cycle at the aconitase or IDH stages during the accumulation of citric acid.

In 1947, Weinhouse and Millington pointed out that citrate in _A. niger_ cannot accumulate unless auxiliary sources
can supply C\textsubscript{4} acids. They proposed two plausible mechanisms for this formation: carboxylation of pyruvate with CO\textsubscript{2} to yield oxalacetate (the Wood and Werkman reaction) and dehydrogenative coupling of 2 acetates to yield succinate (the Thunberg condensation).

\[
\text{CH}_3\text{COOH} + \text{CO}_2 \xrightarrow{\text{carboxylation}} \text{HOOCCH}_2\text{COOCH} \\
2 \text{CH}_3\text{COOH} \rightarrow \text{HOOCCH}_2\text{CH}_2\text{COOH} + 2 \text{H}
\]

Assimilation of carbon dioxide into fumarate and citrate was observed by Foster et al (1941) and fixation of carbon dioxide in citrate was reported by Martin, Wilson and Burris. Evidence for the occurrence of the Thunberg condensation was provided by Foster et al (1949) for the mold Rhizopus nigricans.

Lewis and Weinhouse (1951) studied the formation of citric acid in A. niger using labeled acetate in the medium and suggested that citric acid is an "overflow product" formed by faulty operation of the Krebs citric acid cycle. From the data of Weinhouse and Millington they presented their concept of citrate formation:

\[
\text{CO}_2 + \text{pyruvate} \rightarrow \text{oxalacetate} + \text{acetate} + \text{citrate} \\
\text{citrate} \xrightarrow{\text{degradation}} \text{oxalosuccinate} + \text{α-ketoglutarate} + \text{succinate}
\]
They envisioned a condensation of acetate with oxalacetate, with the latter being supplied from 3 sources; the citric acid cycle (breakdown of citrate through α-ketoglutarate), the Wood and Werkman reaction, and the Thunberg condensation. The extent of participation of these 3 processes in $C_4$ acid formation would vary under different conditions. Under conditions of rapid citrate accumulation, (as in the commercial production from sugars) they assumed that the major sources of $C_4$ acids would be the Wood and Werkman or the Thunberg reaction. They were fairly certain that citric acid is not an end-product of metabolism in molds, but a normal intermediate which accumulates under certain circumstances because of some interference in its further metabolism. Other workers (Clement 1952; Gerhard, Dorell, and Baldwin 1946; Karow and Waksman 1947; Martin and Waters 1952) have also suggested that the production of citric acid by molds, in amounts desired commercially, is not a normal metabolic process; that the accumulation of large amounts of citric acid is possible only when metabolism is impaired by toxic substances or deficient media.

Over production of citric acid may alternatively be due to mutation of regulatory genes controlling the formation of an enzyme after the citrate stage. This possibility has not been
considered by workers in the field of mold metabolism; nevertheless, in direct analogy with other microorganisms which excrete metabolites concomittant to a loss of regulatory genes by mutation. Such consideration is indeed warranted. It has become amply clear in recent years (Umbarger, 1961) that excretion of a normal metabolite in bacteria may be due to interference either with a genetic or a biochemical control mechanism of the cell which regulates the formation of the given metabolite.

Thus excretion of metabolite(s) may be due to a metabolic block, as in sulfonamide inhibition in *E. coli* (Gots, 1950), or it may be due to a genetic deficiency. Gots observed the accumulation of a derivative of 4-amino-5-imidazolecarboxamide by *E. coli* under the influence of a sulfonamide. When he supplemented the medium with preformed purines, he noted that accumulation was less.

Kinoshita et al (1957) isolated an arginine auxotroph of *Micrococcus glutamicus* that accumulated large amounts of glutamate. Udaka and Kinoshita (1958a) studied the biosynthetic pathway of L-ornithine in this mutant, and observed that the block was after ornithine. Study of the control mechanism by them (1958b) revealed that arginine, the endproduct
of the pathway starting with glutamate, prevented the formation of an activated form of N-acetylglutamate, resulting in the accumulation of glutamate. Such a mechanism is known as "negative feedback" or endproduct inhibition, and a combination of negative feedback control mechanism and of mutants with defective amino acid synthesizing systems have been used lately for amino acid fermentations. In the microbiological production of ornithine, lysine, threonine and isoleucine, techniques such as the addition of specific metabolic precursors or the use of various nutritional mutants have already been introduced.

Despite the presence of biosynthetic mechanisms capable of forming the wide variety of required metabolites from a single carbon source and inorganic salts, bacterial cells do not, in general, oversynthesize these metabolites. To maintain this balanced metabolism, two well known phenomena are known to occur in cells: (1) repression, and (2) endproduct inhibition, or negative feedback. These two regulatory mechanisms are barriers to excessive synthesis of metabolites.

In repression, the endproduct of a biosynthetic pathway is able to repress or prevent the formation of each enzyme of the sequence. In contrast to repression, which affects the
formation of all the enzymes in a pathway, endproduct inhibition involves only the activity of the first enzyme of the particular pathway. The typical endproduct not only represses enzyme formation, but also inhibits enzyme action. The earliest examples of such inhibition were found in isoleucine biosynthesis (Umbarger, 1956) and in pyrimidine biosynthesis (Yates and Pardee, 1956).

Endproduct inhibition thus prevents excessive production of not only the endproduct itself but the intermediates as well. It permits the internal pool of the endproduct to remain small but constant even though the demands on it for macromolecule synthesis may fluctuate. This control mechanism is known to occur in most biosynthetic pathways. Repressibility of biosynthetic pathways is not universally found. Alone, it cannot provide an adequate control over a biosynthetic pathway. It provides a sluggish control that can only be evoked to full advantage after several generations. Endproduct inhibition, on the other hand, has a finer control, and its action is immediate. Moyed (1961) has shown that a mutant in which a pathway was no longer sensitive to endproduct inhibition, but which retained repressibility, was unable to prevent oversynthesis.
The first thorough analysis of an interaction between the sensitivity of the first enzyme and its endproduct inhibitor was performed by Gerhart and Pardee (1961, 1962). These workers found that the sensitivity of aspartic transcarbamylase (the enzyme catalyzing the first step in pyrimidine biosynthesis) to the endproduct, cytosine triphosphate, could be abolished by treatment with Hg$. When inhibitor site was destroyed, they observed increase in enzyme activity, indicating a "better fit" of the protein with its substrate.

In a similar study of the mechanism by which isoleucine inhibited threonine deaminase, Changeux (1961) tested the effect of mercury and found the endproduct-sensitive site to be destroyed. He also showed that it was possible to destroy sensitivity to isoleucine, but retain enzymatic activity by heating the enzyme.

In another analysis of an endproduct-sensitive enzyme, Martin (1963) demonstrated that the first enzyme in the biosynthetic pathway of histidine has a catalytic site different from the endproduct-sensitive site.

With the evidence obtained from these three systems, Monod et al (1963) suggested that the sites for inhibitor and
substrate may lie at some distance from each other but indirectly interact with each other through the structure of the protein. Monod and Jacob (1961) have suggested that endproduct inhibition is a specific example of allosteric interaction in which the regulatory molecule need have absolutely no steric relationship with the substrate. The enzyme's activity is regulated by metabolites structurally unrelated to the substrates of the regulated enzyme. The effect of these regulatory agents, or "effectors", appears to result exclusively from a conformational alteration induced in the protein due to the binding of the "effector". Monod et al (1963) hypothesized that these regulatory proteins possess two, or at least two, stereospecifically different, non-overlapping receptor sites. One of these, the active site, binds the substrate and is responsible for the biological activity of the protein. The second receptor site, usually called the allosteric site, is complementary to a structurally unrelated metabolite (allosteric effector) which binds specifically and reversibly to the protein. The formation of the enzyme-allosteric effector complex does not catalyze a reaction involving the effector itself. It is assumed only to bring about a discrete reversible alteration of the
molecular structure of the protein or allosteric transition which modifies the properties of the active site, changing one or several of the kinetic parameters which characterize the biological activity of the protein. This latter characteristic will be mentioned further on.

It is amply clear from the above example that if a regulatory protein intervened somewhere in the TCA cycle in Aspergillus whose activity was controlled by feedback, a pile up of citrate could occur if the activity of this enzyme was inhibited or repressed by environmental conditions or by gene mutations. Out of the many enzymes of the TCA cycle, it seemed that isocitric dehydrogenase could possibly be a regulatory protein in Aspergillus.

The biological conversion of isocitrate to α-ketoglutarate and carbon dioxide was established by the work of Martius and others (Martius 1937; Krebs 1937; Adler, Euler, Gunther, and Plass 1939). Using a TPN-specific isocitric dehydrogenase from pig heart, Ochoa (1945) demonstrated that this conversion was the result of two reversible steps with oxalosuccinate as the intermediate product. Kornberg and Pricer (1951) isolated both TPN- and DPN-specific isocitric dehydrogenases from yeast. The latter enzyme had a specific requirement for AMP. Plaut and
Sung (1954) first showed the presence of a DPN-specific isocitric dehydrogenase in a variety of animal tissues. This system, unlike that in yeast, did not require AMP but is now known to be activated by ADP (Chen and Plaut 1963; Chen, Brown, and Plaut 1964). Martin (1955) reported the occurrence of TPN and DPN-specific isocitric dehydrogenases in cell-free extracts of *A. niger*. Sanwal *et al* (1963) showed the occurrence of two IDH in *Neurospora crassa* distinguished on the basis of their coenzyme requirement.

Both enzymes, the NADP- and NAD-specific IDH catalyze the reaction

\[
\text{D-isocitrate} + \text{NAD} \quad \xrightarrow{\text{Mn}^{++}} \quad \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \quad \text{or} \quad \text{NADH} + \text{H}^+ \\
\text{or} \quad \text{NAD} \quad \xrightarrow{\text{Mg}^{++}} \quad \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADH} + \text{H}^+
\]

However, the NADP-isocitric dehydrogenase differs from the NAD-isocitric dehydrogenase in that the former decarboxylates oxalosuccinate, while the latter does not. The NAD-specific enzyme has an absolute and specific requirement for AMP, except for those found in animal tissues. Kornberg and Pricer (1951) thought that the endproduct, \(\alpha\)-ketoglutarate, was not reduced by the NAD-dependent IDH under conditions favorable for its reduction by the NADP enzyme. Hathaway and Atkinson (1963)
demonstrated that the reaction is reversible at pH 6.5 but very weakly so at pH 7.5. Sanwal et al. (1964) showed that with their *Neurospora* enzyme, at pH 6.5, a decrease in absorbancy at 340 μm was obtained at a linear rate. From their kinetics studies (1963, 1964), they suggested that the NAD-specific IDH possibly fulfills a regulatory function in the TCA cycle. Their NAD-specific enzyme did not obey Michaelis-Menten kinetics at its pH optimum of 7.6. At this pH, the enzyme was inhibited by α-ketoglutarate (endproduct) and activated by citrate (precursor). Precursor activation has been shown to occur in several regulatory proteins by many workers (Changeux, 1962; Gerhart and Pardee, 1962; Algranati and Cabib, 1962; Leloir, Olavaria, Goldemberg, and Carminatti, 1959; Leloir and Goldemberg, 1960; Glaser and Brown, 1957). At pH 6.5, the enzyme was shown to exhibit classical Michaelis-Menten kinetics. Plots of initial velocity against concentration yield a normal hyperbolic curve. But at pH 7.6, a similar plot yield a sigmoidal curve. At this pH optimum, double reciprocal plots of substrate concentration against initial velocity gave a parabolic curve. Such curves have been shown to be characteristic of regulatory proteins such as aspartate trans-carbamylase (Gerhart and Pardee, 1962), threonine deaminase
(Monod, Changeux, and Jacob 1963; Changeux 1961, 1962), and
hemoglobin (Benesch, Ranney, Benesch, and Smith 1961).
METHODS AND MATERIALS

Cultures

Aspergillus niger P.R.L. No. 558, a citrate accumulating strain, was obtained from the Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan. A laboratory strain of Aspergillus flavus, which does not excrete any citrate, was obtained from the culture collection of the Department of Microbiology, University of Manitoba.

Inoculum

A. niger stock cultures were maintained in Difco Potato Dextrose Agar in 250 ml Erlenmeyer flasks. A. flavus stock cultures were maintained in a solid medium containing

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<th>Amount</th>
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<tr>
<td>glucose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>peptone</td>
<td>1.75 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>yeast extract</td>
<td>2.5 g</td>
</tr>
<tr>
<td>agar</td>
<td>12.5 g</td>
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distilled water to a total of 500 ml
For inoculation into liquid medium, the conidia were washed from the surface of the agar with sterilized distilled water containing one or two drops of Tween-80. Five milliliters of a heavy suspension of the conidia was used to inoculate 500 ml of liquid medium in 2-liter Erlenmeyer flasks.

**Liquid Medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tr>
<td>sucrose</td>
<td>150.0 g</td>
</tr>
<tr>
<td>urea</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.08 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.15 g</td>
</tr>
<tr>
<td>MnSO$_4$.4H$_2$O</td>
<td>0.02 g</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.01 g</td>
</tr>
</tbody>
</table>

Distilled water to a total of 1 liter

The liquid medium was autoclaved for 20 mins. at 15 lbs per square inch pressure. The urea was sterilized by millipore filter technique, and added to the liquid medium after it had been cooled to room temperature.

The fungi were grown on a New Brunswick Scientific rotary shaker at 170 revolutions per minute in a 28° C
incubator room for 48 hours for *A. niger*, and for 72 hours for *A. flavus*.

**Preparation of Cell-free Extracts**

The mycelium, after 48 hours growth (72 hours in the case of *A. flavus*), was harvested on two layers of muslin, washed repeatedly with twice the volume of distilled water. The excess water was then squeezed off. All the operations after this step were conducted at 0-3°C. The nearly dry mass of cells was mixed with 1.5 times their weight of acid-washed levigated alumina and ground in a prechilled mortar till the whole mass acquired a 'sticky' consistency (5-7 minutes). The disrupted cells were extracted with 3 volumes of 0.1 M KH$_2$PO$_4$ buffer, pH 6.5, containing 10$^{-3}$ M EDTA, 10$^{-3}$ M GSH, and 10$^{-3}$ M AMP. The alumina and cell-wall debris were removed by centrifugation in a RC-2 Sorval centrifuge at 12,100 x g for 10 minutes. The opalescent supernatant solution was used for assay and subsequent purification.

**Chemicals**

The following chemicals were obtained from commercial sources:
Before use, isocitric acid lactone was hydrolyzed in alkali (Deutsch and Phillips, 1957). Different batches of isocitric acid were assayed before use by means of the enzymic method of Ochoa (1948).

Enzyme Assay

The NAD-specific IDH catalyzing the conversion of d-isocitrate to α-ketoglutarate and CO₂ was assayed by
following the rate of appearance of reduced DPN at 340 mμ on a Model 2000 Gilford Recording Spectrophotometer.

The reaction mixture contained: 5 mM dl-isocitrate; 1.66 mM AMP; 0.5 mM DPN; 3.3 mM MgSO₄·7H₂O; and 0.2 M KH₂PO₄ buffer (pH 6.5) to a final volume of 3.0 ml. Cuvettes of 10 mm light path were used. The temperature of assay was 24-25°C. The reaction was started by the addition of 0.1 ml of a properly diluted enzyme. Under the assay conditions, the rate of reaction was linear for approximately 2 minutes.

A unit of IDH activity is defined as the amount of enzyme causing an increase in optical density of 0.001 per minute at 340 mμ. Specific activity is expressed as units per milligram of protein. Protein was determined by the method of Lowry et al (1951).

Purification of the NAD-specific IDH from A. niger

Solid (NH₄)₂SO₄ was added to the cell-free extract continuously over a 10 minute period, with constant stirring, to 45% saturation and stirred at 4°C for another 10-15 minutes. The mixture was centrifuged at 12,100 x g for 10 minutes and the precipitate was discarded. The supernatant fraction was brought to approximately 70% saturation by the
addition of more solid (NH₄)₂SO₄. After 15 minutes, the suspension was centrifuged at 12,100 x g for 10 minutes and the pellet was dissolved in one-tenth of the original volume of 0.1 M KH₂PO₄ buffer, pH 6.5, containing 10⁻³ M EDTA, 10⁻³ M GSH and 10⁻³ M AMP.

To this concentrated enzyme solution, ammonium sulfate was added to 40% saturation and was allowed to stir for 10-15 minutes at 4°C. The precipitate, obtained after centrifugation at 12,100 x g for 10 minutes, was discarded. To the supernatant fraction, ammonium sulfate was added to yield 55% saturation and the precipitate allowed to form for 10 minutes. The small pellet was obtained by centrifugation at 12,100 x g for 10 minutes and dissolved in one-tenth of the original volume with 0.1 M KH₂PO₄ buffer, pH 6.5, containing 10⁻³ M EDTA, 10⁻³ M GSH, and 10⁻³ M AMP. At this stage the enzyme preparation was extremely labile to dialysis in 0.05 M KH₂PO₄ buffer, pH 6.5, containing 10⁻³ M EDTA, 10⁻³ M GSH, and 10⁻³ M AMP. Under the above experimental conditions, the NAD-specific enzyme could not be completely separated from the NADP-specific enzyme. The same difficulty was experienced by Ramakrishnan and Martin (1955). The enzyme preparation was free from DPNH oxidase, and NAD- and NADP-specific glutamic
dehydrogenases. The outlines of the purification procedure are given in Table 1.

Further purification of fraction IV (see Table 1) with alcohol was as follows: To this fraction, alcohol was added to a concentration of 40% with stirring at 0°C. The precipitate was discarded after centrifugation at 12,100 x g for 8 minutes. The supernatant was made to 50% concentration with alcohol and the pellet obtained after centrifugation was dissolved in 5 to 6 ml of 0.1 M KH₂PO₄ buffer, pH 6.5, containing 10⁻³ M EDTA, 10⁻³ M GSH, and 10⁻³ M AMP.

**Purification of the NAD-specific IDH of A. flavus**

The NAD-specific IDH of *A. flavus* proves to be more labile than the corresponding enzyme in *A. niger* in a partially purified state. The procedures outlined for *A. niger* IDH could not be used for *A. flavus* IDH. The following steps were applied for partial purification of IDH from *A. flavus*.

To the cell-free extract, ammonium sulfate was added to yield 40% saturation. The precipitate was allowed to form for 10-15 minutes with constant stirring at 40°C and was discarded after centrifugation at 12,100 x g for 10 minutes. The enzyme fraction in the supernatant was used for assay and
kinetic studies. Purification procedures for \textit{A. flavus} IDH are given in Table 2.
RESULTS

Lability of the Enzymes

A 40-fold purification of NAD-IDH of *A. niger* was obtained with a combination of \((\text{NH}_4)_2\text{SO}_4\)-alcohol procedure. With alcohol precipitation, the enzyme seemed to be unstable and lost more than half its activity in an hour. Therefore the \((\text{NH}_4)_2\text{SO}_4\) fraction was used for study purposes. Attempts to purify the enzyme with alumina Cγ, sephadex G100, G200, and DEAE-cellulose were unsuccessful. Table 1 gives the purification steps of *A. niger* NAD-IDH.

The same purification procedure of \((\text{NH}_4)_2\text{SO}_4\) precipitation could not be used for the NAD-IDH of *A. flavus* since the enzyme proved to be more labile than the corresponding one in *A. niger*. In this regard the enzyme seems to be comparable to the NAD-specific isocitric dehydrogenase obtained from plants (Davies, 1955).

pH Optimum

The pH optimum of NAD-specific IDH of *A. niger* was determined in a series of \(\text{KH}_2\text{PO}_4\) and Tris-HCl buffers. From
Table 1

Purification of NAD-specific isocitric dehydrogenase from *Aspergillus niger*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units</th>
<th>Total units</th>
<th>Protein mg/ml</th>
<th>Specific activity units/mg protein</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>665</td>
<td>39,900</td>
<td>7.82</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>I. 0-45% (NH₄)₂SO₄ sup.</td>
<td>450</td>
<td>27,000</td>
<td>3.0</td>
<td>150</td>
<td>68%</td>
</tr>
<tr>
<td>II. 45-70% (NH₄)₂SO₄ ppt.</td>
<td>2660</td>
<td>15,960</td>
<td>13.69</td>
<td>194</td>
<td>40%</td>
</tr>
<tr>
<td>III. 0-40% (NH₄)₂SO₄ sup.</td>
<td>1460</td>
<td>8,760</td>
<td>4.95</td>
<td>295</td>
<td>22%</td>
</tr>
<tr>
<td>IV. 40-55% (NH₄)₂SO₄ ppt.</td>
<td>1540</td>
<td>9,240</td>
<td>6.6</td>
<td>233</td>
<td>23%</td>
</tr>
</tbody>
</table>
### Table 2

Purification of NAD-specific isocitric dehydrogenase from *Aspergillus flavus*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units</th>
<th>Total units</th>
<th>Protein mg/mL</th>
<th>Specific activity units/mg protein</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>800</td>
<td>24,000</td>
<td>10.8</td>
<td>78.7</td>
<td></td>
</tr>
<tr>
<td>0-45% (NH₄)₂SO₄ sup.</td>
<td>465</td>
<td>13,950</td>
<td>6.1</td>
<td>77.5</td>
<td>98.5%</td>
</tr>
</tbody>
</table>
pH 6.0 to pH 7.0, phosphate buffer, and from pH 7.5 to pH 8.5, Tris-HCl buffer was used. The optimum pH for the enzyme was approximately 7.3 (Fig. 1).

Effect of Stabilizing Agents on NAD-specific IDH of *A. flavus*

*A. flavus* IDH was found to be more labile than the corresponding one in *A. niger*. It appeared more stable at pH 6.5 than at pH 7.0 to pH 7.5. The addition of both AMP and GSH at a concentration of $1 \times 10^{-3}$ M seemed to stabilize the enzyme, however, each alone was not as effective. This is shown in Fig. 2.

**Thermo-inactivation Study**

Heat inactivation study was carried out with the NAD-specific IDH of *A. niger*. The enzyme appeared extremely labile at $55^\circ$ C and $60^\circ$ C. At $45^\circ$ C and $50^\circ$ C, activity decreases with time as indicated in Fig. 3.

**Effect of Inhibitors on NAD-specific IDH of *A. niger***

Table 3 shows the various inhibitors used to study their effects on the reduction of dl-isocitric acid. The results indicate that EDTA (a chelating agent) does not
Fig. 1. Optimum hydrogen ion concentration of IDH of \textit{A. niger}. Plots of specific activity versus hydrogen ion concentration. The reaction mixture contained: 5.0 mM dl-isocitrate, 0.83 mM AMP, 0.5 mM DPN, 3.3 mM MgSO$_4$, 0.18 mg/ml of enzyme, and enough 0.2 M of the proper buffer to a total of 3.0 ml.
Fig. 2. Thermo-inactivation of NAD-IDH of A. niger. The enzyme was heated in water baths of 45°C, 50°C, 55°C, and 60°C. At various time intervals samples were withdrawn and cooled immediately to 0°C. The reaction mixture contained: 5.0 mM dl-isocitrate, 0.83 mM AMP, 1.0 mM DPN, 3.3 mM MgSO₄, 0.15 mg/ml of enzyme, and enough 0.2 M KH₂PO₄ buffer (pH 6.5) to a total of 3.0 ml.
Fig. 3. Effect of stabilizing agents on NAD-IDH of *A. flavus*. Curve: (1) no AMP or GSH, (2) + AMP, (3) + GSH, (4) + AMP and GSH, all at concentrations of 1.0 x 10^{-3} M. The reaction mixture contained: 5.0 mM dl-isocitrate, 0.83 mM AMP, 1.0 mM DPN, 3.3 mM MgSO_{4}, 0.22 mg/ml of enzyme, and enough 0.2 M KH_{2}PO_{4} buffer (pH 7.15) to a total of 3.0 ml.
Table 3

Effect of inhibitors on NAD-specific IDH of *Aspergillus niger*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final Concentration (M)</th>
<th>Units</th>
<th>Specific Activity (units/mg protein)</th>
<th>Per cent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>1750</td>
<td>407</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>$1.0 \times 10^{-2}$</td>
<td>1560</td>
<td>363</td>
<td>89</td>
</tr>
<tr>
<td>EDTA</td>
<td>$3.0 \times 10^{-2}$</td>
<td>495</td>
<td>115</td>
<td>28</td>
</tr>
<tr>
<td>Na azide</td>
<td>$1.0 \times 10^{-2}$</td>
<td>1780</td>
<td>414</td>
<td>100</td>
</tr>
<tr>
<td>Na azide</td>
<td>$5.0 \times 10^{-2}$</td>
<td>1215</td>
<td>283</td>
<td>69</td>
</tr>
<tr>
<td>Na fluoride</td>
<td>$1.0 \times 10^{-2}$</td>
<td>1885</td>
<td>439</td>
<td>100</td>
</tr>
<tr>
<td>Na fluoride</td>
<td>$10.0 \times 10^{-2}$</td>
<td>1215</td>
<td>283</td>
<td>69</td>
</tr>
<tr>
<td>pCMBA</td>
<td>$1.0 \times 10^{-4}$</td>
<td>1565</td>
<td>366</td>
<td>90</td>
</tr>
<tr>
<td>pCMBA</td>
<td>$1.0 \times 10^{-3}$</td>
<td>685</td>
<td>159</td>
<td>39</td>
</tr>
<tr>
<td>Na molybdate</td>
<td>$5.0 \times 10^{-2}$</td>
<td>1357.5</td>
<td>315.7</td>
<td>78</td>
</tr>
<tr>
<td>Na molybdate</td>
<td>$10.0 \times 10^{-2}$</td>
<td>903.8</td>
<td>210.2</td>
<td>52</td>
</tr>
</tbody>
</table>

Assay conditions: The reaction mixture contained 0.83 mM AMP, 0.5 mM NAD, 3.3 mM MgSO₄, 0.14 mg/ml of enzyme, inhibitor, and KH₂PO₄ buffer (pH 6.5) to a total of 3.0 ml. The mixture was incubated at room temperature for 3 minutes, and the reaction was started with the addition of 5.0 mM dl-isocitrate.
significantly inhibit at low concentration, but at higher concentrations is a potent inhibitor. The NAD-specific IDH is very sensitive to pCMBA. The sensitivity to this reagent suggests that -SH groups are necessary for enzymic activity.

**Kinetics of Isocitric Dehydrogenase from A. niger**

**Initial velocity analysis at pH 6.5**

When isocitrate was used as a variable substrate at several fixed concentrations of NAD, the double reciprocal plots (Fig. 4) were linear and excellent fits were obtained to equation 1.

\[
v = \frac{VS}{K + S}
\]

(1)

(where \(v\) = initial observed velocity, \(V\) = max. velocity, \(S\) is substrate concentration and \(K\) is the Michaelis concentration for the substrate). Replots of values for slopes and intercepts obtained from fits to equation (1) against reciprocals of the concentrations of NAD were likewise linear (Fig. 5a and 5b). With NAD as the variable, and isocitrate as the changing fixed substrate, the double reciprocal plots (Fig. 6) as well as the replots of \((1/V)\) and \(K/v\) were linear (Fig. 7a and 7b). In the presence of NAD, and isocitrate, the
Table 4

Kinetic constants of *Aspergillus niger* NAD-specific isocitric dehydrogenase

<table>
<thead>
<tr>
<th></th>
<th>pH 7.6</th>
<th>pH 7.6 (citrate)</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$</td>
<td>$-$</td>
<td>$0.25$</td>
<td>$0.23$</td>
</tr>
<tr>
<td>$K_{ia}$</td>
<td>$2$ mM</td>
<td>$-$</td>
<td>$2$ mM</td>
</tr>
<tr>
<td>$K_p$</td>
<td>$-$</td>
<td>$1.8$</td>
<td>$1.6$ mM</td>
</tr>
</tbody>
</table>
Fig. 4. Double reciprocal plots of velocity versus varying dl-isocitrate concentrations with NAD as fixed variable. NAD concentrations: (1) 0.4 mM, (2) 0.6 mM, (3) 1.2 mM, (4) 2.4 mM. The reaction mixture contained: 1.66 mM AMP, 3.3 mM MgSO_4, 0.14 mg/ml of enzyme, and enough 0.2 M KH_2PO_4 buffer (pH 6.5) to make a total of 3.0 ml.
Fig. 5a. Replot of slopes versus reciprocal of NAD concentrations from Fig. 4 in which dl-isocitrate was varying substrate, and NAD the fixed variable.

Fig. 5b. Replot of intercepts versus reciprocal of NAD concentrations from Fig. 4 in which dl-isocitrate was varying substrate, and NAD the fixed variable.
Fig. 6. Double reciprocal plots of velocity versus varying NAD concentrations with dl-isocitrate as fixed variable. DL-isocitrate concentrations: (1) 0.83 mM, (2) 0.996 mM, (3) 1.328 mM. The reaction mixture contained: 1.66 mM AMP, 3.3 mM MgSO₄, 0.1 mg/ml of enzyme, and enough 0.2 M KH₂PO₄ buffer (pH 6.5) to a total of 3.0 ml.
Fig. 7a. Replot of intercepts versus reciprocal of dl-isocitrate from Fig. 6 in which NAD was varying, and dl-isocitrate the fixed variable.

Fig. 7b. Replot of slopes versus reciprocal of dl-isocitrate from Fig. 6 in which NAD was varying, and dl-isocitrate the fixed variable.
kinetic data fit equation (2) which is given by all sequential mechanisms (Cleland, 1963).

\[ v = \frac{V_A B}{K_{i_A} K_b + K_a B + K_b A + AB} \]  

(2)

(where A = NAD; B = isocitrate, \( K_a \) and \( K_b \) are Michaelis constants of A and B; \( K_{i_A} \) is the inhibition constant for A).

Fit to equation (2) rules out a Ping-pong mechanism (Cleland, 1963) because in this mechanism there is no \( K_{i_A} K_b \) term in the denominator of initial velocity equation.

From the analysis given above, it is difficult to tell apart A from B, but since all pyridine nucleotide linked dehydrogenases worked out so far bind the coenzyme to the free enzyme form (Sund, Diekmann and Wallenfels, 1964), it is assumed that such is also the case here. The various kinetic constants in equation (2) can be calculated from Figures 5a, 5b, 6, 7a and 7b. The horizontal intercept of the \((1/V)\) replot, for instance, yields the Michaelis constant of the changing fixed substrate while the ratio of vertical intercept of \((1/V)\) and \((K/V)\) replot gives the Michaelis constant of the varied substrate. The \( K_{i_A} \) value can be calculated from a fit to equation (3).
\[ v = \frac{v_{S(isocitrate)}}{K(1 + A/K_{is}) + S(isocitrate) \left( 1 + \frac{A}{K_{ii}} \right)} \]  

(3)

(where A is the reciprocal of NAD concentration, \( K_{is} = \) slope inhibition constant, and \( K_{ii} = \) intercept constant). Thus the horizontal intercept of the \((K/V)\) replot (Fig. 5a) of data where isocitrate was the variable, and NAD the changing fixed substrate (Fig. 4), also yields \( K_{ia} \).

It may be mentioned that equation (2) also applies to Theorell-Chance and Rapid equilibrium random mechanisms (Cleland, 1963), but to distinguish between these mechanisms product inhibition studies are necessary which have not been attempted in this thesis. However, for evaluating results (reported later) obtained at pH 7.6, kinetic analysis at pH 6.5, reported above was considered necessary to exclude at least random mechanisms or mechanisms having alternate pathways. Since the replots were all linear, it can be concluded that the substrates bind at only one fixed active site. Binding at sites other than the active one would lead to curvatures of the replots.

**Initial Velocity Analysis at pH 7.6**

In contrast to results obtained at pH 6.5, when
isocitrate was varied at several fixed levels of NAD the double reciprocal plots were markedly curved and could only be approximated by parabolas \((y = an^2 + bn + c)\). The lower-most curve when fitted by a least square method (Snedecor, 1948) gave a good fit and the repression coefficients \(b\) and \(c\) were significant at 1\% and 5\% level. Since no weighing factors were used in arriving at these values, an absolute reliance cannot be placed on these figures. To interpret the curves given in Figure 8, a suggestion given by Sanwal et al (1964) was utilized in deriving a rate equation which would fit the data reported here. According to the views of these authors, the parabolic type of curves may be due to the binding of substrate at two different sites, one active and another activating, and further, that a reversible connection may exist between the two binding sites.

Assuming that the substrate is an activator and that there is no binding at the active site unless one molecule of substrate has been bound at the activated site, we can conceive the following mechanism:

\[
E \rightleftharpoons EA \rightleftharpoons EAB^1 \rightleftharpoons EAB^1B \rightarrow \text{Products}
\]

(where \(E\) is the free enzyme, \(A\) is NAD, \(B^1\) is isocitrate at
Fig. 8. Double reciprocal plots of velocity versus varying dl-isocitrate concentrations with NAD as fixed variable. NAD concentrations: (1) 0.4 mM, (2) 0.6 mM, (3) 1.2 mM, (4) 2.4 mM. The reaction mixture contained: 1.66 mM AMP, 3.3 mM MgSO₄, 0.15 mg/ml of enzyme, and enough 0.2 M Tris-acetate buffer (pH 7.6) to a total of 3.0 ml.
activating site and B at the active site).

Defining $K_1$ as dissociation constant of $B^1$ from $EAB^1$

$$K_1 = \frac{(EA)(B^1)}{(EAB^1)}$$

also, $EA + EAB^1 = E_t$

$$\frac{EA}{E_t} = \frac{K_1}{K_1 + B} \quad \text{and} \quad \frac{EAB}{E_t} = \frac{B}{K_1 + B}$$

Since, according to our assumptions only form $EAB^1$ has activity, we can multiply the initial velocity equation (1) by the factor

$$\frac{B}{K_1 + B}$$

and this is (4)

$$v = \frac{VS^2}{K_mK_1 + (K_1 + K_m)S + S^2} \quad (4)$$

and in a reciprocal form (5)

$$\frac{1}{v} = \frac{K_mK_1}{v} 1^2 + \frac{K_1 + K_m}{v} \frac{1}{S} + \frac{1}{v} \quad (5)$$

which is identical in form to $y = an^2 + bn + c$. In the presence of A (NAD) and B (isocitrate) the equation is (6):

$$v = \frac{VAB^2}{K_{ia}K_{b}K_1 + K_{a}K_1B + K_{b}K_{a}A + K_{1}AB + K_{ia}K_{b}B + K_{a}B^2 + K_{b}AB + AB^2} \quad (6)$$
This equation predicts not only the parabolic type of double reciprocal plots when isocitrate is varied, but also predicts the kind of curves one would obtained when NAD is the variable substrate and isocitrate is the changing fixed one. Rearranging equation (6) in a reciprocal form for varying A ( = NAD) conditions, we have equation (7):

\[
\frac{1}{v} = \frac{K_a}{v} \left[ 1 + \frac{K_i + K_i a K_b}{K_a} + \frac{K_i a K_b K_i}{K_a B^2} \left( \frac{1}{A} + \frac{1}{v} \right) \right] + \frac{K_i + K_b}{B} + \frac{K_i K_b}{B^2} \quad (7)
\]

In accord with equation (7) when NAD is varied at several fixed concentrations of isocitrate, the double reciprocal plots are linear (Fig. 9) and the lines intersect above the horizontal axis. Replots of slopes and intercepts are parabolic functions of 1/isocitrate (Fig. 10).

In summary of the above analysis, then, most observed facts are explicable on the basis that at its pH optimum, the enzyme has one active site for NAD and one activating and another active site for isocitrate. Since at pH 6.5, the analysis confirms to equation (1) we can only assume that at this pH the activating site becomes 'inoperative' due
Fig. 9. Double reciprocal plots of velocity versus varying NAD concentrations with dl-isocitrate as fixed variable. DL-isocitrate concentrations: (1) 0.83 mM, (2) 0.996 mM, (3) 1.328 mM, (4) 4.15 mM. The reaction mixture contained: 1.66 mM AMP, 3.3 mM MgSO_4, 0.1 mg/ml of enzyme, and enough 0.2 M Tris-acetate buffer (pH 7.6) to a total of 3.0 ml.
Fig. 10. Replot of intercepts versus reciprocal of dl-isocitrate from Fig. 9 in which NAD was varying and dl-isocitrate the fixed variable substrate.
perhaps to ionization of critical binding groups at the activating site.

**Effect of Citrate on the Velocity of the Reaction**

The effect of citrate on the velocity of the reaction at pH 7.6 is represented in Figure 11. At pH 6.5, citrate has no activating effect. It will be noted from Figure 8 that the usual parabolic double reciprocal plots become linear in the presence of citrate. The conclusion seems inescapable that citrate binds at the activating site of the enzyme and since this site is now occupied, the substrate (isocitrate) can only bind at the active site. Presumably because only one molecule of the substrate binds in the presence of citrate, usual linear plots showing fit to equation (2) are obtained. The $V_{\text{max}}$ does not change in the presence of the activator and as seen from Table 4 the kinetic constants obtained in its presence are essentially similar to those obtained at pH 6.5. This is enough justification for the assumptions used in deriving equation (5) and (6).
Fig. 11. Lineweaver-Burk plot of velocity versus varying dl-isocitrate concentrations, with constant citrate, and NAD as fixed variable. NAD concentrations: (1) 0.1 mM, (2) 0.2 mM, (3) 0.4 mM, (4) 0.8 mM. The reaction mixture contained: 0.83 mM AMP, 3.3 mM MgSO₄, 0.523 mM citrate, 0.11 mg/ml of enzyme, and enough 0.2 M Tris-acetate buffer (pH 7.6) to a total of 3.0 ml.
Fig. 12a. Replot of slopes versus reciprocal of NAD from Fig. 11 in which dl-isocitrate was varying, citrate was constant, and NAD the fixed variable.

Fig. 12b. Replot of intercepts versus reciprocal of NAD from Fig. 11 in which dl-isocitrate was varying, citrate was constant, and NAD the fixed variable.
Fig. 13. Double reciprocal plots of velocity versus varying NAD concentrations with dl-isocitrate as fixed substrate at 5.0 mM. The reaction mixture contained 0.83 mM AMP, 3.3 mM MgSO₄, 0.2 mg/ml of enzyme, and enough 0.2 M KH₂PO₄ buffer (pH 6.5) to a total of 3.0 ml.
Fig. 14. Double reciprocal plots of velocity versus varying dl-isocitrate concentrations with NAD as fixed substrate at 1.0 mM. The reaction mixture contained: 0.83 mM AMP, 3.3 mM MgSO₄, 0.2 mg/ml of enzyme, and enough 0.2 M KH₂PO₄ buffer (pH 6.5) to a total of 3.0 ml.
Fig. 15. Double reciprocal plots of velocity versus varying dl-isocitrate concentrations with NAD fixed (1.0 mM). The reaction mixture contained: 0.83 mM AMP, 3.3 mM MgSO₄, 0.2 mg/ml of enzyme, and 0.2 M Tris-acetate buffer (pH 7.6) to a total of 3.0 ml.
A. flavus

Fig. 16. Double reciprocal plots of velocity versus varying dl-isocitrate concentrations, with citrate, and NAD fixed (1.0 mM). The reaction mixture contained 0.83 mM AMP, 3.3 mM MgSO$_4$, 0.66 mM citrate, 0.2 mg/ml of enzyme, and 0.2 M Tris-acetate buffer (pH 7.6) to a total of 3.0 ml.
DISCUSSION

This investigation was prompted by observations of several workers that certain strains of *A. niger* are citric acid excretors, while strains of *A. flavus* are not. Production of citric acid by *A. niger* has been of industrial importance, and through the years, several workers have proposed hypothesis for the causes of citrate accumulation. Lewis and Weinhouse (1951) noted that citric acid was not an endproduct of metabolism in *A. niger*, but a normal intermediate which accumulated under certain circumstances because of some interference in its further metabolism. They suggested that citric acid is an "overflow product" formed by the faulty operation of the Krebs' citric acid cycle. Ramakrishnan, Steel, and Lentz (1955) from studies of the variation of activities of several enzymes of Krebs' tricarboxylic acid cycle, suggested an interruption of this cycle at the aconitase or isocitric dehydrogenase stages.

At the time the investigators cited above (see Historical) proposed their hypotheses, a significant facet of metabolic control mechanisms was not known, namely, control of the activity of certain regulatory enzymes by
the endproduct of the sequence in which they participate. For instance, studies of the accumulation of glutamate by an arginine auxotroph of Micrococcus glutamicus by Udaka and Kinoshita (1958b) revealed an operation of "negative feedback" mechanism. Arginine, the endproduct of the pathway starting with glutamate, prevented the formation of an activated form of N-acetylglutamate, resulting in the accumulation of glutamate. Moyed (1961) pointed out that mutants in which a pathway was no longer sensitive to end-product inhibition (due to mutational events) was unable to prevent oversynthesis of its metabolites and this led to secretion of such metabolites in the culture fluid.

With the discovery that NAD-specific isocitric dehydrogenase of another mold, Neurospora crassa (Sanwal et al., 1963, 1964) was an allosteric protein, specifically activated by citrate, an explanation of citrate accumulation in diverse Aspergilli seemed feasible (cf., Introduction). One could reasonably assume that citrate secretors had an isocitric dehydrogenase which could not be activated by citrate owing possibly to a loss of the allosteric site by mutation. This assumption was strengthened by the nature of pathway between citrate and α-ketoglutarate in the Krebs'
cycle:

\[ \text{Citrate} \xrightarrow{1} \text{cis-aconitate} \xrightarrow{2} \text{isocitrate} \xrightarrow{3} \alpha\text{-ketoglutarate} \]

It is well known from work on aconitase, catalyzing steps 1 and 2 that equilibrium of the reaction strongly favours the formation of citrate, and at equilibrium in a system containing citrate, aconitate and isocitrate, over 82% is present in the form of citrate (Krebs, 1953). This unfavorable equilibrium is compounded by the fact that the affinity of isocitrate for isocitric dehydrogenase (NAD-specific) from microbial sources (Kornberg and Pricer, 1951) is usually high.

To overcome this unfavorable circumstance and for the smooth running of the TCA cycle, it is necessary to have a mechanism whereby the equilibrium can be shifted to the formation of \( \alpha \)-ketoglutarate. Activation by citrate of step 3 has perhaps been evolved in the molds to achieve precisely this end. This also explains, teleonomically speaking, the special construction of the crucial enzyme, NAD-specific isocitric dehydrogenase. It is at once clear that if the allosteric site or isocitric dehydrogenase were lost by mutation or became inoperative due to its specific inhibition by environmental factors (included are trace elements, carbon-
nitrogen ratio and other factors leading to a secretion of citric acid, citrate would not be able to overcome the unfavorable equilibrium of the pathway, would pile up in the cells, and would eventually be excreted.

With all this theoretical reasoning behind the investigation undertaken here, the NAD-specific isocitric dehydrogenase of A. flavus and A. niger were partially characterized and were found to be allosteric in nature (in complete agreement with results obtained in Neurospora crassa by Sanwal et al. (1964). However, our results indicated that since the NAD-IDH from both strains of Aspergillus responded to citrate activation at pH 7.5, the mechanism of citrate excretion in A. niger may not be due to a mutational loss of the distinct 'allosteric' site. This leaves us with the possibility that the allosteric site of citrate excreting strains may be unusually sensitive to some factors in the growth medium normally used to induce citrate accumulation. It is a common experience that addition of ferricyanide, for instance, to fermentation broth (Martin and Waters, 1952; Steel et al., 1954, 1955; Martin, 1955) leads to an accumulation of large excess of citrate. That there are strain differences in the nature of NAD-specific dehydrogenase can be readily
seen from our results. The enzyme of *A. flavus*, for instance, is extremely labile as compared with that of *A. niger*. It is conceivable that the allosteric site of the citrate secreting strain, *A. niger*, is easily 'densensitized' (or becomes inoperative) by some unknown factors in the growth medium (possibly ferricyanide or just iron) so that citrate is unable to activate the enzyme *in vivo*. It is one of the characteristic properties of allosteric enzymes, like aspartate transcarbamylase (Gerhart and Pardee, 1962) and threonine deaminase (Changeux, 1962) that the allosteric site on the enzyme surface is 'fragile' and can easily be 'densensitized' by treatment with heavy metal ions, urea, mild heat treatment, and thiol reagents. All our attempts at desensitization of the enzymes from Aspergilli failed owing to the extreme lability of the isocitric dehydrogenases. Further work remains to be done, but the suggestion that citrate accumulation may be due to 'densensitization' of the allosteric site of isocitric dehydrogenase by environmental factors remains indeed an attractive possibility.
REFERENCES


