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To the Committee on Graduate Study:

I am submitting to you a dissertation written by John Francis Christman entitled "The Relationship between Biotin and the Coenzyme of Aspartic Acid, Serine and Threonine Deaminases." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Bacteriology.

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THE RELATIONSHIP OF BIOTIN WITH THE COENZYME OF ASPARTIC ACID, SERINE AND THREONINE DEAMINASES

A DISSERTATION

Submitted to
The Committee on Graduate Study
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

---

by

John Francis Christman

June 1950

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CHAPTER I

HISTORICAL INTRODUCTION

A. Metabolism of Bacterial Cells

In any study of enzymatic action in the living cell there can be no limited definition of the influences measured, since there are numerous interrelated chemical reactions, both assimilative and dissimilative, which are in a constant state of equilibrium. This circumstance has been adequately described by Dixon (1949) who states that the living cell exists as a system of unstable catalysts which in turn exist because of the occurrence of the reactions which they catalyze. Each enzyme does not maintain itself; it is rather a collective effort by a certain minimum number of enzymes which brings about the necessary series of reactions for resynthesis. Thus it becomes necessary to study enzymatic reactions as a means of viewing life processes, but at the same time, it remains essential to study these reactions, not only as isolated crystalline moieties, but also as a particular link in a large chain of reactions.

Kluyver (1931), upon the introduction of the concept of comparative biochemistry, created a scientific attitude that the basic enzymatic mechanisms of all cells are similar, regardless of the origin of the cell. The microbiol-

ogist, then, is justified in the study of the nutrition and metabolism of a microorganism since this knowledge readily adapts itself to the nutrition and metabolism of the higher plants and animals. In the last fifteen years the microbiologist, by taking advantage of the principle of comparative biochemistry, has demonstrated a number of vitamins and enzymatic systems important to both microbial and higher forms of life. In the same vein the microbiologist has made major contributions in the study of photosynthesis and the genetic effects of radiations. The investigator of enzymatic pathways is benefited considerably by the use of microorganisms because of the inexpensive, easily reproducible, rapidly multiplying nature of the cell source.

The early observations of cellular metabolism in bacteria were obtained by chemical analyses of the products of putrefaction of unidentified media by mixed cultures of organisms. Although this work revealed the nature of some of the compounds produced by biological action, it was essentially impossible to attain reproducibility of results. One step towards control was the use of pure cultures of bacteria; later, chemically defined media were introduced. These modifications of the earlier techniques proved valuable in the determination of many metabolic products. However, this was a meager picture of true metabolism, for as Gale (1940) pointed out, "We may have recorded as products of . . . metabolism, substances that are really produced in stages at

different times during the incubation period, under different environmental conditions and by what are, biochemically speaking, different organisms."

Quastel and Whetham (1924, 1925a,b) introduced the use of washed cell suspensions of microorganisms, which served as concentrates of active enzyme preparations stable over a period of several hours. This resting cell technique has been used extensively during the last twenty-five years in conjunction with manometric, colorimetric and other techniques of biochemistry in the study of relative rates and the quantitative nature of bacterial enzyme attack. The optimum conditions under which the enzymes involved are active and the conditions under which they are formed can be measured easily. Despite some native limitations, such as restriction and action by other enzymes present in the cell, permeability of the cell membrane to the enzyme substrate, etc., this technique, particularly as advanced by the Cambridge school, has produced some of the most important results in bacterial metabolism.

A specific account of the properties of any enzyme can come only by observation away from the intact cell. However, until the wet-crushing mill was developed (Booth and Green, 1938) there was no way of obtaining intracellular bacterial enzymes in a cell free state. A number of bacterial enzymes have been studied in this manner. For the majority of enzyme studies cell-free extracts are not nearly

so desirable as one might expect, since in the destruction of cell structure, many enzymes are destroyed and many others are rendered inactive. For those enzyme systems that are stable to vacuum drying of washed cell suspensions, this technique is preferred because permeability of the cell membrane is increased over that of the normal wet cell. Up to the present time there has been no report of the successful crystallization of a bacterial enzyme, and in this respect the microbiologist is handicapped.

Elsden and Pirie (1949), in their obituary of Dr. Marjory Stephenson, stated that at the inaugural meeting of the Society for General Microbiology Dr. Stephenson

> . . . analysed the steps in the development of research in the field of bacterial metabolism, and pointed out that research took place at a series of levels. At the first level the worker was concerned with mixed cultures; at the second with pure cultures growing in complex media; at the third with pure cultures growing in a chemically defined media; at the fourth with washed cell suspensions from pure cultures; and finally at the fifth level with cell-free enzyme preparations. She concluded that no one level was, by itself, adequate; and for an understanding of bacteria as they are found in Nature, research must occur at all levels.

B. Metabolism of the Amino Acids

The metabolism of amino acids long has been a subject of investigation by the biochemist since analysis of proteins revealed that these chemical compounds were the apparent starting materials in the formation of the protein mole-

cules. The processes of formation and degradation of the amino acids have been studied in a large number of species of animals and plants, and once again the microbiologist has made significant contributions to the general problem.

Of the infinite number of amino acids whose chemical preparation is theoretically possible, some twenty-five have been found as component parts of proteins. They all are related in spatial configuration to the L-isomer of glyceraldehyde, and hence are referred to as the L-amino acids. These amino acids are components of nearly all cells; in the philosophy of comparative biochemistry cells require all of these amino acids, and if a cell does not need one or more of these materials from an exogenous supply, then it must be capable of synthesizing those which it can apparently "do without."

The majority of animal species probably require exogenous supplies of about ten of the amino acids; green plants obviously require none of them. Bacteria, on the other hand, vary tremendously from species to species, and even from strain to strain, in their amino acid requirements. Some, like *Escherichia coli* and *Aerobacter aerogenes*, are capable of synthesizing all of the essential amino acids from an inorganic nitrogen source, such as ammonium ion, and from carbon-containing residues obtained on the degradation of glucose. Other bacteria, particularly the members of the family *Lactobacteriaceae*, require an out-

side supply of nearly all of the naturally occurring amino acids. Particular use is made of these fastidious bacteria in the microbiological assay of the amino acids, similar to the microbiological assay of the vitamins to be discussed in Chapter II.

In order to understand the pathways of synthesis of the amino acids, the immediate degradations of the amino acids by bacterial enzymes should be considered. If these are typical of most enzymatic reactions they will be reversible, and therefore could lead to synthesis. Five methods of amino acid degradation appear possible:

(1) Removal of the alpha-amino group directly, producing ammonia and an acid: if this reaction is carried out oxidatively, as in the case of glutamic acid, the product will be a keto acid, which is the general rule. This process is referred to as deamination.

(2) Removal of the terminal carboxyl group, producing a primary amine and carbon dioxide; this is referred to as decarboxylation.

(3) Simultaneous decarboxylation and deamination, which results in ammonia, carbon dioxide and a hydrocarbon (with respect to the alpha-carbon atom) as end products.

(4) Degradation of the amino acid into two products, without affecting the alpha-carbon constituents, resulting in a new amino acid.

(5) Transamination of a keto acid (A) and an amino

acid (B) resulting in a keto acid (B') and an amino acid (A').

Of these processes only transamination and deamination have been demonstrated to be reversible, and both of these are limited, by our present knowledge, to a few of the naturally occurring amino acids.

Apparently the cell is capable of synthesizing a few key amino acids, and is then able to produce the remaining amino acids by several types of transaminating and condensing mechanisms. For example, there exist certain mutants of Neurospora which require a pre-formed source of organic nitrogen. Almost any of the amino acids can serve as this source of nitrogen. From the standpoint of single gene mutation studies it would appear that the primary synthetic mechanism has been lost in the mutation, and once this product or a potential precursor of this product is available, the cell is able to grow and reproduce (Fincham, 1950). Yeasts, such as Saccharomyces cerevisiae require just one amino acid, such as aspartic acid, and from this initial source of organic nitrogen, the cells are capable of synthesizing the remainder of the required amino acids (Snell, et al., 1940).

Of the amino acids that can be formed enzymatically from an inorganic nitrogen source, there appear to be three that are prominently involved. These are aspartic acid (Cook and Woolf, 1928; Woolf, 1929; Gale, 1938; Virtanen and

Erkama, 1938; Lichstein and Umbreit, 1947a), glutamic acid (Dewan, 1938; Adler, et al., 1937, 1938) and alanine (Konikova, et al., 1949). The formation and degradation reactions are shown in Figure 1. These three amino acids are directly converted to one another through the transaminases (Cohen, 1942; Herbst, 1944; Green, et al., 1945; Lichstein and Cohen, 1945; Lichstein, Gunsalus and Umbreit, 1945) as shown in Figure 2.

Until recently ammonification or transamination could account for only three amino acids. However, Wood and Gunsalus (1950) have discovered the existence of several other transaminases in bacteria. They found that the amino acids which are active in transferring the amino group to alpha-ketoglutarate include valine, leucine, isoleucine, norleucine, methionine, tryptophane, tyrosine, phenylalanine, histidine and lysine. The tyrosine and phenylalanine transaminases have been shown to be reversible. Apparently much of the difficulty can be ascribed to technical problems, rather than to the actual lack of the transaminating enzymes in the cell.

C. Metabolism of Aspartic Acid

Since aspartic acid is reversibly deaminated in many cell systems, and apparently undergoes transamination readily, considerable interest has been centered on the metabol-

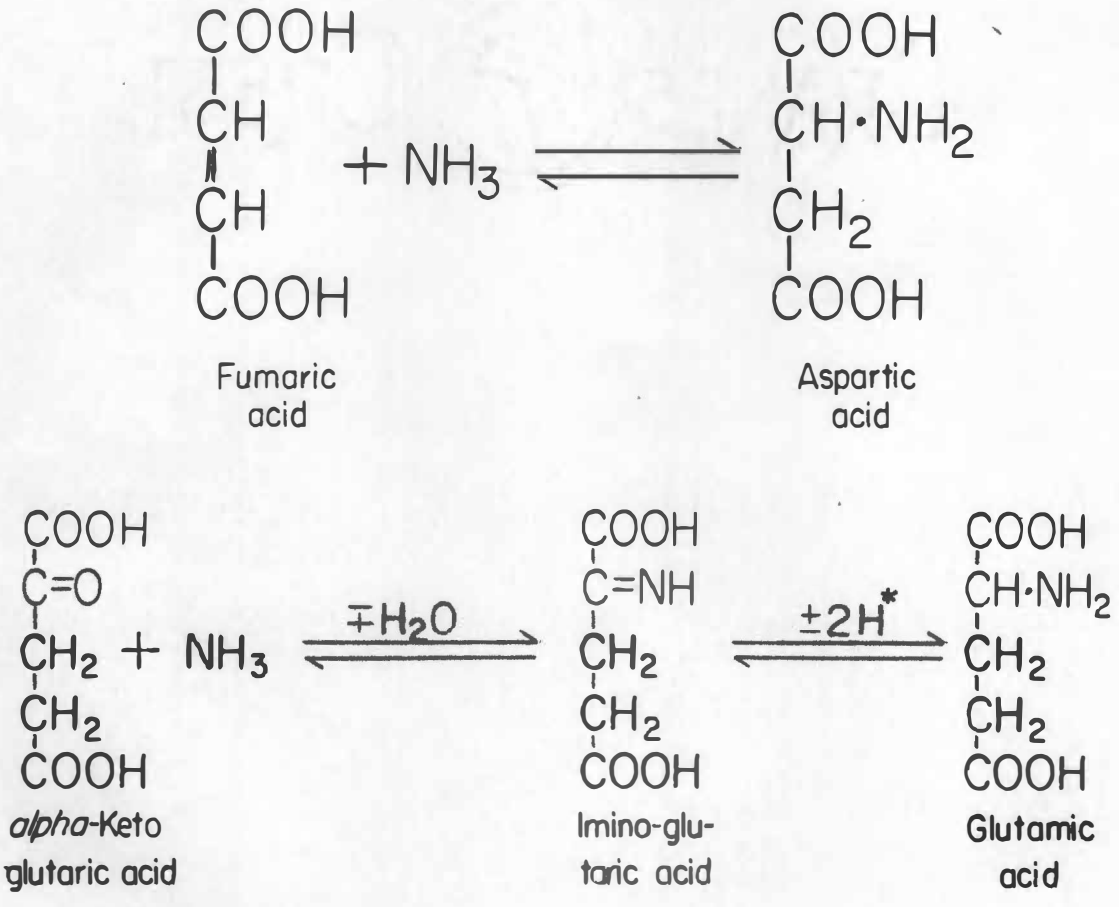
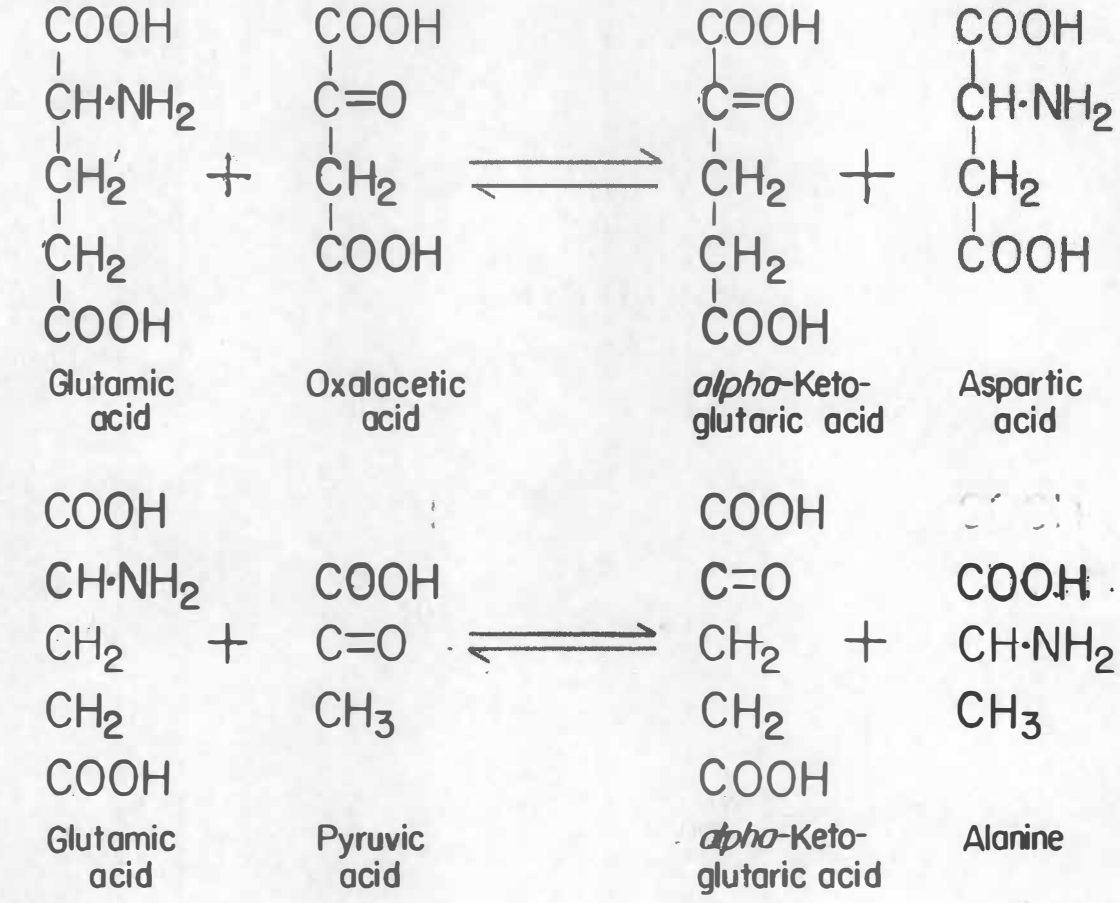


Figure I: Direct enzymatic ammonification of organic acids to produce aspartic acid, glutamic acid and alanine

TRANSAMINATION REACTIONS:



TRANSAMINASE CYCLE:

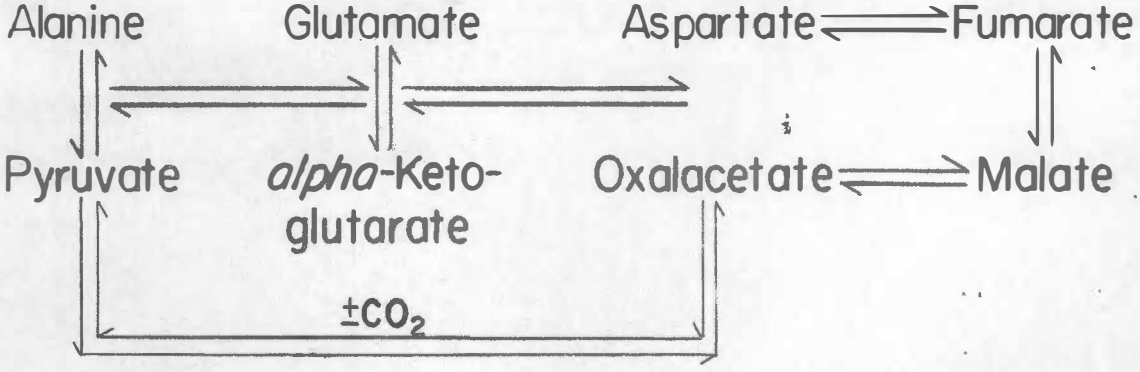


Figure 2: The transamination reactions and the transaminase cycle

ism of this amino acid. Much of the early data concerning the degradation of aspartic acid are confused and contradictory.

Aspartic acid deaminase, often referred to by British workers as aspartase, was first demonstrated by Harden (1901) who showed that E. coli growing in a glucose broth with added aspartic acid produced large quantities of succinic acid. Quastel and Woolf (1926) showed that in the absence of an inhibitor, washed cell suspensions of E. coli produced succinic acid from aspartic acid, but that in the presence of an inhibitor, such as toluene, an equilibrium mixture resulted containing aspartic acid, fumaric acid and ammonia. Woolf (1929) later showed that this reaction was not quite so simple in inhibited cell suspensions, and that actually the equilibrium mixture also contained malic acid. By using a series of inhibitors which were specific for certain reaction sites, he was able to show that the primary product of deamination of aspartic acid was fumaric acid, which in the presence of the enzyme fumarase was converted to malic acid; if a hydrogen donor was present, succinic acid was formed, enzymatically catalysed by succinic dehydrogenase.

Gale (1938) rather confused the picture by fractionation of aspartic acid deaminase from cell-free extracts of E. coli. By a study of the loss and recovery of activity of these fractionated enzymes he discovered that the rate of

deamination of one of the two fractions was greatly increased by the addition of adenosine or inosine. He concluded that one enzyme (aspartase I) was stable to incubation with toluene, and was unaffected by the presence of adenosine; the second enzyme (aspartase II) was inactive in the absence of some coenzyme which could be replaced *in vitro* by adenosine, and completely inactivated by toluene treatment. Both enzymes were optimally active at pH 7.5. Aspartase I contained no fumarase, and produced fumaric acid and ammonia from aspartic acid; aspartase II contained some active fumarase, and produced a mixture of fumaric and malic acids and ammonia from an aspartic acid substrate.

Lichstein and Umbreit (1947a) studied the aspartic acid system and were able to demonstrate that vacuum dried cells of *E. coli* produced fumaric acid from aspartic acid. The enzyme malic dehydrogenase was absent in these cells, and the enzyme fumarase was either absent or very weak.

Although the two-enzyme theory as presented by Gale (1938) apparently conflicts with the other data, evidence will be presented later which indicates that in all probability instead of isolating two enzymes, Gale actually isolated the apo-enzyme which could then be reactivated by the coenzyme, or coenzyme precursor. The work of Virtanen and Erkama (1938) can also be explained on this premise.

Thus from the data available it would appear that the deamination of aspartic acid proceeds through fumaric acid,

and the fate of the fumaric acid is dependent upon the conditions in the cell. The aspartic acid cycle of E. coli Gratia is presented diagramatically in Figure 3 (Lichstein and Umbreit, 1947a).

D. The Deaminases of Serine and Threonine

Gale and Stephenson (1938) studied the serine deaminase system in E. coli. They showed that although the activity of this enzyme is very high in washed cell suspensions, it will decrease rapidly on standing. The activity can be restored by the addition of boiled cells, glutathione or formate ion, all in the presence of phosphate ion; phosphate ion alone will cause some reactivation. Ammonia production was used as a measure of deamination. Further consideration will be made of these data later in this chapter.

Chargaff and Sprinson investigated the mechanism of deamination of serine (1943a) and of serine and threonine (1943b) and concluded that the deamination occurs as presented in Figure 4. They reached this conclusion by measuring the effects of washed cell suspensions of E. coli on the two amino acids and their O-substituted derivatives. By blocking the hydroxyl group they successfully prevented deamination both aerobically and anaerobically. Good yields of the end products varying from 10 to 40 per cent of the theoretical, were obtained from the unsubstituted amino acids.

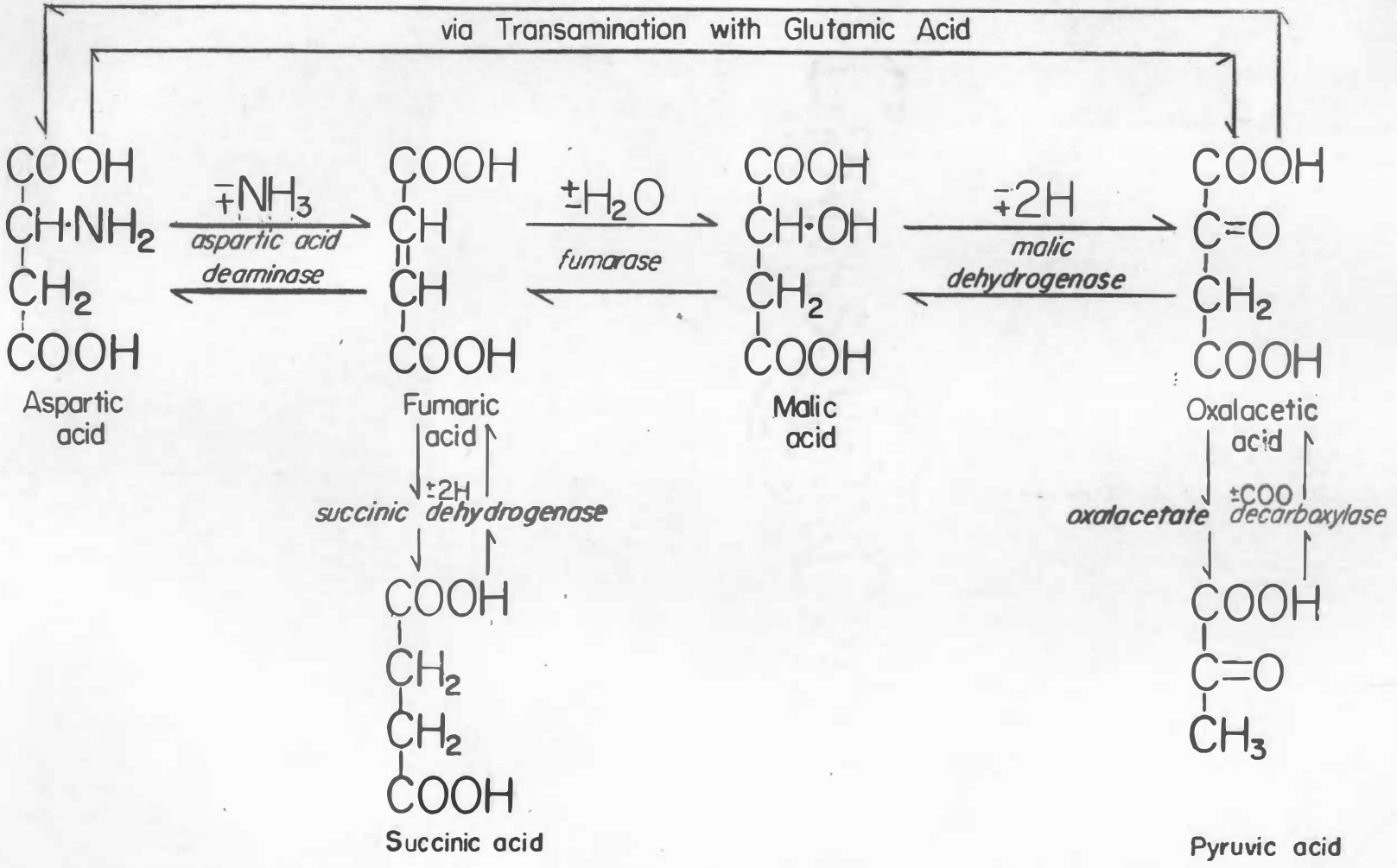


Figure 3: The aspartic acid cycle of *E. coli* Gratia (Lichstein and Umbreit, 1947a)

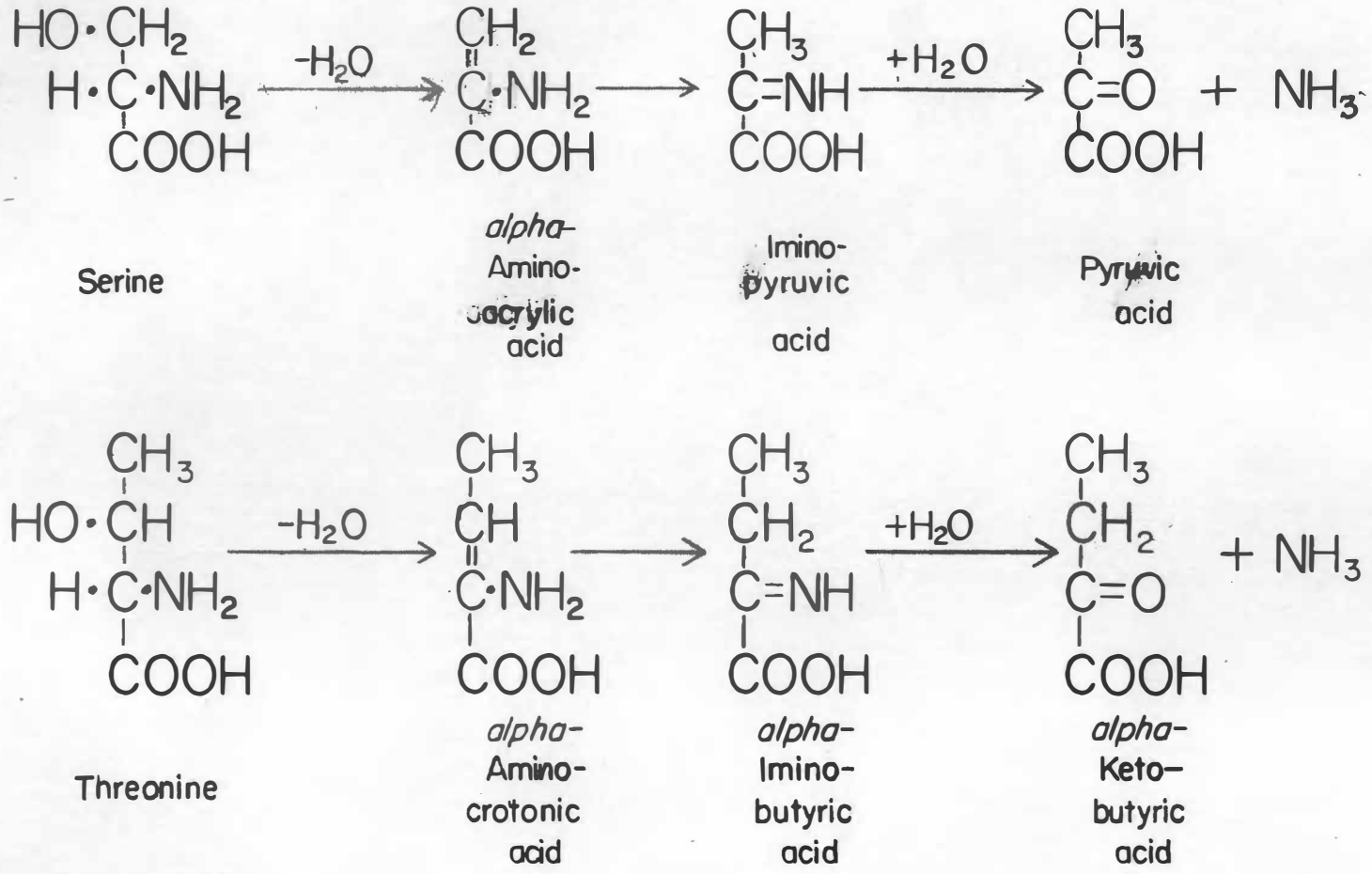


Figure 4: The enzymatic mechanisms of serine and threonine deaminases (non-reversible)

Wood and Gunsalus (1949) in a cell-free study of these deaminases stated that

> . . . since serine and threonine deaminases occurred in the extracts (of bacterial cells under fractionation) in approximately the same ratio as in the dried cells, were activated by the same concentrations of adenylic acid and glutathione, and threonine deaminase disappeared when serine deaminase was inactivated. . . (then there is the suggestion that) . . . both substrates may be activated by a single enzyme.

However, this "same ratio," which was to remain constant, varied between 0.77 and 1.42 for the various enzyme fractions. It would appear that rather than the same enzyme being responsible for the two reactions, the same coenzyme maybe activating different enzymes.

Unpublished data of Christman (1948) and Cardella (1949) show that some organisms, particularly A. aerogenes contain a potent serine deaminase, while a threonine deaminase was not demonstrable. For the most part the minimal pH of activity for serine deaminase is five, while threonine deaminase is inactive at pH 5, and is active at pH 6 and higher. From these data it would appear that Wood and Gunsalus (1949) should have declared that the enzymes were different, while the activators were the same. Data are presented in Chapter III which indicate more clearly that the coenzyme for these reactions is the same.

E. Growth Factors Involved in Aspartic Acid, Serine and Threonine Metabolism

In 1942 Koser, Wright and Dorfman (1942) reported that the yeast, Torula cremoris, required biotin for growth, and that this need for biotin could be greatly reduced when aspartic acid was added to the medium. They pointed out that aspartic acid did not completely replace the biotin requirement, and concluded that biotin must play not only some role in the metabolism of aspartic acid, but also some additional role in the metabolism of the cell.

Winsler, Burk and duVigneaud (1944) showed that biotin deficient yeast cells could be stimulated to assimilate ammonia on the addition of biotin, but they came to no conclusions as to which reaction or reactions were being affected.

Stokes, Larsen and Gunness (1947a,b) showed that in a variety of biotin requiring organisms such as Lactobacillus arabinosus and Streptococcus faecalis biotin could substitute completely for aspartic acid. They showed that the biotin-aspartic acid relationship was specific: riboflavin, pantothenic acid, thiamine, para-aminobenzoic acid and pyridoxamine could not replace biotin and that fourteen other essential amino acids could not be replaced by biotin. They elaborated several mechanisms as possibilities for the locus of biotin activity as follows:

(1) mechanisms employing transamination:

(a) glutamic acid plus oxalacetic acid to yield aspartic acid and alpha-ketoglutaric acid

(b) alanine plus oxalacetic acid to yield pyruvic and aspartic acids

(c) cysteic acid plus oxalacetic acid to yield aspartic acid and alpha-keto-beta-sulfopropionic acid (sulfopyruvic acid)

(2) mechanism involving ammonia incorporation: fumaric acid plus ammonia to yield aspartic acid

(3) mechanism concerned in oxalacetic acid formation: pyruvic acid plus carbon dioxide to yield oxalacetic acid which in turn could be converted to aspartic acid by either mechanism (1) or (2).

In their second paper, Stokes, et al., (1947b) examined these possibilities to determine if the locus of biotin activity could be placed in one or more of these roles. It appeared from these data that there was no influence of biotin on the transaminases; since Lichstein, et al., (1945) showed that pyridoxal phosphate was the coenzyme of the bacterial transaminases, it would have been strange if biotin would have been found to be active in these mechanisms.

They attempted to demonstrate the involvement of biotin in the deaminase reaction (reaction 2) and could obtain no conclusive data. They recognized, however, that the lactic acid organisms with which they were working did not con-

tain this particular enzyme, and thus they did not eliminate this as a possible site of activity.

They were able to show that oxalacetic acid replaced biotin to some extent for cell growth and concluded that biotin was not concerned in the transaminases, and that biotin might be concerned in either or both of the other two reactions.

Four laboratories were able to demonstrate that biotin was concerned in oxalacetic acid decarboxylase by means of four very widely varied techniques, all within a month of one another in 1947; this occurrence is probably unparalleled in metabolic studies. In chronological order Lardy, et al., used a medium deficient in both biotin and aspartic acid and showed that the growth of L. arabinosus could be stimulated by the addition of oxalacetic acid or bicarbonate ion. Shive and Rogers (1947) used inhibition analysis techniques and demonstrated that the inhibition caused by an analogue of biotin on the growth of E. coli could be overcome by the addition of alpha-ketoglutaric acid. This observation, along with the elusive and still unpublished work of Garrison and Eakin, led these authors to conclude that the site of biotin activity was in the carbon dioxide fixation reaction with pyruvic acid.

Lichstein and Umbreit (1947a) used resting cells of E. coli, which were grown on a complex medium containing biotin. These cells, when harvested, were "aged" in one

molar phosphate buffer for a short time; after "aging" the cells had lost the ability to produce carbon dioxide from added aspartic acid, and this lost activity could be specifically restored by added biotin. Oxalacetic acid or malic acid could be substituted for the aspartic acid. The "aging" technique was novel, at least as far as biotin metabolism was concerned, and will be discussed in greater detail later. The authors concluded that biotin must somehow be concerned as the coenzyme of oxalacetic acid decarboxylase.

Ochoa, et al., (1947) used the more time-honored method of producing vitamin deficiency, by growing turkeys on a biotin deficient diet. They were able to show that the malic dehydrogenase and oxalacetic acid decarboxylase activities were both markedly decreased in these deficient cells and concluded that biotin was somehow concerned in this latter reaction.

Although all four groups used different techniques, each concluded that biotin was concerned in this one reaction. This not only adds weight to the fact that biotin must be concerned with the reaction, but also that all four techniques can, and do, give similar results under proper treatment.

Lichstein and Umbreit (1947b) used the "aging" technique and presented preliminary evidence that biotin was also concerned in the deaminases of aspartic acid, serine and threonine. Later Lichstein and Christman (1948) were able

to demonstrate the "aging" phenomenon in a variety of organisms such as Proteus vulgaris, Bacterium cadaveris, A. aerogenes and several different strains of E. coli under limited conditions. They presented data from which they concluded that:

(1) biotin was definitely concerned with aspartic acid, serine and threonine deaminases;

(2) adenylic acid was also involved in these reactions;

(3) both biotin and adenylic acid were involved in the reversal of aspartic acid deamination. i.e. the reaction of fumaric acid with ammonia to form aspartic acid;

(4) at pH 7 numerous biological materials caused stimulation of the aged cells, but at pH 4 only biotin and adenylic acid caused stimulation; and

(5) the effects of biotin and adenylic acid were independent, and occasionally additive.

Lichstein (1949a) later found that occasionally systems could be obtained, both by aging and by cell-free preparations from dried cells, that could be stimulated by biotin and adenylic acid together and also by yeast extract; on refrigeration of these systems the activity caused by biotin with adenylic acid was lost, whereas the yeast extract still caused marked activation of the aged cells. This evidence, coupled with the observed hundred-fold difference in the

minimal concentration of yeast extract that caused activation of the aged cells and the minimal concentration of yeast extract that would support the growth of S. cerevisiae in a biotin deficient medium, led him to express the opinion that a biotin-containing preformed coenzyme of aspartic acid deaminase exists in yeast extract and that adenylic acid is somehow concerned in its formation. Lichstein and Christman (1949) presented confirmatory evidence for these observations and this work will be presented in detail in Chapter III.

Wright, et al., (1949) confirmed the findings of Lichstein and Umbreit (1947b) and Lichstein and Christman (1948, 1949) when they announced that they had been successful in being able to age cells of E. coli, and restore the activity of aspartic acid deaminase specifically with biotin.

F. Aging as a Technique of Obtaining an Apoenzyme

The problem of obtaining an apoenzyme is difficult in that no one method is certain of producing positive results. There are several techniques which have been employed such as the growth of the organism in a medium deficient in a particular vitamin (Gunsalus, et al., 1944a,b), the use of biochemical mutants (Beadle, 1946, 1948), the use of autolysis, the use of metabolite inhibitors (Shive, 1946), growth replacement studies (Koser, et al., 1942) and finally

that of aging. It is this last technique with which we are concerned.

Aging differs from normal autolysis or dialysis in that the vitamin in question is apparently destroyed or degraded to a useless form, either with or without the need of an enzyme. The vitamin after aging can not be detected either in the cell or the supernatant aging fluid. The first mention of aging was made by Gale and Stephenson (1938) and Gale (1938) who apparently degraded aspartic acid and serine deaminases. They were able to restore activity with adenylic acid and adenosine, and since these substances were shown to be involved in the same reactions by Lichstein and Christman (1948), it would appear that these later materials were somehow concerned with the coenzyme, and the protein that was activated was at least a partially resolved enzyme system.

Lichstein and Umbreit (1947a) introduced the term "aging" for the process of holding bacterial cell suspensions in molar one phosphate buffer at pH 4 for a short period of time. This process effectively decreased the activity of oxalacetic acid decarboxylase, and this activity could be restored specifically with biotin. Lichstein and Umbreit (1947b) extended the aging technique to include the deaminases of aspartic acid, serine and threonine, which were also activated with biotin. Lichstein and Christman (1948) extended phosphate aging to include adenylic acid, and later

Lichstein (1949a) showed that a coenzyme form which probably contained biotin existed in yeast extract.

Considerable difficulty was noted in the attempted reproduction of the data of Lichstein, et al., (1947a,b, 1948, 1949). Most of these difficulties were reported by means of private communications; however, Axelrod, et al., (1948) announced that although they could definitely decrease activity of cell suspensions with phosphate at pH 4, they were not able to replace that activity with biotin. Lichstein (1949b) showed that at least part of the difficulty encountered by the other group was in the use of certain different materials in the growth medium, and although definite conditions are still to be established, it would appear that these difficulties are merely technical. At least one laboratory (Wright, et al., 1949) has reported the successful demonstration of aging and reactivation with biotin, and at least five different individuals working in Lichstein's laboratory have been able to reproduce this effect.

Aging with respect to folic acid, has been carried out in at least three laboratories. Nimmo-Smith, Lascelles, and Woods (1948) showed that cell suspensions of Streptobacterium (Lactobacillus) plantarum rapidly resynthesized this vitamin when para-aminobenzoic acid was added. This work was reproduced, at least in part, by Lichstein and White (1950).

From the evidence that has accumulated in the last three years concerning aging, it will have to be placed among the other techniques as a valid method for the production of a stable apoenzyme.

G. Bound Forms of Biotin as Potential Coenzymatic Forms of Biotin

Since the introduction of a method of biotin assay by Snell, Eakin and Williams (1940) a considerable number of investigators have studied the problem of biotin content of natural materials. Snell, et al., used S. cerevisiae as their assay organism, and with their techniques were able to assay for biotin in the range of 25 to 150 micromicrograms (10^{-12} g.). They demonstrated the presence of biotin in a variety of natural materials, nearly all of which have been substantiated by later investigators. A year later Thompson, Eakin and Williams (1941), used L. casei as an assay organism in a study of hydrolysates of natural tissues both for biotin content and for degree of biotin liberation. They recommended 6 N sulfuric acid for two hours at 121 C as the ideal hydrolysing conditions, and concluded their paper with the comment: "Biotin appears to occur naturally in different combinations which are broken down with varying degrees of ease."

Lampen, Bahler and Peterson (1942) studied the occur-

rence of free and bound forms of biotin in various materials and they showed that organisms responded differently to these materials with regard to biotin content. They showed also that while yeast extracts were slowly inactivated with respect to biotin content on hydrolysis in 4N sulfuric acid, there was no corresponding destruction in liver extracts. They showed also that there was some substance in both yeast and liver extracts which was extremely resistant to acid hydrolysis.

Hertz (1943) modified the medium of Snell, et al., to increase the sensitivity to biotin and bound biotin forms.

Wright and Skeggs (1944) used L. arabinosus as an assay organism for biotin and showed a difference in the level of biotin concentration using the three assay organisms. L. casei and S. cerevisiae both gave approximately the same biotin values in the assay of natural materials, but L. arabinosus gave values that were distinctly lower. On acid hydrolysis all three organisms gave about the same values showing that there must be some acid labile substance present in yeast extract which is assayable (available for growth) by L. casei and S. cerevisiae but which is unavailable for the growth of L. arabinosus. Wright, et al., (1949) isolated this material, named biocytin, in crystalline form from yeast extract; they stated that biocytin was the only bound form of biotin which they could demonstrate in yeast extract. They reported some activity of this sub-

stance toward the partially resolved aspartic acid deaminase; however, Lichstein, Christman and Boyd (1950) presented conclusive evidence that biocytin was different from their concentrates of yeast extract which had coenzyme activity.

Bowden and Peterson (1949) studied the conditions for hydrolyses of bound biotin in liver and yeast extracts, and although their data confirm, at least in part, the data of Wright and Skeggs (1944), they found also that considerable biotin destruction occurred on autoclaving samples in sulfuric acid in concentrations as low as 2 N.

Plaut and Lardy (1949) assayed the biotin content of purified enzymes in which biotin apparently functions. One study with oxalacetate decarboxylase showed that the biotin content of this enzyme decreased on increased purification. Since they did not hydrolyse the enzyme preparations, the biotin form of the coenzyme, if it exists, might not be assayable.

CHAPTER II

EXPERIMENTAL METHODS

A. Materials Used

1. Cultures of Microorganisms

The following strains of microorganisms were employed: Bacterium cadaveris (Gale), isolated by Dr. E. F. Gale of Cambridge, England, apparently identical with B. cadaveris ATCC 9760 and B. cadaveris (Gale) NCTC 6578; Saccharomyces cerevisiae (139), the Fleischmann Laboratories strain, apparently identical with S. cerevisiae (Hansen) ATCC 9896; Saccharomyces cerevisiae (Java) apparently the same strain as S. cerevisiae (Hansen) ATCC 4125.

2. Reagents Used

a. Chemicals. All standard chemicals used were of the grade referred to as chemically pure. The synthetic vitamins and amino acids employed had been tested for physiological activity by the manufacturer. The biotin was the free acid, designated D-biotinic acid, prepared synthetically by Hoffman-LaRoche and Co., Nutley, New Jersey.

b. Natural products. The yeast extract was prepared by Difco Laboratories, Inc., and bore one of the following lot numbers: 395778, 402218, 402560, 402561, 402562, 402563 and 402564. The liver extracts were graciously supplied by

Eli Lilly and Co., Indianapolis, Indiana under one of the following lot numbers: E-1417 and E-1418. E-1418 is that portion of a hot water extract of liver which is soluble in 70 per cent ethyl alcohol; E-1417 is somewhat purer.

The adenylic acid was the adenosine-5-phosphoric acid kindly supplied by the Ernst Bischoff Co., Ivoryton, Connecticut.

B. Preparation of Aged Cell Suspensions

1. Maintainence of Stock Cultures

Stock cultures of B. cadaveris were maintained on slants with the following composition: 1 per cent yeast extract, 1 per cent tryptone, 0.5 per cent monopotassium phosphate and 2 per cent agar. The slant was streaked, incubated for twenty-four hours at 30 C and stored at 4-6 C; they were transferred at intervals of six weeks; each six months they were plated out and checked for purity from discrete colony isolates.

Stock cultures of the strains of S. cerevisiae were maintained on a medium of the following composition: 1 per cent yeast extract, 1 per cent glucose and 2 per cent agar. Before sterilization the medium was adjusted to pH 5 with concentrated sulfuric acid. The slants were streaked, incubated for forty-eight hours at 30 C and stored at 4-6 C; the cultures were transferred to fresh slants at three month

intervals, and were checked for purity from single colony isolates every eighteen months.

2. Medium

The organism used in the preparation of the cell suspension was grown in the following broth medium: 1 per cent yeast extract, 1 per cent tryptone, and 0.5 per cent monopotassium phosphate. This medium is referred to as A-C medium.

3. Preparation of the Inoculum

The inoculum was prepared by removing a small quantity of bacterial growth from the stock culture slant and placing it in a 15 X 150 mm. test tube containing 8 ml. of A-C broth. This culture was incubated for twenty-four hours at 30 C, after which it was sub-cultured by removing one loopful to a fresh tube of broth. After five serial subcultures the organism was considered to be physiologically active; during routine work daily transfers were made of the organisms in order that fresh cultures were always available.

4. Growth of the Culture in Large Quantities

A 500 ml. Erlenmeyer flask containing 300 ml. of A-C medium was inoculated with 1 ml. of a twenty-four hour culture of the organism used. The flask was incubated for twelve to sixteen hours at 30 C after thorough mixing of the

inoculum by gentle swirling. When large quantities of cells were desired, two or more flasks of cells were prepared.

5. Harvesting the Culture

After incubation the culture flask was removed from the incubator, swirled to insure good suspension of the cells, and centrifuged in a Servall Angle Head centrifuge at 1800 g. for twenty minutes. The supernatant was carefully decanted; about 50 ml. of distilled water was added to one of the bottles and vigorously shaken to assure complete suspension of the packed cells. This suspension was then added to the second bottle, and again the mixture was shaken; the resulting suspension was diluted with an additional 150 ml. of water, and then recentrifuged at 1800 g. for twenty minutes. The resulting supernatant was discarded, and the cells then aged.

6. Aging the Cell Suspension

About 10 ml. of one M phosphate buffer, pH 4.00 ± 0.05, was added to the centrifuge bottle containing the washed cells; the suspension was thoroughly mixed and aerated by drawing the material into a 10 ml. pipette and rapidly discharging the solution against the bottle wall. The mixture was allowed to stand for thirty to sixty minutes at room temperature (22-27 C) with occasional shaking to insure complete suspension of cells. Although this procedure did not always give reproducible results, it invariably led to

some degradation of the enzyme system, and activity of the coenzyme could be measured. Biotin activity, on the other hand, was very often either slight or negligible.

C. Method of Coenzyme Assay

Aged cell suspensions of B. cadaveris were used in the coenzyme assay. Reaction vessels were 12 X 100 mm. Pyrex test tubes to which the following materials were added in order: phosphate buffer, water, amino acid, biotin, adenylic acid, yeast or liver extract standard and yeast or liver extract fractions to be assayed. When the proper additions had been carried out, the tubes were placed in a large water bath at 37.0 ± 0.5 C and allowed to equilibrate temperature for at least ten minutes. The cell suspension, previously aged, was added, and the tubes thoroughly shaken individually, insuring a nearly constant state of oxygen tension. After suitable incubation, ranging from thirty to one hundred and twenty minutes, the cell activity was stopped by the addition of one-tenth volume of 2 molal trichloroacetic acid. The suspensions were again shaken individually, and centrifuged at 1800 g. for twenty minutes. Following centrifugation, an aliquot of the supernatant was analyzed for ammonia content. Table I presents an outline of a typical assay for coenzyme activity.

TABLE I

TYPICAL EXPERIMENT MASTER SHEET

Reagent	Volume in ml. added Tube No.							
	1	2	3	4	5	6	7	8
Cells; 0.5 mg. N/ml.; pH 4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Buffer; 1 M; pH 4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Substrate; 0.1 M; pH 5	---	---	---	---	0.1	0.1	0.1	0.1
Biotin; 0.1 µg. per ml.; pH 5	---	0.1	---	---	---	0.1	---	---
Adenylic acid 1 mg./ml.; pH 5	---	0.1	---	---	---	0.1	---	---
Yeast extract standard; 10 mg./ml. pH 6.5	---	---	0.1	---	---	---	0.1	---
Yeast extract fraction; 10 mg./ml. pH 6.5	---	---	---	0.1	---	---	---	0.1
Water	0.8	0.6	0.7	0.7	0.7	0.5	0.6	0.6
Total volume	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

D. Analysis for Ammonia Nitrogen

1. Preparation of Nessler Reagent

The method used for the ammonia analyses (Umbreit, Burris and Stauffer (1945) is specific for ammonium ion, with slight interferences by tetravalent organic ammonium ions. The principle involved is that of the Nessler reaction in the formation of a colored mercuri-ammonium complex. The reagent was prepared by grinding ten grams of potassium iodide in a mortar to a very fine powder to prevent difficulties in dissolving the material. Five grams of mercuric iodide was added and the mixture ground to a light red powder. Fifteen milliliters of water was added to the mortar; the resulting solution was a light green color. This solution was washed into a flask and diluted to 1000 ml.

A 1.5 per cent gum arabic, gum ghatti or gum acacia solution was prepared by placing 6 g. of the powered gum in a mortar and adding a small amount of water. This was vigorously ground to a thick paste and diluted to 400 ml.

To prepare the reagent 1000 ml. of the potassium iodide-mercuric iodide solution was mixed with 320 ml. of the gum solution, and diluted to 3000 ml. If prepared correctly, no color was discernible, and no precipitate formed on standing. The solution stored in a brown stoppered bottle or in the dark was stable for several months.

2. Analysis of the Samples

The following additions were made to a series of 15 X 150 mm. test tubes: 3 ml. of a 10 per cent sodium hydroxide solution, 1 ml. of the sample to be analyzed, 2 ml. of Nessler reagent, and 4 ml. of water. A blank, using water instead of an ammonia sample, served as a zero standard. The solutions were mixed thoroughly by inversion of the capped tubes, allowed to stand for ten minutes, and then read in a Klett-Summerson photoelectric colorimeter equipped with a 4200 A. filter. The same colorimeter tube was used throughout one set of readings; this tube was rinsed with water between each reading. The readings were converted to micrograms of ammonia with a standard curve (Figure 5) which was derived from cumulative data of five separate assays. According to Umbreit, et al., (1945) this technique is sensitive to ± 0.5 micrograms; somewhat better sensitivity is common in routine analysis.

E. Method of Measuring Cell Suspensions

Cell suspensions were standardized turbidimetrically against nitrogen content for convenience. A suspension of cells was prepared as described; the turbidity of a suitable series of dilutions was read with the Klett-Summerson photoelectric colorimeter with the 4200 A. filter. Each of the dilutions was analyzed for nitrogen content by incubating

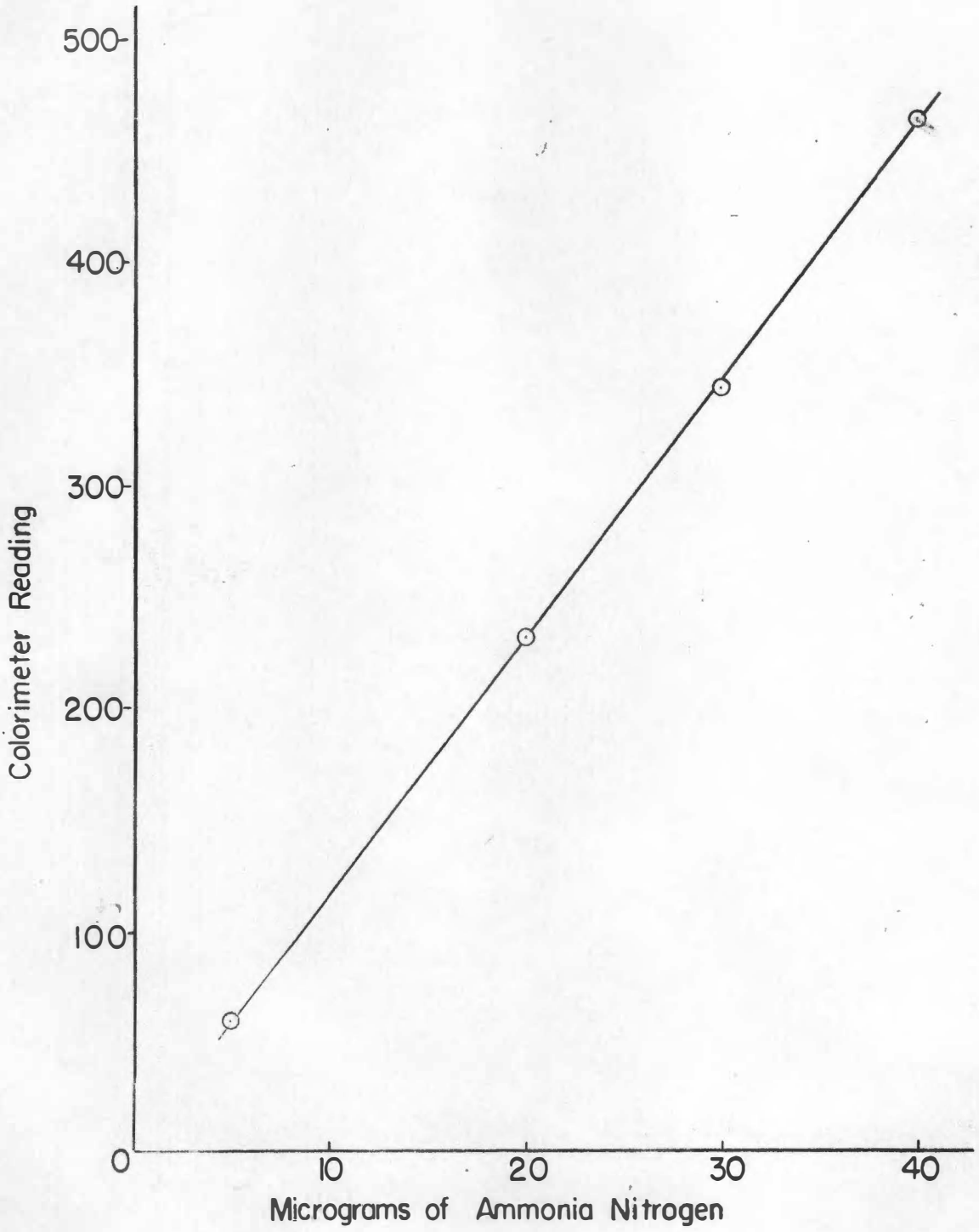


Figure 5: Standard curve for ammonia analyses

1 ml. of the suspension with 1 ml. of concentrated sulfuric acid for eighteen hours at 110 C. At the end of this time two drops of 30 per cent hydrogen peroxide (Superoxal) were added, and the mixture reincubated for four hours at 110 C. The solutions were cooled and made distinctly alkaline with saturated sodium hydroxide solution. Two milliliters of Nessler reagent was added to each tube, the volume was increased to ten milliliters with water, and the tubes were read in the colorimeter. By suitable calculation a standard curve was prepared (Figure 6) which shows the relationship between concentration of nitrogen per milliliter of cell suspension and the turbidity.

F. Microbiological Assay of Biotin

1. Introduction

Although several methods of assay for biotin are available (Snell, et al., 1940; Lampen, et al., 1942; Wright and Skeggs, 1944), each with its particular advantages, the general method of Snell, et al., (1940) was the most practical for our use. This method involves the use of S. cerevisiae as the test organism, and turbidity as the indicator of growth. It is a convenient method from the standpoint of speed of assembly, ease of reading, and the medium is considerably less expensive that the corresponding synthetic media required for the growth of the various strains

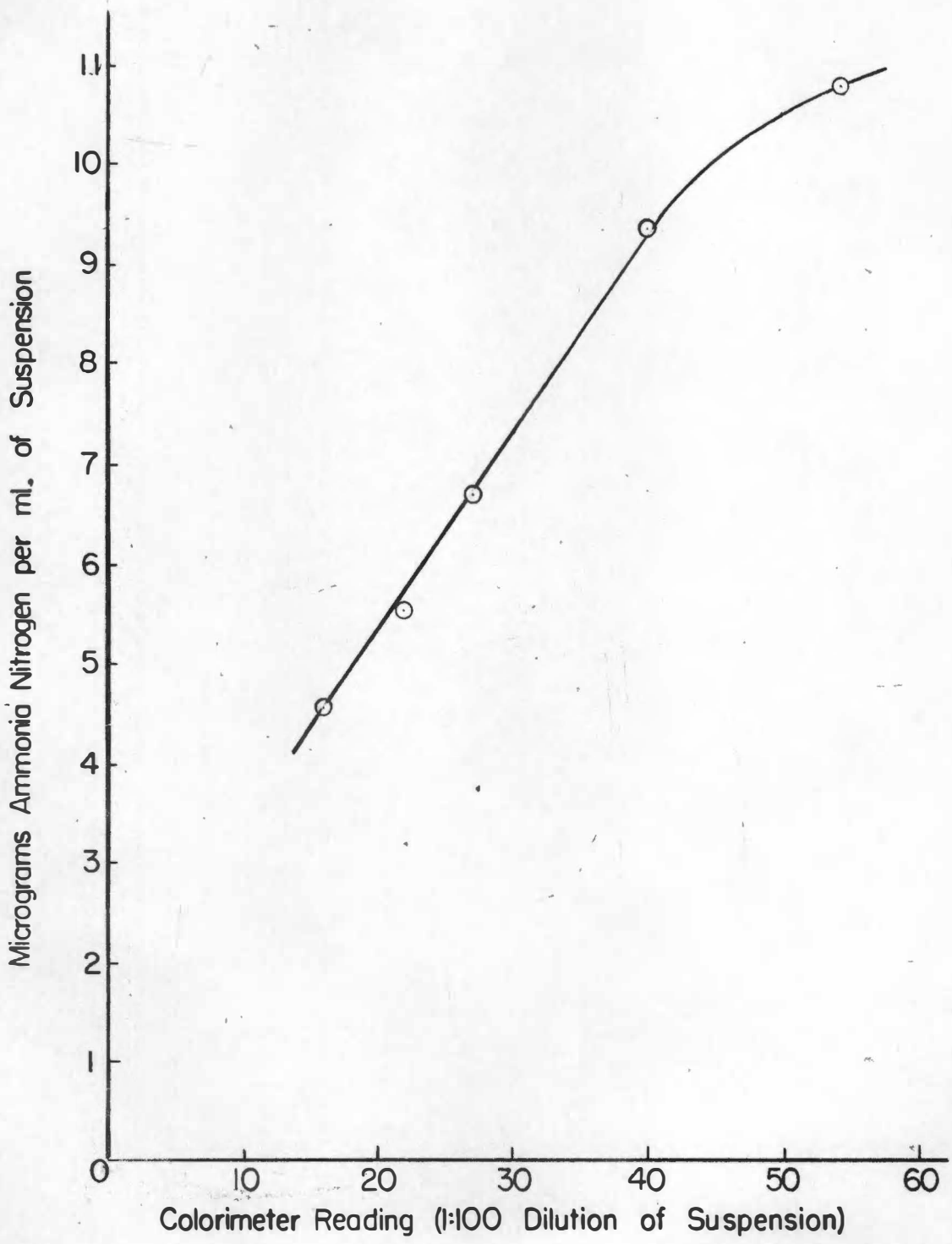


Figure 6: Turbidity of cellular suspension versus nitrogen content

of Lactobacilli. Since few amino acids and vitamins are in the medium only a limited number of organisms are able to grow; therefore, there is no need to use aseptic harvesting techniques, which simplifies the procedure.

2. Medium

The medium of Snell, et al., (1940) was modified considerably during the course of the investigation. Although this medium is extremely simple with respect to the amino acid requirements, it appears to contain the necessary factors for good growth of most strains of S. cerevisiae. The medium most frequently used is given in Table II; the concentration of inositol was changed on several occasions in order to employ the maximum concentration of this material. S. cerevisiae (Java) requires a tremendous amount of inositol for growth; S. cerevisiae (139) on the other hand does not require this factor for growth.

3. Preparation of Inoculum

The inoculum was prepared by subculturing the desired strain of S. cerevisiae in a tube containing about 8 ml. of A-C medium fortified with one drop of 20 per cent glucose solution. This culture was incubated eighteen hours at 30 C. After incubation, the cells were harvested by centrifugation in a 50 ml. plastic centrifuge cup (not sterile) at about 1200 g. for ten minutes. The cells, obtained by decantation of the supernatant, were suspended in 10 ml. of

TABLE II

A SYNTHETIC ASSAY MEDIUM FOR BIOTIN

Sucrose	20.0 g.
Ammonium sulfate	3.0 g.
Monopotassium phosphate	2.0 g.
Inositol	2.0 g.[1]
Calcium chloride dihydrate	0.25 g.
Magnesium sulfate heptahydrate	0.25 g.
DL-Aspartic acid	0.20 g.
Boric acid	1.0 mg.
Zinc sulfate	1.0 mg.
Manganese sulfate	1.0 mg.
beta-Alanine	1.0 mg.
Ferric chloride	0.5 mg.
Cupric sulfate pentahydrate	0.1 mg.
Potassium iodide	0.1 mg.
Nicotinic acid (synthetic)	100 µg.
Pyridoxine hydrochloride (synthetic)	20 µg.
Thiamine hydrochloride (synthetic)	20 µg.
Water	1000 ml.

[1] Occasionally this value was 2.0 mg. instead of 2.0 g.

water and recentrifuged. This process was repeated twice to give three washings of the cells. Ten milliliters of water was added to the washed cells, the cells were suspended by vigorous agitation, and a dilution was made by adding 0.05 ml. of the suspension to 5 ml. of water. One-tenth of a milliliter of the dilute suspension was added to each 100 ml. of assay medium to be used. This technique resulted in assays with negligible growth in those tubes containing no biotin, even after forty-eight hours incubation.

4. Method of Assay

The growth chambers employed were 25 X 250 mm. Pyrex test tubes. Additions of biotin in graded concentrations were made to provide a standard curve with each assay, since the turbidity levels varied somewhat in each assay; accurate determinations of concentrations of biotin in the unknown could not be made without the use of a specific standard curve. Appropriate quantities of the unknown were also added to similar tubes, and the final volume in all tubes was made to one milliliter. The tubes were steamed for ten minutes in order to kill any vegetative cells which might be present, and allowed to cool. After cooling, 5 ml. of synthetic medium, previously inoculated, was added to each tube. The tubes were incubated for a suitable time at 30 C; they were checked periodically, and when the tube containing no biotin began to show a small quantity of growth, the in-

cubation period was considered complete.

After incubation, the tubes were shaken vigorously to insure complete suspension of the packed cells, and the turbidities read in a Klett-Summerson photoelectric colorimeter equipped with a 4200 A. filter. Concentrations of biotin in the unknown was determined by the graphic method, having previously plotted the standard curve of the assay on semi-logarithmic paper. A typical standard curve is given in Figure 7.

G. Techniques for the Separation of the Coenzyme

1. General Techniques

In order to establish certain physico-chemical characteristics, the normal techniques of solvent solubility, acid and base stability, and absorption characteristics were investigated and will be described briefly under the experimental procedures in question.

2. Chromatographic Techniques

a. General description. The ascending paper partition chromatographic technique of Horne and Pollard (1948) was used. In this procedure a two-phase solvent system ascends a hanging paper strip through capillarity. It is readily adaptable to a large variety of solvents and is considerably simpler to manipulate than the descending technique outlined by Consden, Gordon and Martin (1944).

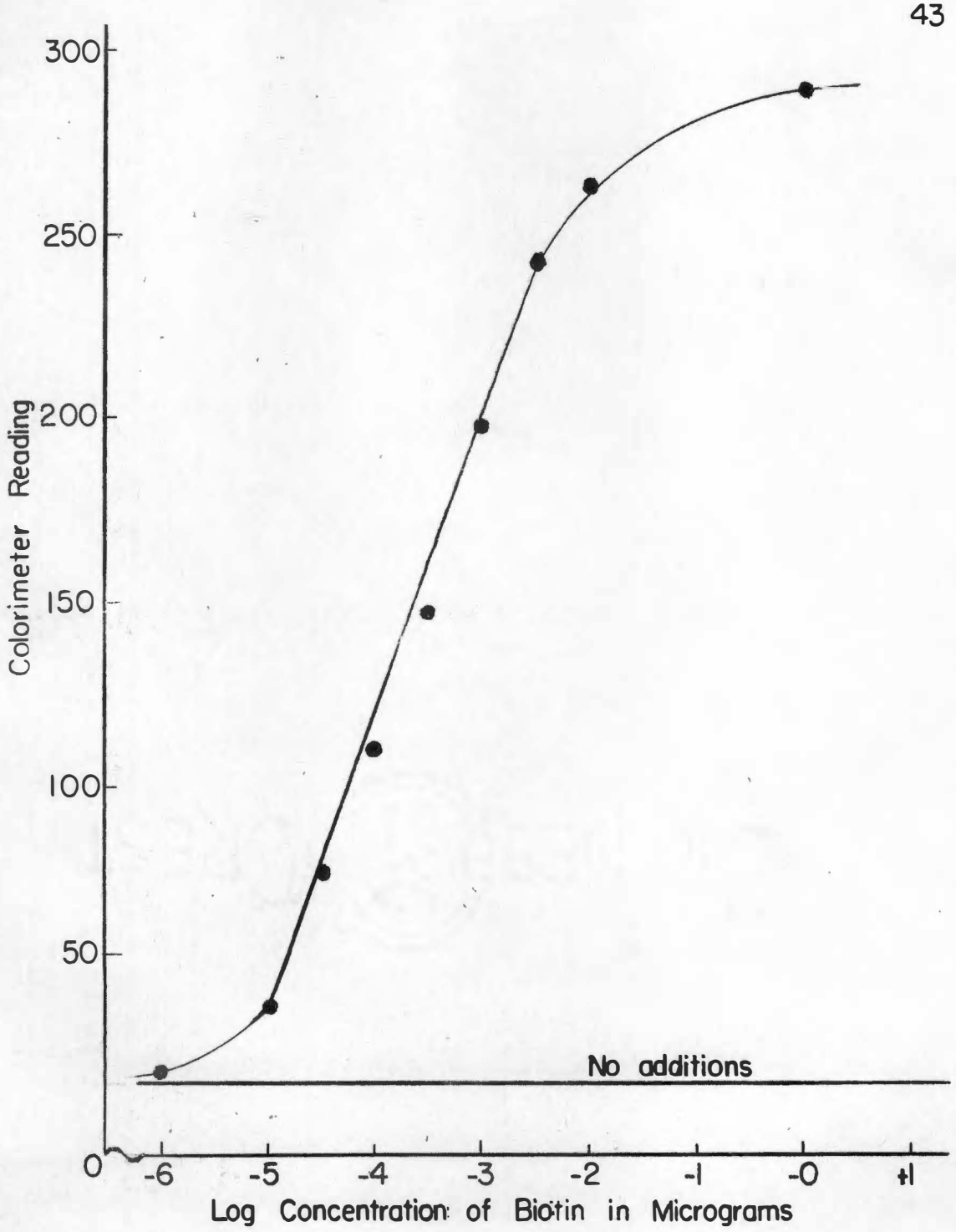


Figure 7: Typical standard growth response of *Saccharomyces cerevisiae* (139) to gradient concentrations of biotin

b. Paper. The paper used in these experiments was Whatman No. 1 filter paper, supplied in sheets measuring 18 X 22.5 in. Each sheet was cut with a razor blade into strips measuring 3 X 18 in. A thin pencil line, drawn at a point 2 in. from one end of the paper, was used as a primary reference line upon which the material was deposited. The strips were handled with forceps to prevent the deposition of materials by the hands.

c. Solvents. A large number of solvent systems can be used to separate materials by paper partition chromatography. The solvent systems used in this work were:

(i) water; this system was the simplest of the solvent systems, and employed distilled water as both the mobile (ascending) and stationary (saturating) phase.

(ii) phenol-water; the mobile phase was composed of a solution of 75 ml. of melted phenol mixed with 25 ml. of water. The phenol-water solution was colorless and, if used within twenty-four hours after preparation, gave reproducible results, with little discoloration of the strips by the phenol. After use, the solution was distilled to recover the phenol. During distillation, the phenol-water azeotrope was collected as it came over at 99-100 C and saved. When several liters of this azeotrope had been collected, the solution was saturated with sodium chloride, the phenol layer recovered, and redistilled. The pure phenol distilled at 182 C and when cool was stored in brown narrow-mouth bottles

until ready for use. The stationary phase for this system was water.

(iii) butanol-water; this system consisted of a mobile phase of butanol saturated with water, and a stationary phase of water or 50 per cent ethanol-50 per cent water.

(iv) isoamyl alcohol-phosphate; the mobile phase of this system consisted of a 5 mm. layer of isoamyl alcohol over a 5 per cent solution of disodium phosphate. The stationary phase was water.

(v) ethanol-citric acid; the mobile phase was a solution made of 200 ml. of ethyl alcohol mixed with 40 ml. of 5 per cent citric acid solution adjusted to pH 3.78 with sodium hydroxide. No stationary phase was used with this system.

d. Preparation of the strips. The sample of the material to be chromatogramed was dissolved in water so that 0.05 ml. contained from 0.3 to 30 mg. The paper strip was placed on a hot plate set at 300 F so that the reference line was across the center of the hot plate. By rapid addition of the solution from a serological pipette a smooth deposit containing about 0.05 ml. of material could be made across the reference line. The dried strip was removed from the hot plate and stored. In this way samples of material could be stored for future use quite easily.

e. Development of the chromatogram. The chambers used were either single units consisting of 6 X 18 in. bat-

tery jars, or large circular aquaria 13 X 24 in. These were equipped with small dishes in which the mobile phase was placed; the stationary phase was placed in the bottom of the chamber surrounding the central dish. In the small chambers the strip was hung from a wire brace fastened to a flat glass top; the larger chambers held a wooden rack which suspended a circular aluminum brace around which seven strips could be hung at one time. To run a chromatogram the stationary phase was added to the chamber to a depth of about 10 mm. the mobile phase placed in the central dish to about 15 mm. in depth, the strip hung from the brace fastened with a wire clip, and the top put into position. The strip was so adjusted that about 5 mm. of the end of the strip was immersed into the mobile phase solvent. The chamber was placed in an incubator to avoid drafts and temperature changes. After twelve to twenty-four hours, the solvent front had advanced to about 280 mm. from the reference line; the strip was removed from the chamber, and the position of the farthest solvent advancement was marked. The strip was dried at 110 C for thirty to sixty minutes.

f. Measurement of the R_f values. In order to establish the difference in mobility of various materials in the sample, the use of the front ratio (R_f) value was employed. This ratio is defined as:

$$R_f = \frac{\text{Distance traveled by a particular substance}}{\text{Distance traveled by the solvent front}}$$

where both distances are determined from the reference line.

Since there are no known colorimetric methods for detecting the presence of either biotin or the coenzyme of aspartic acid deaminase, these materials were detected by microbiological and enzymatic assays of the water eluates of the strips. The strip was cut in ten equal pieces, starting with the base reference line and continuing to the position of the farthest advance of the solvent front. They were marked as R_f 0.1 to 1.0 in tenths; thus the material having an R_f 0.6 to 0.7 would be found in the fraction labeled R_f 0.7.

g. Elution of the strips. If single strips were to be assayed, the individual pieces were minced with scissors and placed in test tubes; 3 to 5 ml. of water was added to each, and the sludge was allowed to stand one hour. Assays were then made on the eluting fluid. If large numbers of strips were to be pooled (as many as 200 strips were pooled in one experiment) all those pieces with the same R_f value were placed in a Waring blendor, covered with water, and minced. The sludge was allowed to stand for at least six hours, filtered to remove the paper, and evaporated to dryness. During evaporation some material would precipitate which could not be redissolved in water. This material was removed from the water soluble portion by re-solution in a small volume of water, filtering the mixture, and evaporating the filtrate to dryness. The samples were weighed, and

a standard solution containing 1 mg. per 0.1 ml. was prepared. This solution was assayed both for biotin content, by measuring the growth response of S. cerevisiae; and for coenzyme activity, by measuring the aspartic acid deaminase activation of aged cell suspensions of B. cadaveris.

CHAPTER III

EXPERIMENTAL DATA

A. Demonstration of the Coenzyme Present in Yeast Extract

The evidence presented by Lichstein (1949a) indicated that the coenzyme of aspartic acid deaminase was present in yeast extract. This conclusion was reached because of the nature of the stimulation in comparison with biotin or adenylic acid stimulations; the assay of yeast extract by biotin assaying techniques and enzyme assaying techniques showed a hundred-fold difference in the levels of stimulation.

Clearer demonstration of the existence of the coenzyme is given in the following data: the response of the cells is directly proportional to the concentration of yeast extract added in aspartic acid deaminase assays (Figure 8); the stimulation caused by a given amount of yeast extract is in direct relation with the concentration of the substrate added (Figure 9); and stimulation of cells could be obtained after aging on nearly every attempt. That this substance is not identical with either biotin or adenylic acid (both of which are present in yeast extract) can be seen from the data presented in Figure 10. Because of the interest of many investigators in the field of amino acid metabolism, it

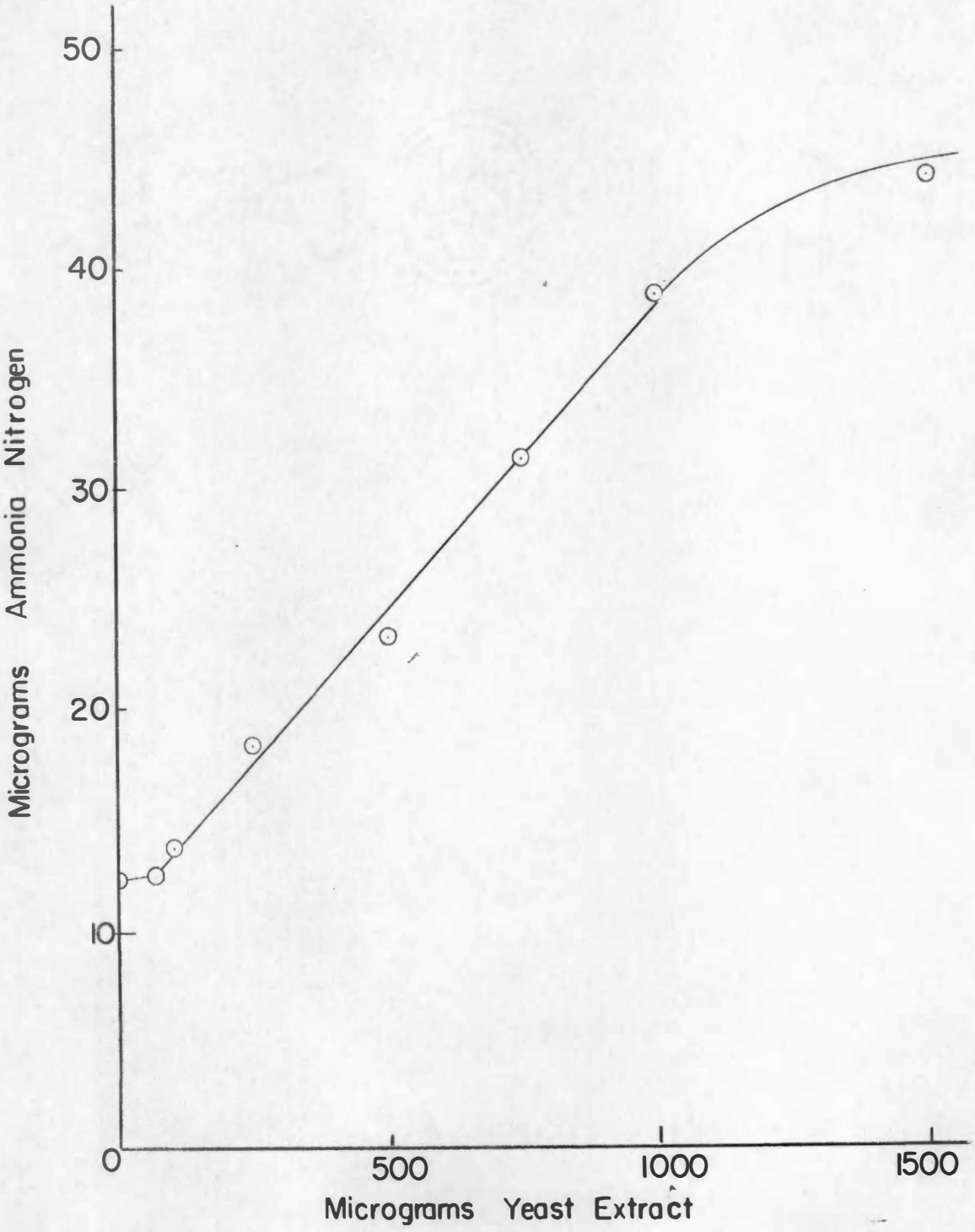


Figure 8: The stimulation of aged cells of *B.cadaveris* by gradient concentrations of yeast extract

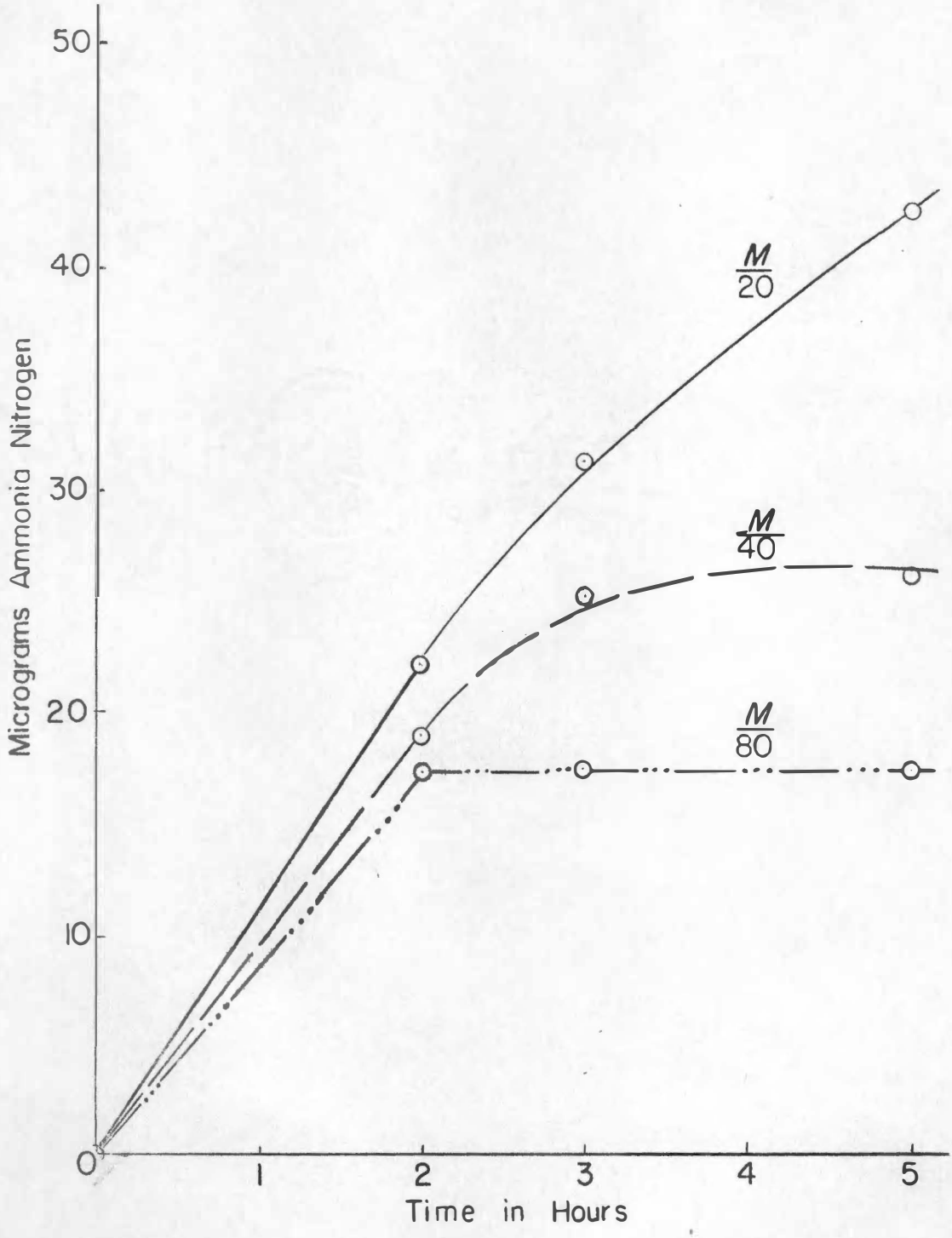


Figure 9: The effect of varying concentrations of aspartic acid on aged cell suspensions of *B. cadaveris* stimulated by equal quantities of yeast extract

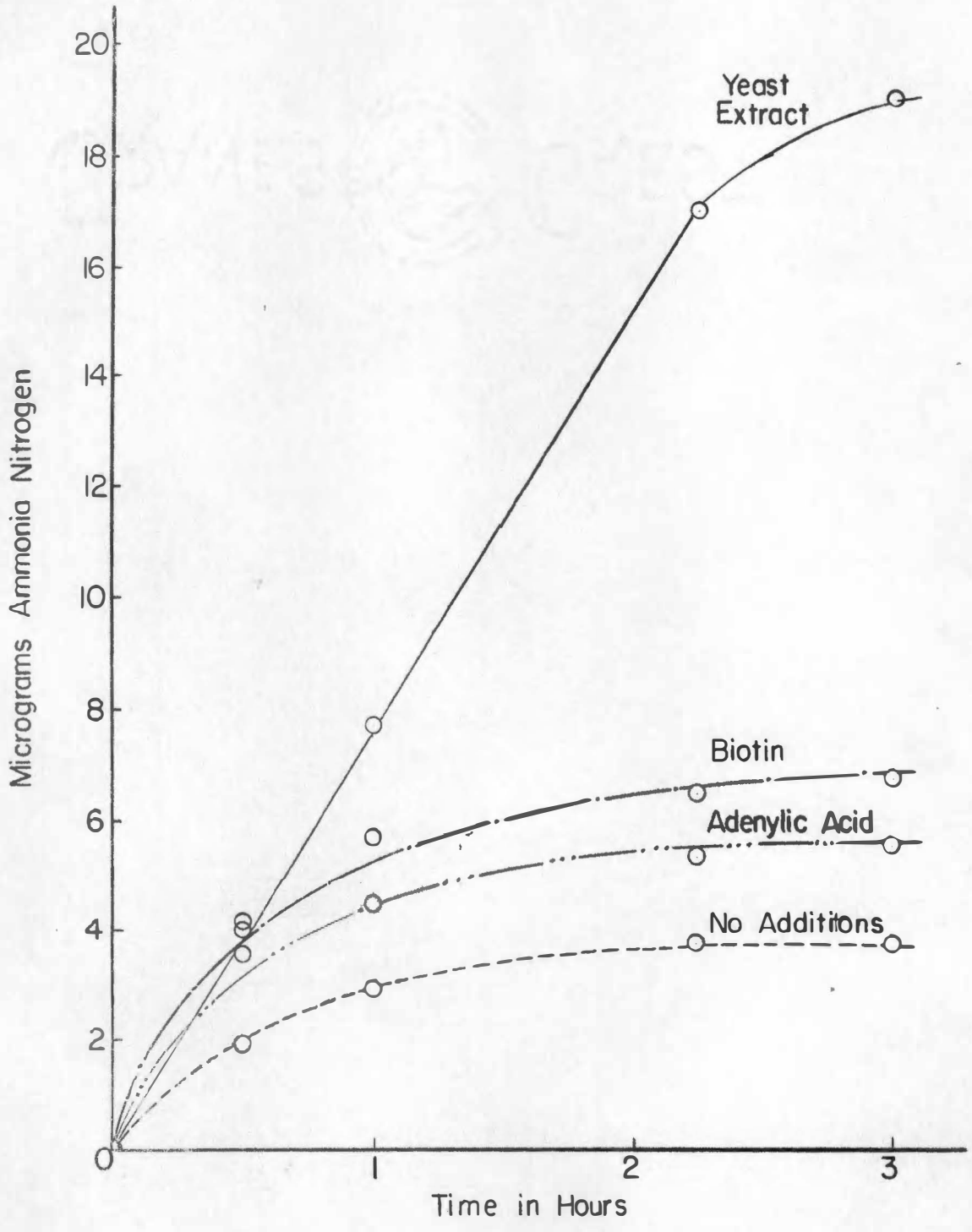


Figure 10: The relative stimulatory effects of biotin, adenylic acid, and yeast extract on aspartic acid deaminase

seemed that characterization of the coenzyme would be an important contribution to the knowledge of amino acid metabolism.

B. Resistance of the Coenzyme to Physical and Chemical Manipulation

1. Solution and Stability in Water

The coenzyme was remarkably stable in water solutions in concentrations up to 10 per cent. These solutions when stored at 4-6 C would remain stable for several months.

2. Stability to Heat

a. Autoclaving. The coenzyme activity of a 1 per cent solution of yeast extract is not appreciably altered by autoclaving at 15 pounds pressure (121 C) for fifteen minutes (Table III).

b. Evaporation to dryness. A 1 per cent solution was evaporated to dryness at 110 C, and redissolved in water. Repeated solution and evaporation to dryness had little or no effect on the coenzyme (Table III).

c. Incineration. One gram of yeast extract was placed in a crucible, and ignited carefully. After initial ashing, the crucible was allowed to cool and 5 ml. of concentrated nitric acid added. The solution was boiled for thirty minutes, allowed to cool, and 2 ml. of 50 per cent perchloric acid added. The solution was reheated until

TABLE III

RESPONSE OF AGED CELL OF BACTERIUM CADAVERIS TO SAMPLES OF YEAST EXTRACT THAT HAVE BEEN SUBJECTED TO DIFFERENT TREATMENTS

Additions	Experiment Number 17-19	17-22	17-26	17-32
	Micrograms Ammonia Nitrogen[1]			
None	3.5	5.9	6.1	4.2
Biotin, 0.1 µg.	4.0	7.8	6.1	5.9
Yeast extract, 1mg., treated as follows:				
None	9.5	19.7	15.1	7.7
Autoclaved 1 hr.	13.7	23.2	10.1	7.7
Repeated evaporation to dryness	13.8	15.8	----	---
Incineration	3.2	5.7	----	---
Acid hydrolysis	13.6	39.4	23.5	19.2
Alkaline hydrolysis	9.7	19.7	----	----

[1]The values given are corrected for the activity of duplicate samples prepared and incubated identically with the experimental tubes but lacking added aspartic acid

white fumes were given off, allowed to cool, and added to 60 ml. of water. The mixture was neutralized with 10 per cent sodium hydroxide, diluted to 100 ml; filtered, and the filtrate assayed for coenzyme activity (Table III). It is evident from the data presented that incineration destroys the coenzyme, and it was concluded that the coenzyme was organic in nature.

3. Stability to Acid Hydrolysis

One gram of yeast extract was dissolved in 50 ml. of water and 2 ml. of concentrated hydrochloric acid added; the mixture was refluxed for thirty minutes, cooled, neutralized, and diluted to 100 ml. Assay for coenzyme content (Table III) shows an increase in activity. This may be due to a degradation of more complex forms of the coenzyme to simpler substances more available to the enzyme system.

4. Stability to Alkaline Hydrolysis

One gram of yeast extract was dissolved in 50 ml. of water and 5 ml. of a 10 per cent sodium hydroxide solution added. The mixture was refluxed for thirty minutes, cooled, neutralized, and diluted to 100 ml. Assay revealed that there was no pronounced effect on the coenzyme activity by this treatment (Table III).

C. Solvent Solubility of the Coenzyme

1. Solubility Tests

To determine the solubility of the coenzyme in a particular solvent 1 g. of yeast extract was placed in an Erlenmeyer flask and 100 ml. of solvent added. The mixture was heated to boiling, cooled and filtered. The precipitate was dried at 110 C; the filtrate was evaporated to dryness at the same temperature. Assays (Table IV) revealed that the coenzyme was insoluble in commercial or absolute diethyl ether, ethyl acetate, n-butyl alcohol, carbon tetrachloride and chloroform; it was only slightly soluble in iso-propyl alcohol; it was relatively soluble in methyl alcohol and 95 per cent and absolute ethyl alcohol; it was completely soluble in water. No good fractionation of yeast extract was obtained by this technique.

2. Solvent Extraction Results

Since the coenzyme was soluble, at least to some extent, in ethyl alcohol, extraction of yeast extract with this solvent was attempted. Both Soxhlet and reflux extraction precedures were used. However, no clear cut extraction could be made with absolute ethyl alcohol, even after three separate extractions of the same material.

TABLE IV

SOLUBILITY OF THE COENZYME OF ASPARTIC ACID DEAMINASE IN VARIOUS ORGANIC SOLVENTS

Additions	Experiment Number 17-45	17-32	17-26
	Micrograms Ammonia Nitrogen[2]		
None	5.5	4.2	6.1
Biotin, 0.1 µg.	5.5	5.9	4.6
Yeast extract, 1 mg., treated as follows:			
None	14.3	7.7	15.1
Sol. in methanol	19.4	---	----
Ins. in methanol	8.5	---	----
Sol. in 95% ethanol	14.8	---	----
Ins. in 95% ethanol	10.3	---	----
Sol. in abs. ethanol	18.2	---	----
Ins. in abs. ethanol	9.5	---	----
Sol. in isopropyl alc.	----	---	9.8
Ins. in isopropyl alc.	----	---	11.2
Ins. in coml. ether[3]	----	10.6	----
Ins. in abs. ether[3]	----	9.5	----
Ins. in n-butyl alcohol[3]	20.5	----	----
Ins. in ethyl acetate[3]	14.3	----	----
Ins. in chloroform[3]	15.4	----	----
Ins. in carbon tetrachloride[3]	15.4	----	----

[2]See footnote 1, Table III

[3]No soluble material could be isolated

D. Chromatographic Characteristics of the Coenzyme

Ten milligrams of yeast extract was dissolved in 0.1 ml. of water and placed on a paper strip 1½ X 18 in. A number of these strips were prepared and chromatographically developed using the various solvent systems described.

1. Water Mobile Phase

With water as the mobile phase, a strip containing yeast extract was developed, dried, sectioned, eluted with water, and tested for biotin content and coenzyme activity. The results (Figure 11) indicate that both biotin and the coenzyme are deposited in the same area. However, a control using pure biotin shows a distinctly different R_f value. These data suggest either that no free biotin is present in yeast extract or that the water mobile phase is not satisfactory as a chromatographic solvent in this case. In order to check these hypotheses, chromatograms of yeast extract and yeast extract containing added biotin were run and assayed. Figure 12 shows that there are only slight differences between the two curves and thus it would appear that water as a mobile phase is an unsatisfactory solvent for chromatographic development of yeast extract.

2. Butanol-Water Mobile Phase

With strips prepared as previously described and chromatographically developed using butanol saturated with

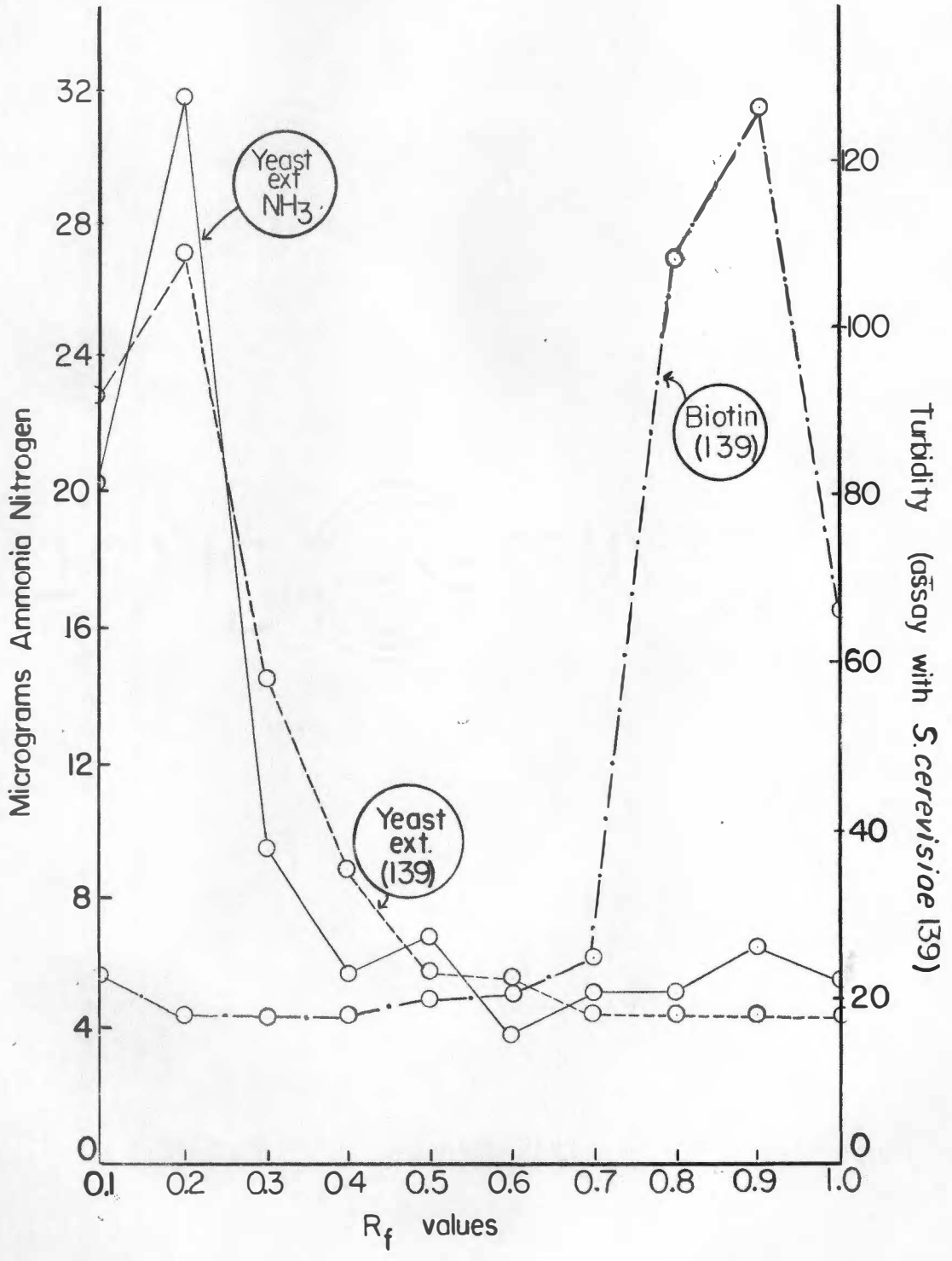


Figure II: Distribution of yeast extract on a paper strip developed in a water mobile phase as compared to distribution of free biotin

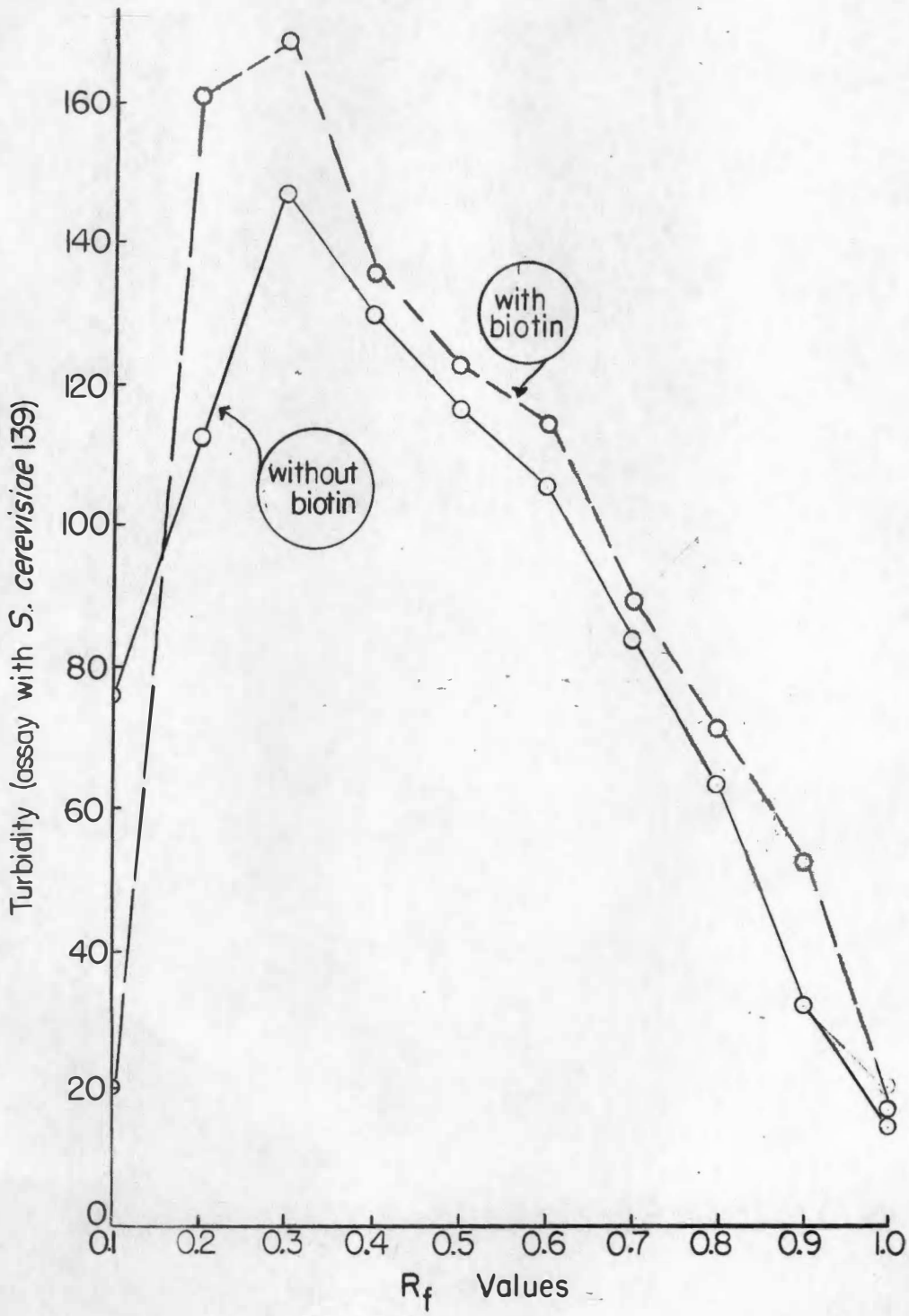


Figure 12: Distribution of yeast extract and yeast extract fortified with free biotin on paper strips developed in a water mobile phase

water as the mobile phase a chromatogram resulted, which when assayed (Figure 13) gave results similar to that obtained with water.

3. Phenol-Water Mobile Phase

A paper strip impregnated with yeast extract was chromatographically developed with phenol-water as the mobile phase. It was evident from the data (Figure 14) that successful separation of biotin from the coenzyme had been effected; it can also be seen that S. cerevisiae does not respond to the coenzyme fraction. Because of the involvement of biotin in serine and threonine deaminases (Lichstein and Umbreit, 1947b; Lichstein and Christman, 1948) the coenzyme fraction, apparently pure with respect to free biotin, was tested against these deaminases. Figure 15 shows that the coenzyme material stimulates all three deaminases.

Although the involvement of biotin in these deaminases does not necessarily mean that it is the coenzyme or even part of the coenzyme, detection of biotin in the coenzyme fraction would give added weight to the hypothesis that the coenzyme is an active form of biotin. As already stated, S. cerevisiae (139) does not respond to this fraction; two possibilities were thus presented, either there is no biotin in this fraction, or the biotin, if present, is not available to this strain.

Experimentally the problem was attacked from two

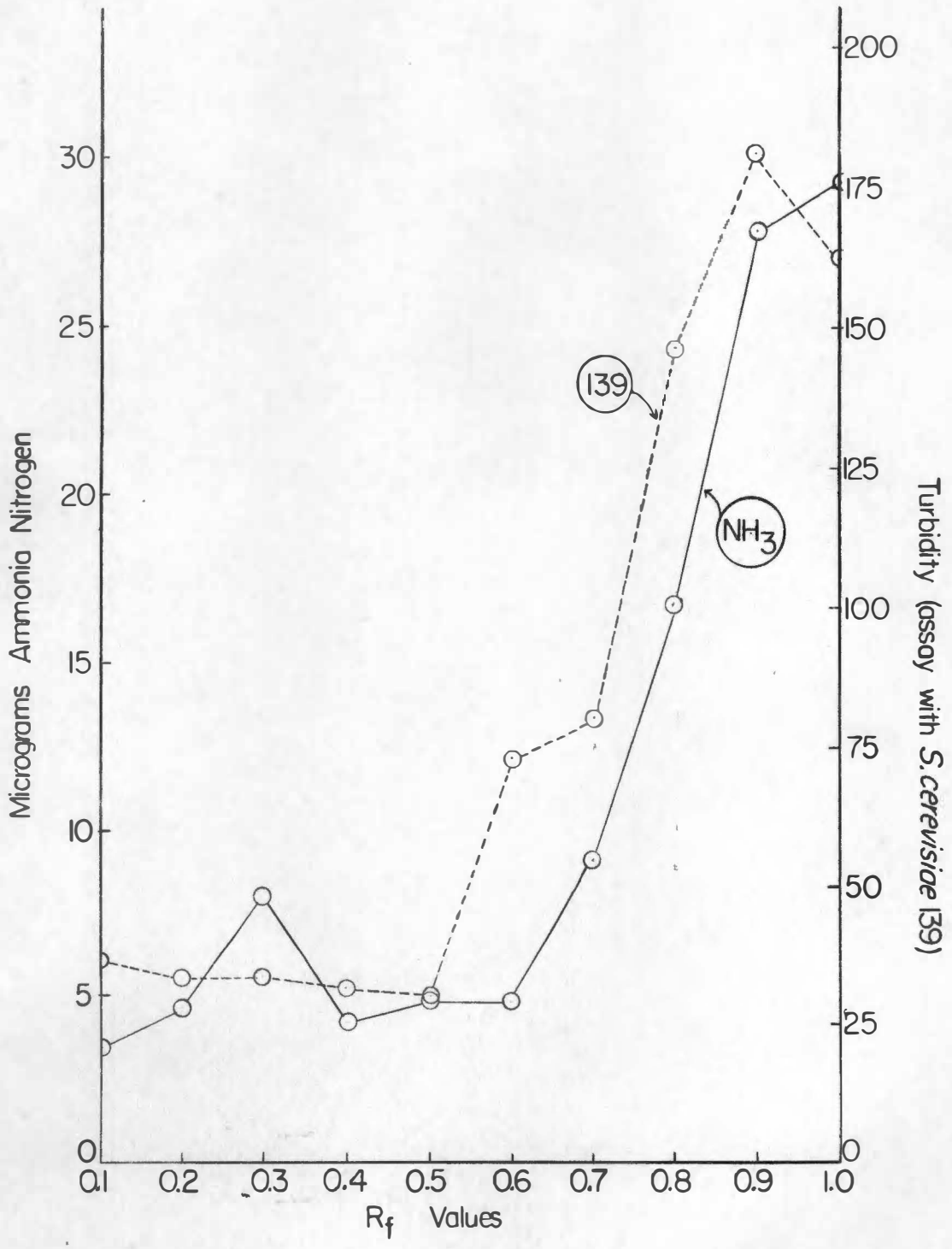


Figure 13: Distribution of yeast extract on a paper strip developed in a butanol-water mobile phase

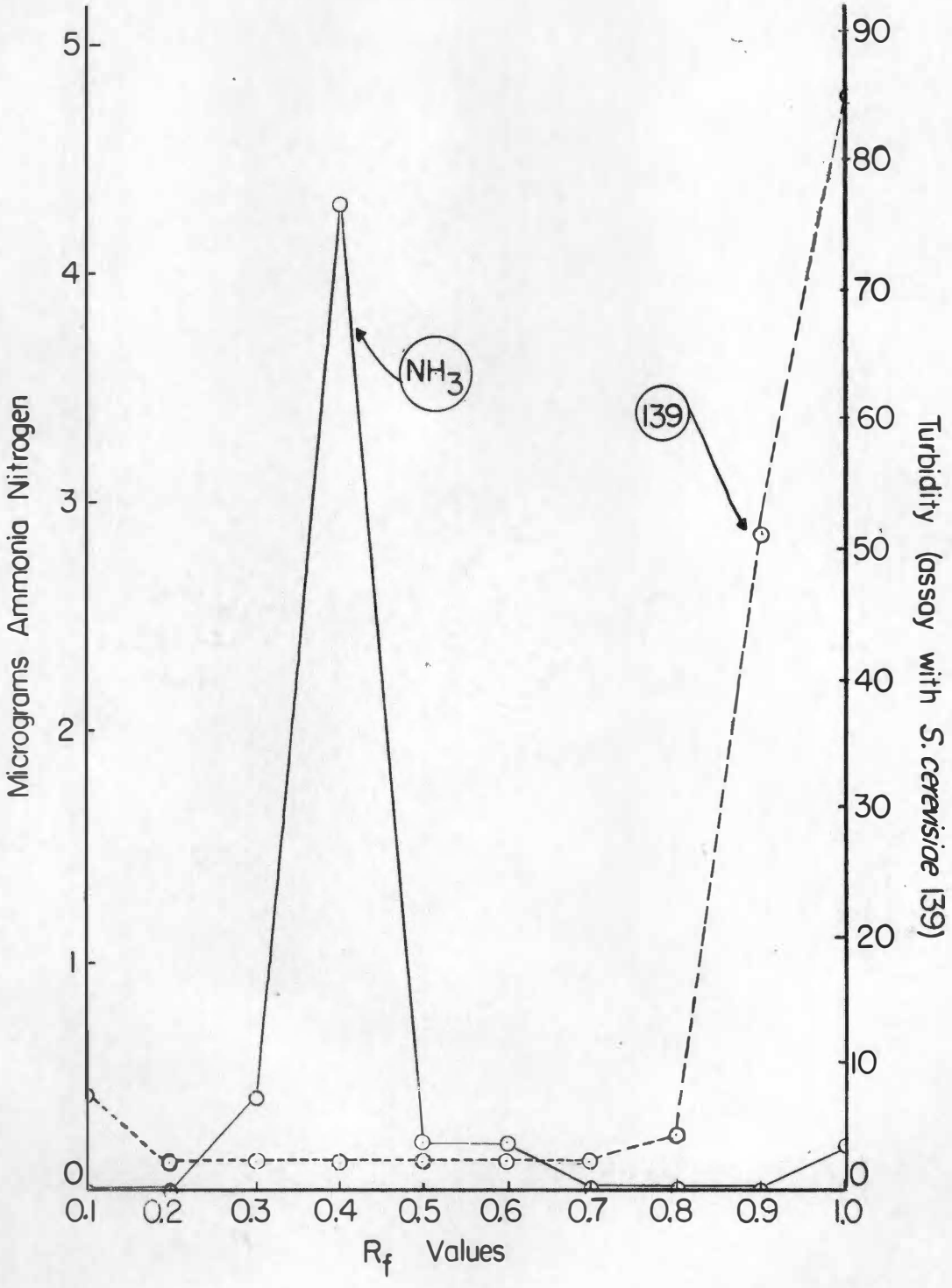


Figure 14: Distribution of yeast extract on paper strip developed in a phenol-water mobile phase

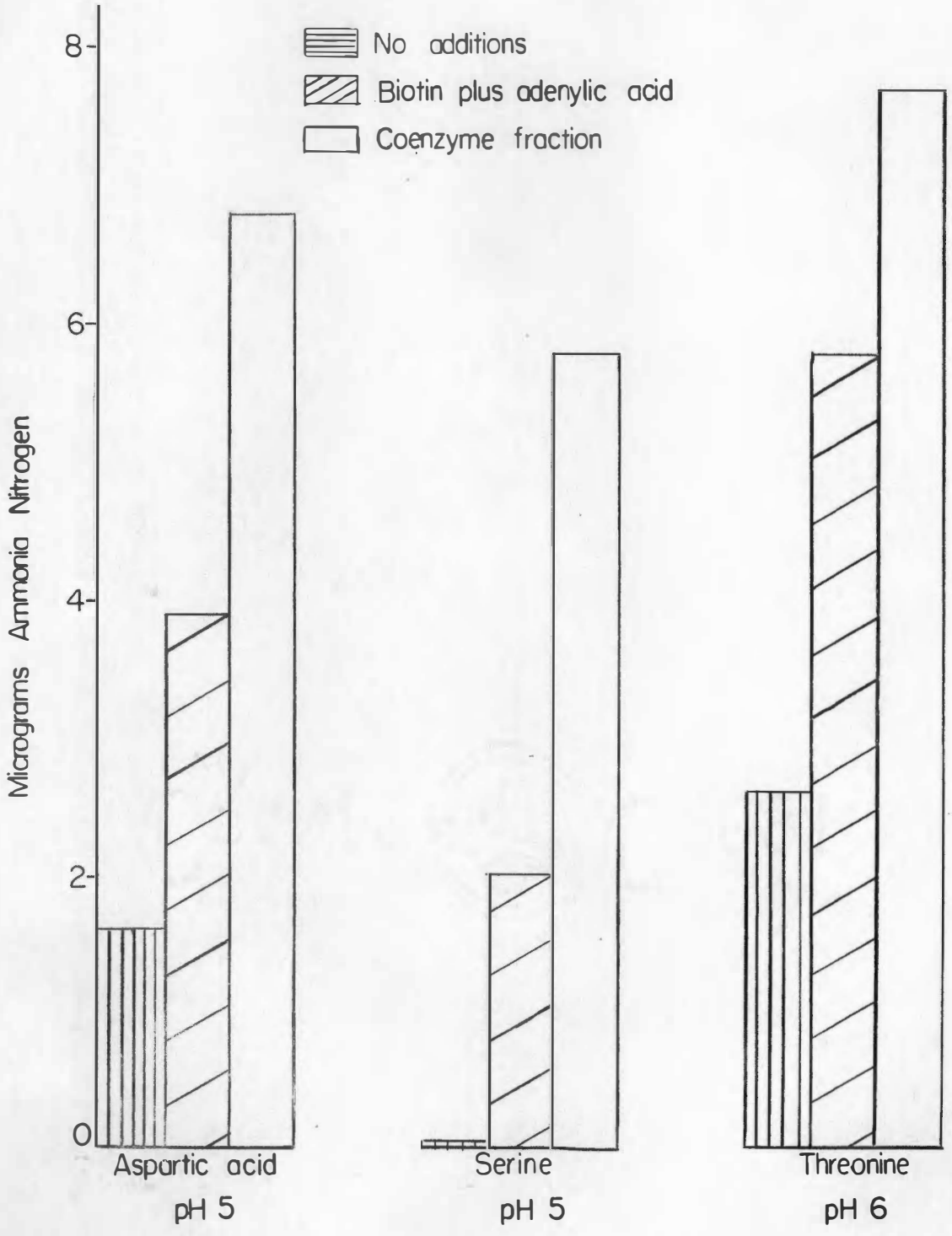


Figure 15: Relative stimulatory effect of biotin with adenylic acid and biotin-free coenzyme fraction of yeast extract on partially resolved aspartic acid, serine and threonine deaminases

directions. A survey was made of various cultures of Saccharomyces that might respond to the coenzyme fraction in a biotin-free medium; the coenzyme was hydrolyzed to determine whether biotin could be made available to S. cerevisiae (139). The results of such an investigation are presented in Table V. It can be seen that S. cerevisiae (Java) does respond to the coenzyme fraction before hydrolysis; S. cerevisiae (139) does not respond to this material. Further, hydrolysis of the coenzyme fraction liberates biotin, or a substitute of biotin, which supports the growth of both S. cerevisiae (139) and S. cerevisiae (Java). Fractions from the same strip that do not contain the coenzyme show identical responses by S. cerevisiae (Java) and S. cerevisiae (139).

The fact that bound biotin was contained in large quantities only in those portions of the strip which contained the coenzyme could be interpreted in two ways. Either the coenzyme is a bound form of biotin, or the association is one of similar physico-chemical properties and thus can be separated on further purification.

TABLE V

RELATIVE EFFECT ON GROWTH OF TWO STRAINS OF SACCHAROMYCES CEREVISIAE BY YEAST EXTRACT AFTER ONE STEP PURIFICATION ON PHENOL-WATER CHROMATOGRAM

SAMPLE	TURBIDITY	
	Strain 139	Strain Java
No additions	30	30
Biotin, 0.00001 µg.	32	33
Biotin, 0.001 µg.	90	90
Biotin, 0.1 µg.	239	154
Biotin, 10.0 µg.	242	160
Pooled R_f 0.1, 0.2, 0.6, 0.7 and 0.8 fractions	35	40
Same fraction hydrolysed[1]	28	32
Pooled R_f 0.3, 0.4, and 0.5 fractions	35	65
Same fraction hydrolysed[1]	50	48
Pooled R_f 0.9 and 1.0 fractions	90	85
Same fraction hydrolysed[1]	26	25
Biotin, 1.0 µg.	260	175
Same hydrolysed[1]	145	140

[1]Hydrolyses carried out in 6N sulfuric acid, 121 C, for two hours

E. Preparation of Samples Purified with Respect to Free Biotin

1. Preparation of Sample 20-85

Thirty-eight paper strips each containing 20 mg. of yeast extract were run in the phenol-water system. After the solvent had advanced to an average length of 235 mm. they were dried, and a section with Rf values of 0.4, 0.5, and 0.6 were removed. These central portions were chopped into small pieces and eluted with two 100 ml. portions of hot water. The eluates were filtered, and the filtrate evaporated to dryness under infra-red light. The weight of the dried sample was 380 mg.; this was dissolved in a minimum quantity of water and divided among 7 strips. The strips were developed in the phenol-water system; the Rf 0.4, 0.5 and 0.6 fractions were cut as above, extracted with hot water, and concentrated under infra-red light. The 45 mg. resulting from this treatment was deposited on one strip which was run in the phenol-water system. After development this strip was dried, cut in 10 equal pieces, and each eluted with 6 ml. of water.

The distribution of the material stimulatory for aspartic acid deaminase in aged cell suspensions of B. cadaveris shows a greater spread than previously (Figure 16). This spread was probably due to the gross overloading of the strip. However, the greatest concentration of

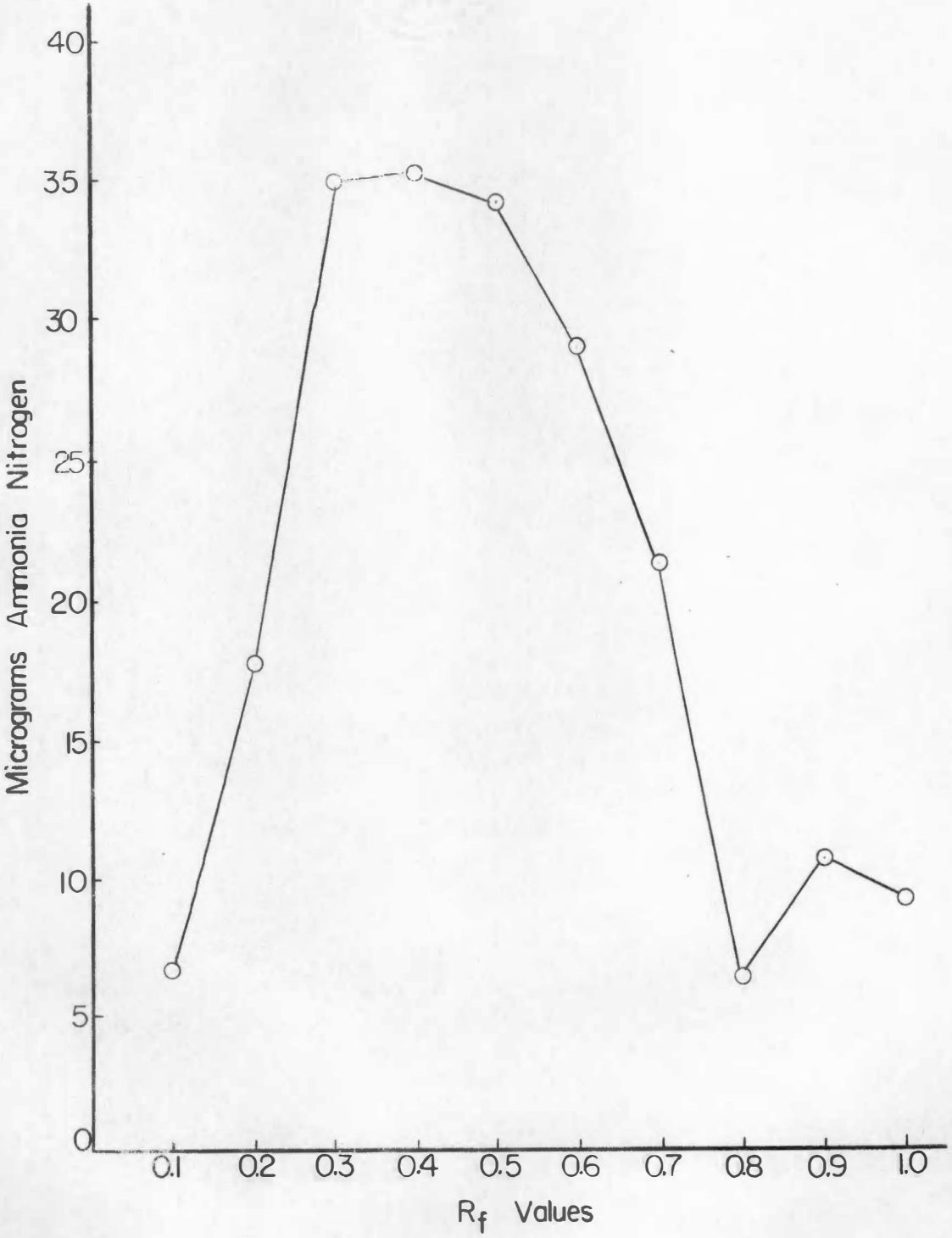


Figure 16: Paper strip chromatogram distribution of coenzyme assayed against aspartic acid deaminase in aged cell suspensions of B. cadaveris after purification 20-85

material was at R_f 0.3 to 0.5, as was experienced with the earlier determinations. The relative stimulatory effect of the R_f 0.4 fraction on partially resolved aspartic, serine and threonine deaminases in B. cadaveris as compared with the effect of biotin and adenylic acid may be seen in Figure 17.

A pooled sample, containing the R_f 0.3 to 0.6 fractions, was assayed against the 139 and Java strains of S. cerevisiae in order to determine the content of bound biotin. In addition duplicate sets of tubes were held in boiling water for varying periods of time to determine if free biotin could be liberated (Table VI). It may be noted that once again strain 139 does not respond to the unhydrolyzed coenzyme fraction, whereas growth of the Java strain is supported by this fraction in a medium deficient in biotin. It may be seen also that boiling in water apparently liberates free biotin which is assayed by both organisms.

The absence of free biotin is more clearly demonstrated by the fact that 0.1 ml. of the pooled sample does not cause any stimulation of the growth of S. cerevisiae (139).

2. Preparation of Sample 20-89

One hundred strips each containing 20 mg. of yeast extract were run with the phenol-water system. The R_f 0.3, 0.4, 0.5 and 0.6 sections were cut, eluted with water,

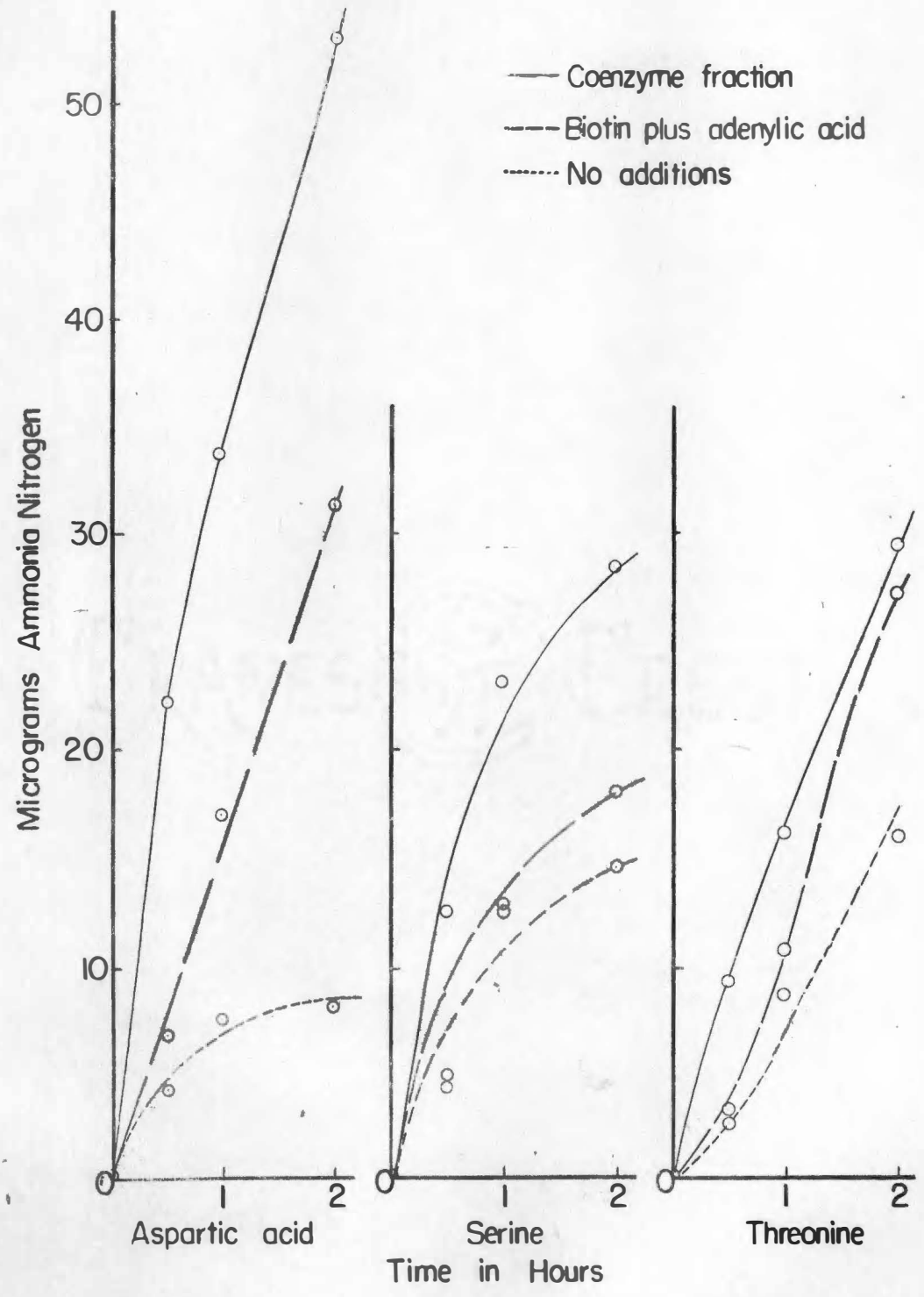


Figure 17: Relative stimulatory effect of biotin plus adenylic acid and biotin-free coenzyme sample 20-85 on aspartic acid, serine and threonine deaminases of aged cell suspensions of B. cadaveris

TABLE VI

RELATIVE EFFECT ON GROWTH OF TWO STRAINS OF SACCHAROMYCES CEREVISIAE BY YEAST EXTRACT AFTER THREE STEP PURIFICATION ON PHENOL-WATER CHROMATOGRAM

ADDITIONS	TURBIDITY			
	Strain 139		Strain Java	
	Exp.1	Exp.2	Exp.1	Exp.2
No additions	22	22	26	54
Biotin, 0.00001 µg.	44	55	50	74
Biotin, 0.001 µg.	103	135	102	115
Biotin, 0.1 µg.	210	278	155	216
Biotin, 10.0 µg.	220	300	146	212
R_f 0.3, 0.4, and 0.5 fractions	30	21	50	75
Same fraction boiled 1 hour	28	38	52	125
Same fraction boiled 2 hours	26	80	65	105
Same fraction boiled 3 hours	42	29	72	90

filtered and evaporated to dryness. The 0.693 g. which was obtained was run on 21 strips, which were eluted, the 0.3 to 0.6 sections were removed, eluted, and dried. The yield of 0.389 g. was rerun on eleven strips. The eluates of the R_f 0.4 to 0.6 sections, all of which showed coenzyme activity, were combined and evaporated to dryness, resulting in a slightly hygroscopic, dark brown mass weighing 77 mg. This material was put on three strips and run in the phenol-water system. An assay of the three strips with B. cadaveris is shown in Figure 18. The coenzyme was contained in the R_f 0.4 to 0.6 sections on all three strips. The eluates, when combined and evaporated to dryness, amounted to 37 mg. and had been considered to have passed through four steps of purification.

Twenty milligrams of this material was placed on a three inch strip and run with iso-amyl alcohol-phosphate as the mobile phase. The strip was sectioned and eluted as usual; assay with B. cadaveris showed the greatest amount of material in the R_f 0.7 to 0.9 range. S. cerevisiae (139) did not respond to any of these fractions.

The concentrate of R_f 0.7, 0.8, and 0.9 of the five step purification was combined with the remainder of the four step purification material and divided into two equal parts. One-half of this material was run in an iso-amyl alcohol-phosphate mobile phase system and the other half was run in a butyl alcohol mobile phase system. From these data

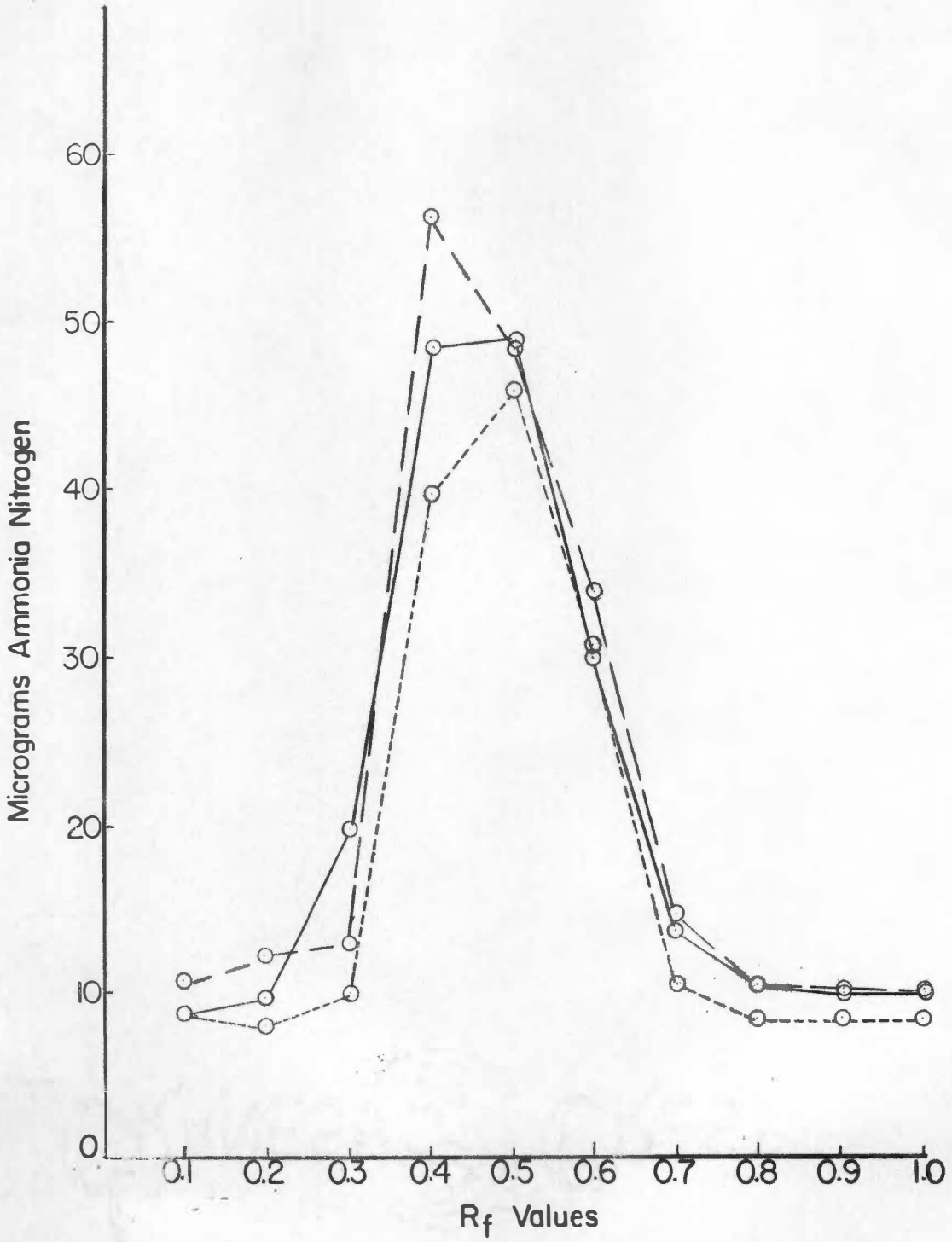


Figure 18: Stimulatory effect of samples 20-88 after phenol-water developments (four step purification) on aspartic acid deaminase of B. cadaveris

(Figure 19 and 20) it would seem that the coenzyme fraction and growth response of S. cerevisiae (Java) are intimately associated. Further indication of this is shown by concentration of the six step purification samples, amounting to 17 mg., which were combined and run in the ethanol-citric acid system. Figure 21 shows a distinct correlation between coenzyme activity and growth response of S. cerevisiae (Java).

F. The Effect of Overloading of the Strip

Comparison of the range of coenzyme activity with varying concentrations of yeast extract placed on the strip show that the ideal concentration is approximately 5 mg. per three inch strip and that extremely wide R_f ranges are obtained if concentrations of 30 mg. or more are used. A considerable amount of the difficulty arising during the previously discussed purification might have been due to overloading the strip. In order that purer samples might be obtained a larger number of strips, each containing a smaller quantity of material, should be run.

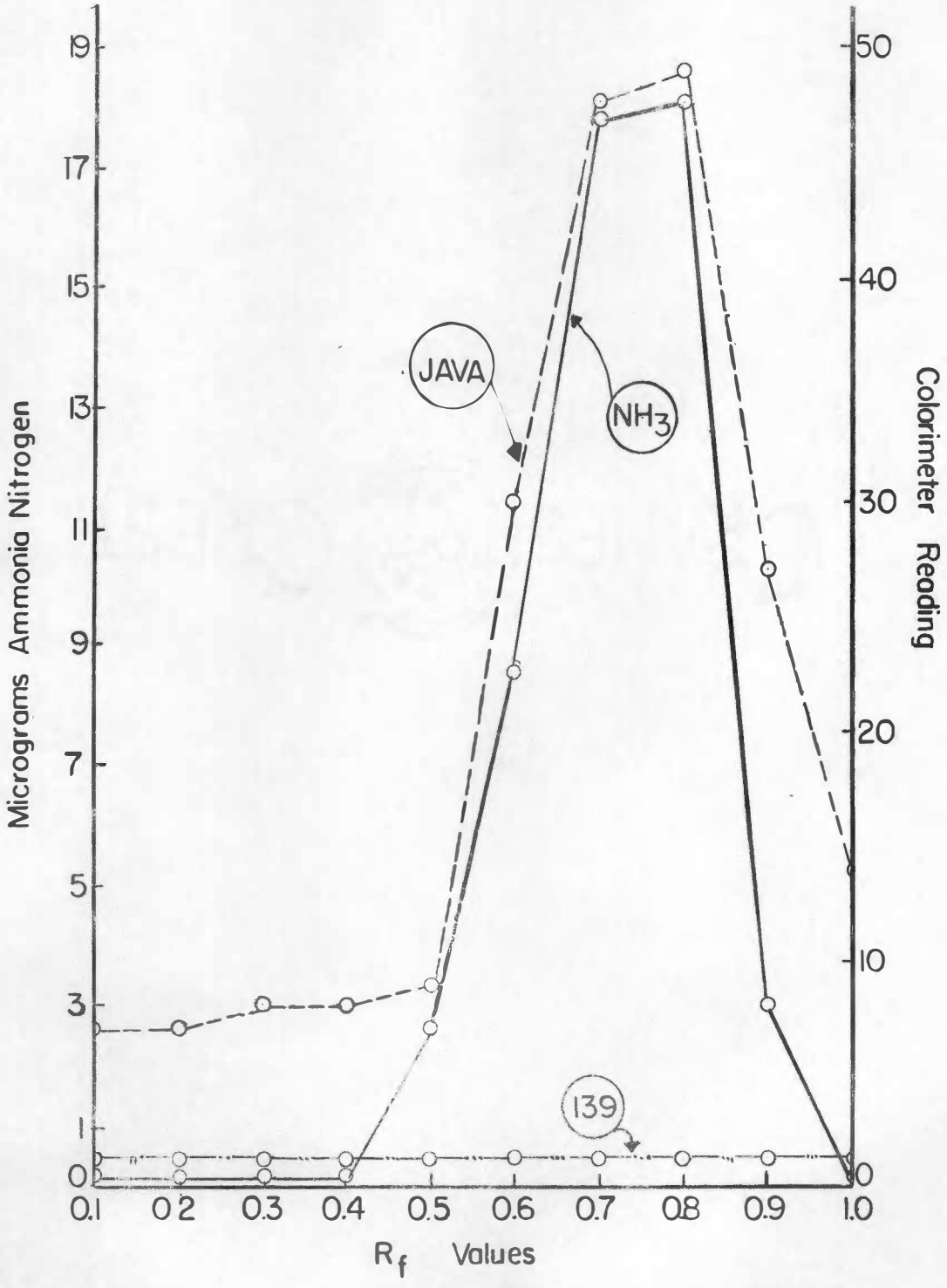


Figure 19: Stimulatory effect of samples 20-88 after butanol-water development (six step purification) on aspartic acid deaminase and growth response of S. cerevisiae

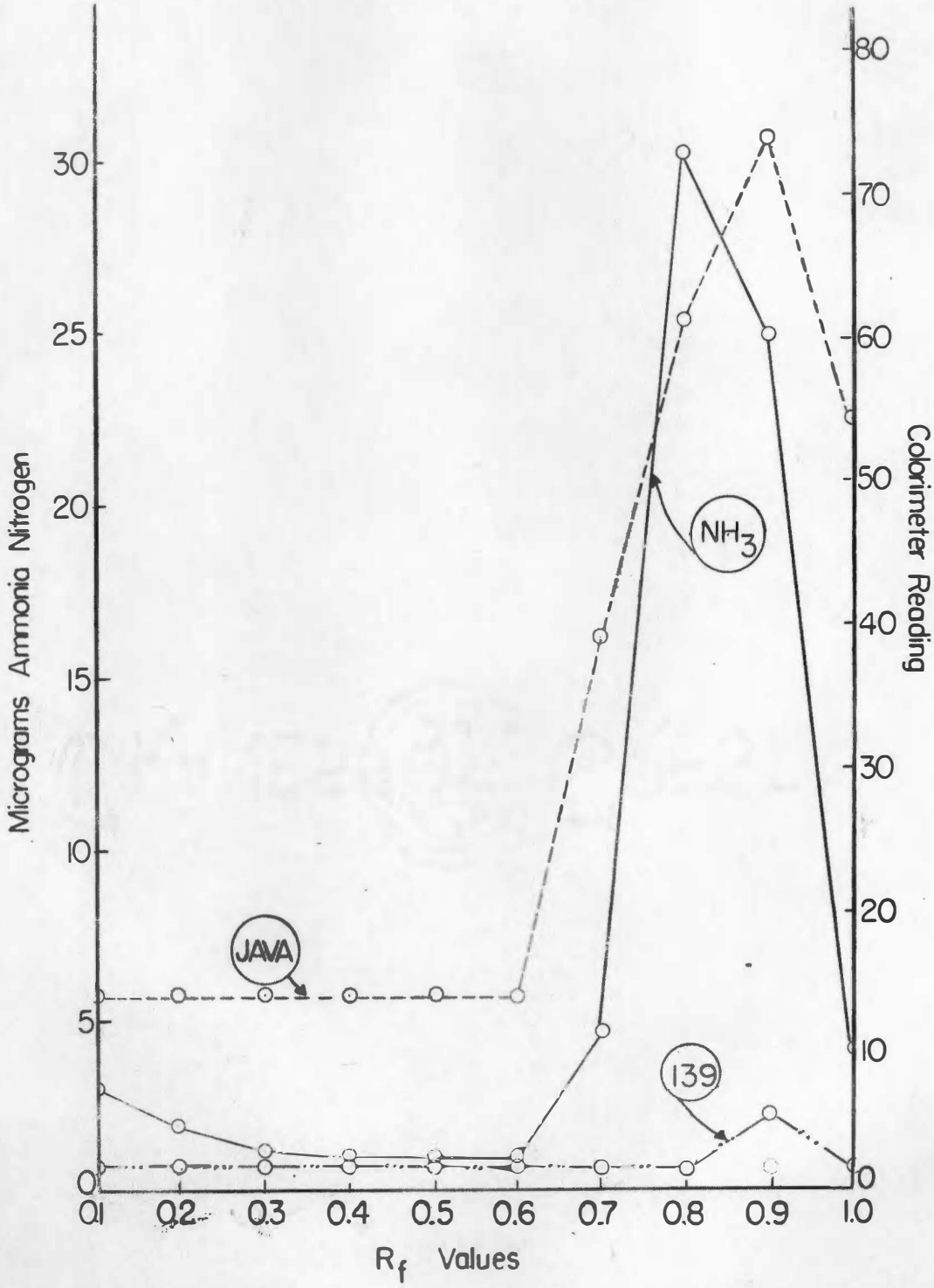


Figure 20: Stimulatory effect of samples 20-88 after iso-amyl alcohol-phosphate development (six step purification) on aspartic acid deaminase and growth response of S. cerevisiae

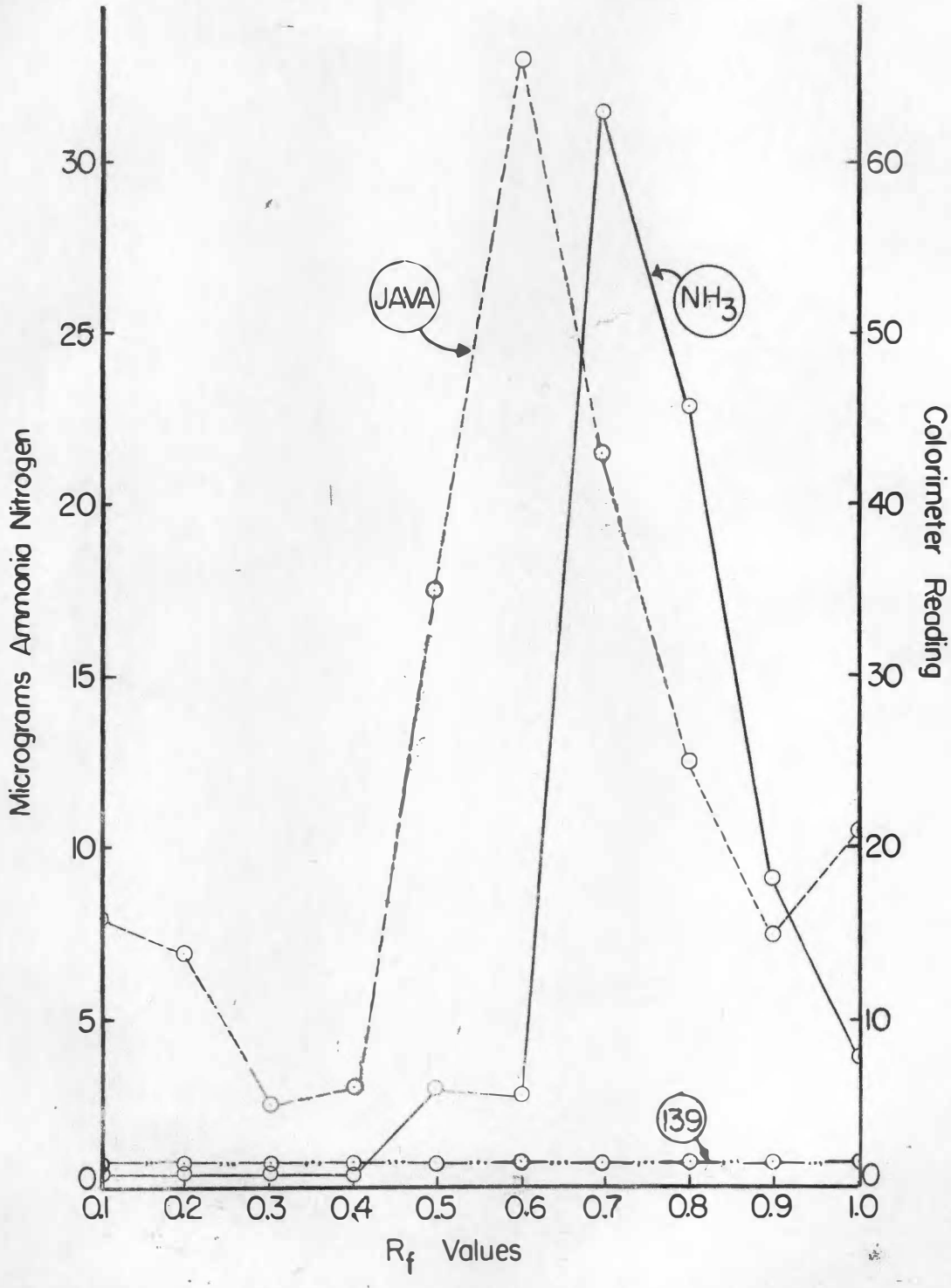


Figure 21: Stimulatory effect of samples 20-88 after ethanol-citric acid development (seven step purification) on aspartic acid deaminase and on growth of S. cerevisiae

G. Distribution of the Coenzyme in Various Yeast and Liver Extract Samples

Data presented in Figure 22 show the comparative effects of equal concentrations of various samples of yeast extract kindly presented to us by Difco Laboratories, Inc., Detroit, Michigan, and liver extract samples supplied through the courtesy of Eli Lilly and Co., Indianapolis, Indiana, on aspartic acid deaminase. The liver extract samples apparently contain considerably more coenzyme than an equivalent weight of yeast extract.

H. Separation of the Coenzyme from Liver Extract

Liver extract fraction E-1418 was filtered to remove any undissolved matter and dried at 110 C. A portion of the solid material, weighing 0.2831 g., was dissolved in 2.8 ml. of water and placed on 45 strips three inches wide. The strips were run in the phenol-water system to an average length of 325 mm. They were sectioned and eluted by blending in a Waring blender with water. The sludge was filtered, the paper washed with a second volume of water and refiltered. The filtrates were combined and evaporated to dryness. The individual samples were weighed; the total mass amounted to 403.4 mg. Since this is an increase in mass of about 120 mg. it would appear that the filter paper contains some water soluble material. However, of this

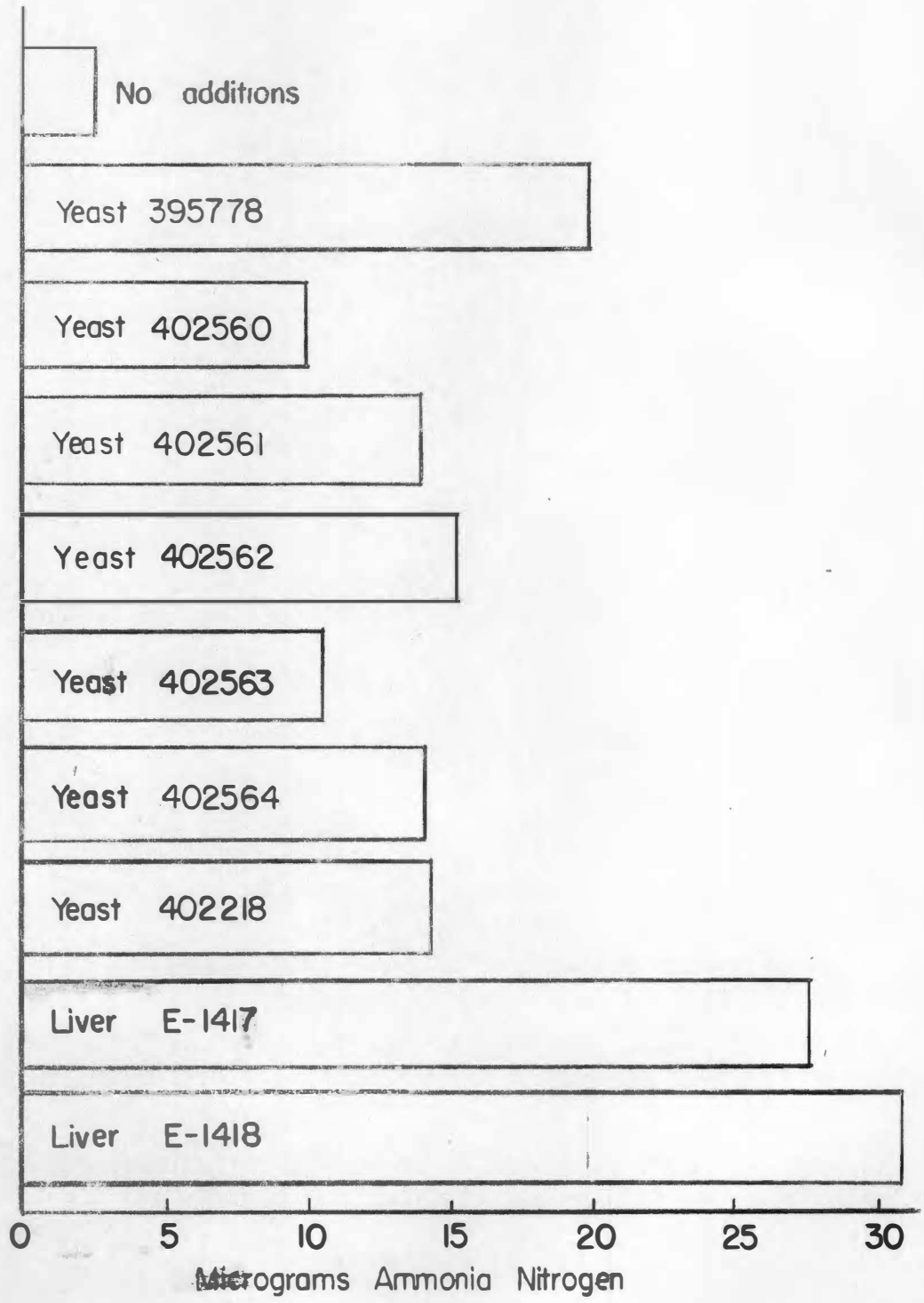


Figure 22: Relative stimulatory effects of various samples of yeast and liver extracts on aspartic acid deaminase of aged cell suspensions of B. cadaveris

total mass only 77 mg., the fractions Rf 0.5, 0.6, and 0.7, were active. The product material when combined contained 1.1×10^{-3}ug. of biotin per mg. as assayed by S. cerevisiae (139) and 2.0×10^{-4}ug. of biotin per mg. as assayed by S. cerevisiae (Java). It may be recalled that heretofore S. cerevisiae (Java) responded to the coenzyme fraction; either the sample no longer contains the substance that stimulated S. cerevisiae (Java), or this organism has undergone a mutation and is no longer able to utilize this material as a source of biotin.

The 77 mg. sample was placed on 16 strips and rerun through the phenol-water system as described. Ten samples, with a total weight of 126.2 mg., were obtained. Of this, 68 mg. was in the Rf 0.4 to 0.7 range, and contained nearly all of the active material. S. cerevisiae (139) showed a biotin concentration of 4.4×10^{-5}ug. per mg. of the combined samples of the coenzyme fractions; S. cerevisiae (Java) gave a biotin concentration of 4.3×10^{-4}ug. per mg. of sample. Although this relation is more in agreement with that of earlier data the magnitude of stimulation of growth of S. cerevisiae (Java) is still considerably less than that previously obtained.

The Rf 0.4 to 0.8 fractions were placed on 85 strips and run in the phenol-water system to an average length of 273 mm. Although this resulted in 10 fractions whose total mass was 286 mg. a large part of this was water insoluble.

By combining fractions R_f 0.4, 0.5, 0.6, and 0.7 by suspending in 10 ml. of water, filtering through two thicknesses of Whatman no. 50 filter paper, and evaporating the filtrate to dryness, it was possible to obtain a water soluble fraction weighing 32.3 mg. A similar treatment was given the coenzyme-free fraction which resulted in 14.8 mg. of material. The two fractions were assayed with S. cerevisiae (139) before and after hydrolysis (Figure 23). It can be seen that the coenzyme fraction contains some substance, liberated on acid hydrolysis, which stimulates the growth of S. cerevisiae (139); this substance does not follow the acid degradation pattern of free biotin or the biotin present in the liver extract fraction containing no coenzyme.

J. The Separation of the Coenzyme from Yeast Extract

Five tenths of a gram of Difco yeast extract, lot no. 395778, was dissolved in 12.5 ml. of water; exactly 0.05 ml. (2.0 mg.) was placed on each of 200 strips. These strips were run in the phenol-water system, dried, sectioned, eluted, and evaporated to dryness. The samples were then assayed for activity against B. cadaveris as showed in Figure 24. It is apparent from these data that both yeast and liver extracts produce similar chromatograms. Assay of the R_f 0.6 and 1.0 fractions before and after hydrolysis reveal astonishingly similar results to that obtained with the

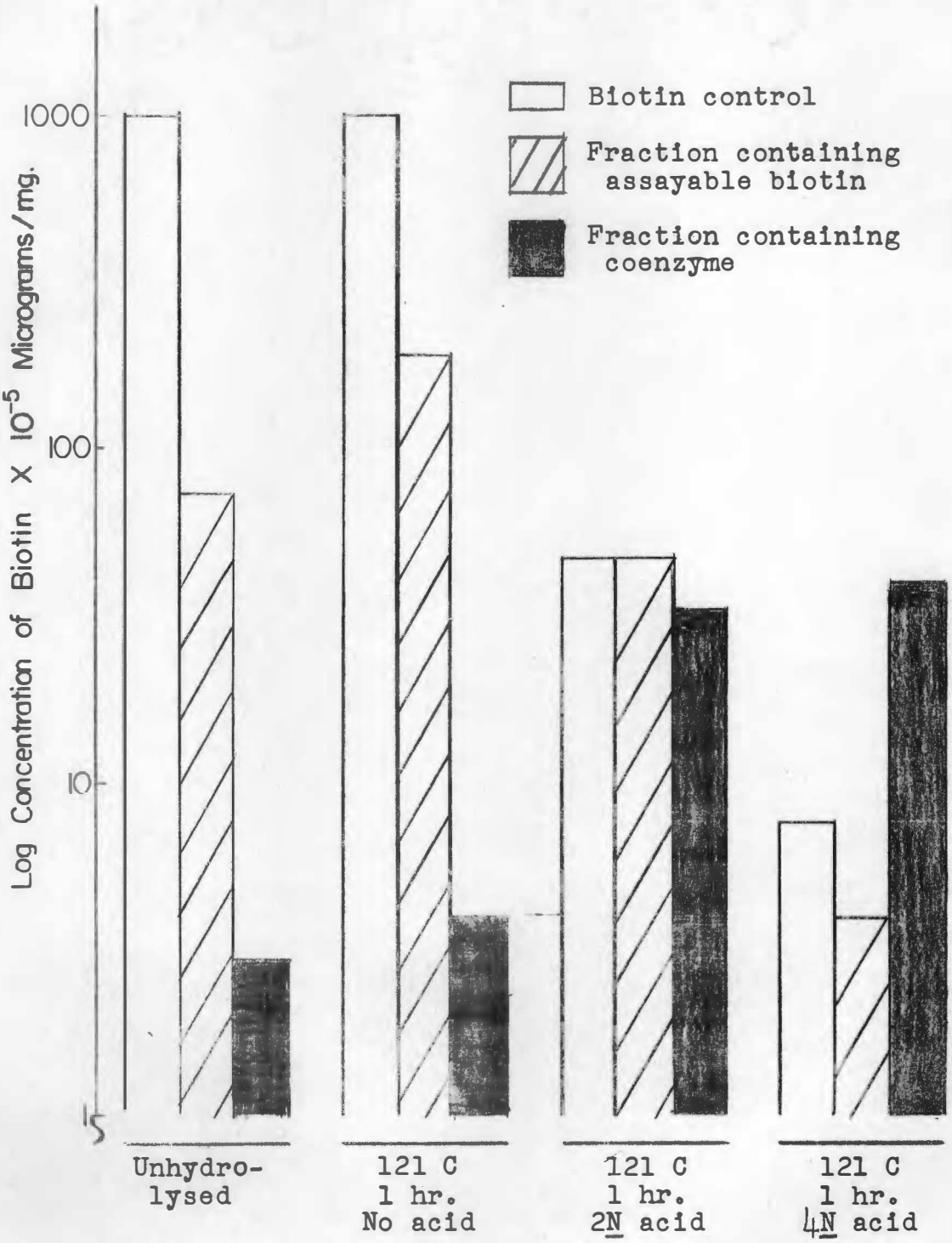


Figure 23: Relative stimulation of growth of S. cerevisiae by biotin, liver extract fraction containing biotin, and liver extract fraction containing coenzyme before and after hydrolysis

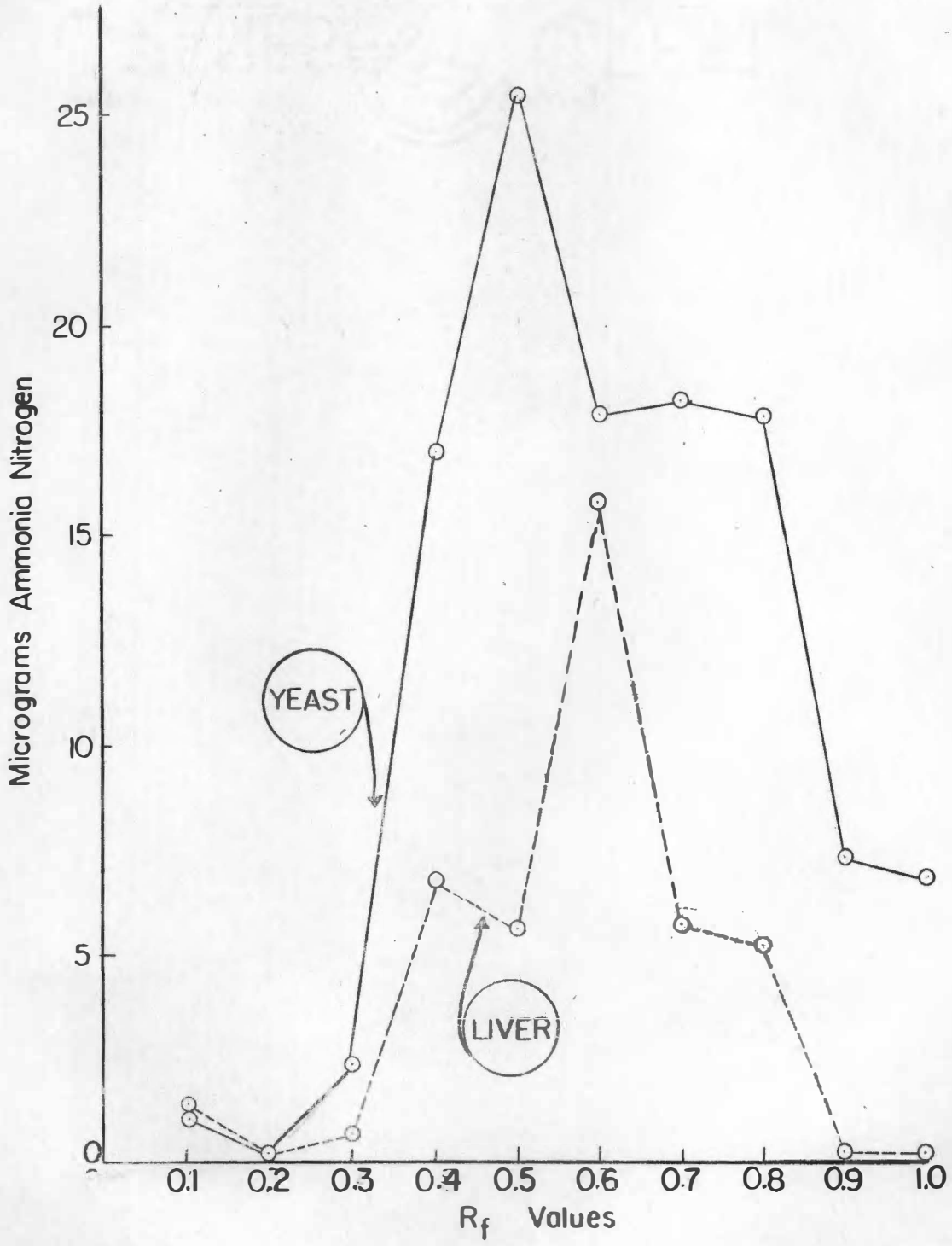


Figure 24: Distribution of the coenzyme in yeast extract (one step purification) and in liver extract (three step purification) after separation by paper strip chromatography in the phenol-water system

liver fractionation (Figure 25). Further, assay of the Rf 0.6 fraction before and after hydrolysis for coenzyme content and ability to stimulate growth of S. cerevisiae (139) show unique correlation. It would appear (Figure 26) that the coenzyme activity is destroyed only by that concentration of acid which will liberate a biotin-like substance than can replace biotin for growth of S. cerevisiae (139).

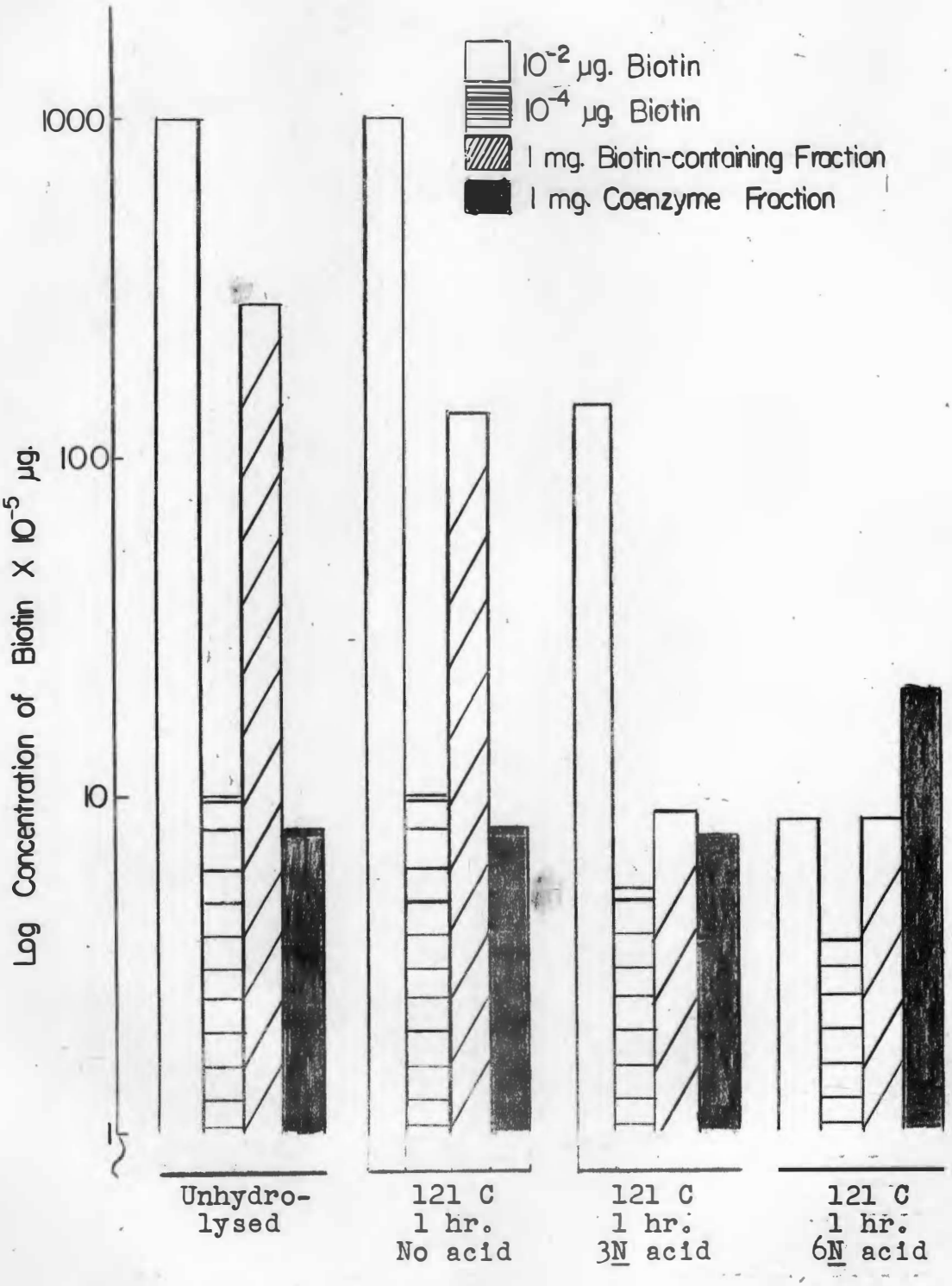


Figure 25: Relative stimulation of biotin, biotin containing yeast extract fraction, and coenzyme containing yeast extract fraction before and after hydrolysis on the growth of S. cerevisiae in a biotin deficient medium

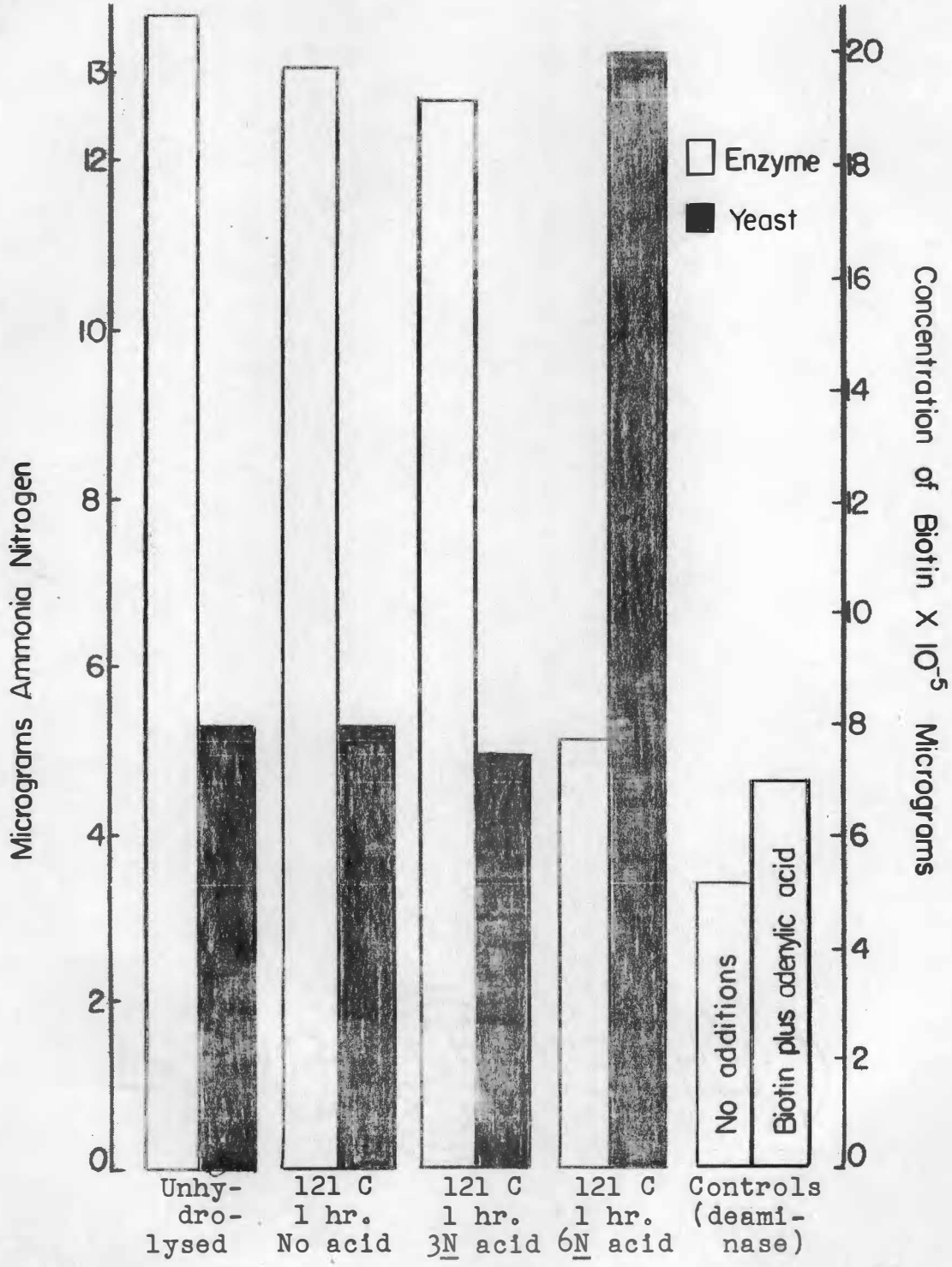


Figure 26: Relative stimulatory effect of the coenzyme fraction (biotin free) of yeast extract on growth of S. cerevisiae and on aspartic acid deaminase before and after hydrolysis

CHAPTER IV

DISCUSSION

A. Summary of Experimental Data

The experimental data presented, along with evidence previously described in the literature concerned with the enzymatic deamination of aspartic acid, serine and threonine, can be summarized as follows:

(1) The exposure of washed cells of B. cadaveris and other Gram negative bacteria to one M phosphate buffer at pH 4 causes a distinct decrease in activity of oxalacetic acid decarboxylase and aspartic acid, serine and threonine deaminases (Lichstein and Umbreit, 1947a,b; Lichstein and Christman, 1948; Axelrod, et al., 1948; Wright, et al., 1949).

(2) These partially resolved enzymes can be reactivated by biotin (Lichstein and Umbreit, 1947a,b; Lichstein and Christman, 1948; Wright, et al., 1949), by adenylic acid (Lichstein and Christman, 1948; Axelrod, et al., 1948), by biotin with adenylic acid (Lichstein and Christman, 1948), by yeast extract (Lichstein, 1949a; Lichstein and Christman, 1949), and by liver extract.

(3) Lichstein and Christman (1948) showed that the biotin content, as assayed by S. cerevisiae, decreased in

cells after phosphate aging.

(4) The character of stimulation of these enzymes caused by the various substances studied is distinctly different. Biotin activation is immediate, even at pH 4; however, the maximum level of activation is decidedly less than the theoretical limit. Adenylic acid stimulation is characterized by a rather high concentration requirement, an initial lag at pH 4, and nearly the same maximum limit shown by biotin. The substance in yeast or liver extract differs from these other stimulants in that the reaction proceeds to the theoretical limit even at pH 4.

(5) The material present in yeast extract causes a response of the apoenzymes of aspartic acid, serine and threonine deaminases. These responses vary linearly with the concentrations of yeast extract or substrate added.

(6) The substance in yeast extract can be destroyed by incineration, but it is stable to weak acid or alkaline hydrolysis, dry or wet heat, and repeated evaporation to dryness from aqueous solutions.

(7) The material can be separated from the free biotin in yeast extract by paper strip chromatography. In the biotin-free state it does not support the growth of S. cerevisiae (139) in a medium deficient in this vitamin. Evidence was presented that at least certain samples of this material did support the growth of S. cerevisiae (Java) in a

biotin deficient medium. Further purification of these samples by chromatography with other solvent systems showed no decrease in the ability to support the growth of S. cerevisiae (Java). A repetition of this work at a later date yielded coenzyme containing fractions which did not support the growth of this organism.

(8) Liver extracts contain a substance probably identical to the coenzyme in yeast extracts, since distribution of the material by paper strip chromatography with the phenol-water system was the same, and stimulations of partially resolved aspartic acid deaminase were comparable.

(9) A fraction containing the coenzyme was isolated from liver extract through four separate chromatographic processes. This highly purified fraction contained only a small amount of biotin as assayed by S. cerevisiae (139). On strong acid hydrolysis a substance was liberated from this fraction which supported the growth of this organism. This material was not D-biotinic acid, since there were large differences between the acid degradation curves of these two substances.

(10) The same acid-stable biotin-like material was also obtained from yeast extract, by the same type of process.

(11) Hydrolysis of the coenzyme fraction sufficient to liberate the biotin-like acid-stable component was accompanied by a loss in coenzyme activity.

B. Theoretical Implications

Although aging is a radical departure from the accepted experimental techniques of producing an apoenzyme, validation of the technique is evident because the results by this method agree with those obtained by other techniques, and the aging method has been reproduced in several different laboratories. The apoenzymes can be reactivated in part by either biotin or biotin with adenylic acid. Complete, or nearly complete, reactivation can be accomplished by the addition of a substance present in either yeast or liver extract. Figure 27 presents a hypothetical scheme of degradation of aspartic acid deaminase by aging.

All four types of aging have been demonstrated experimentally. Type IV aging (total destruction of the coenzyme) occurs most frequently, resulting in a suspension of apoenzyme that can be stimulated only by the addition of coenzyme. If this material is added, then the limiting factor in deamination is the concentration of holoenzyme, and the reaction should proceed to theoretical limits with time. In aging of Types I, II or III biotin, adenylic acid, or both will stimulate the cells; the limiting factor in such cases will be the extent to which the coenzyme can be synthesized from the added reagents and the fragments left from aging. Gale (1938) produced Types II and IV aging by the addition of toluene to washed cell suspensions.

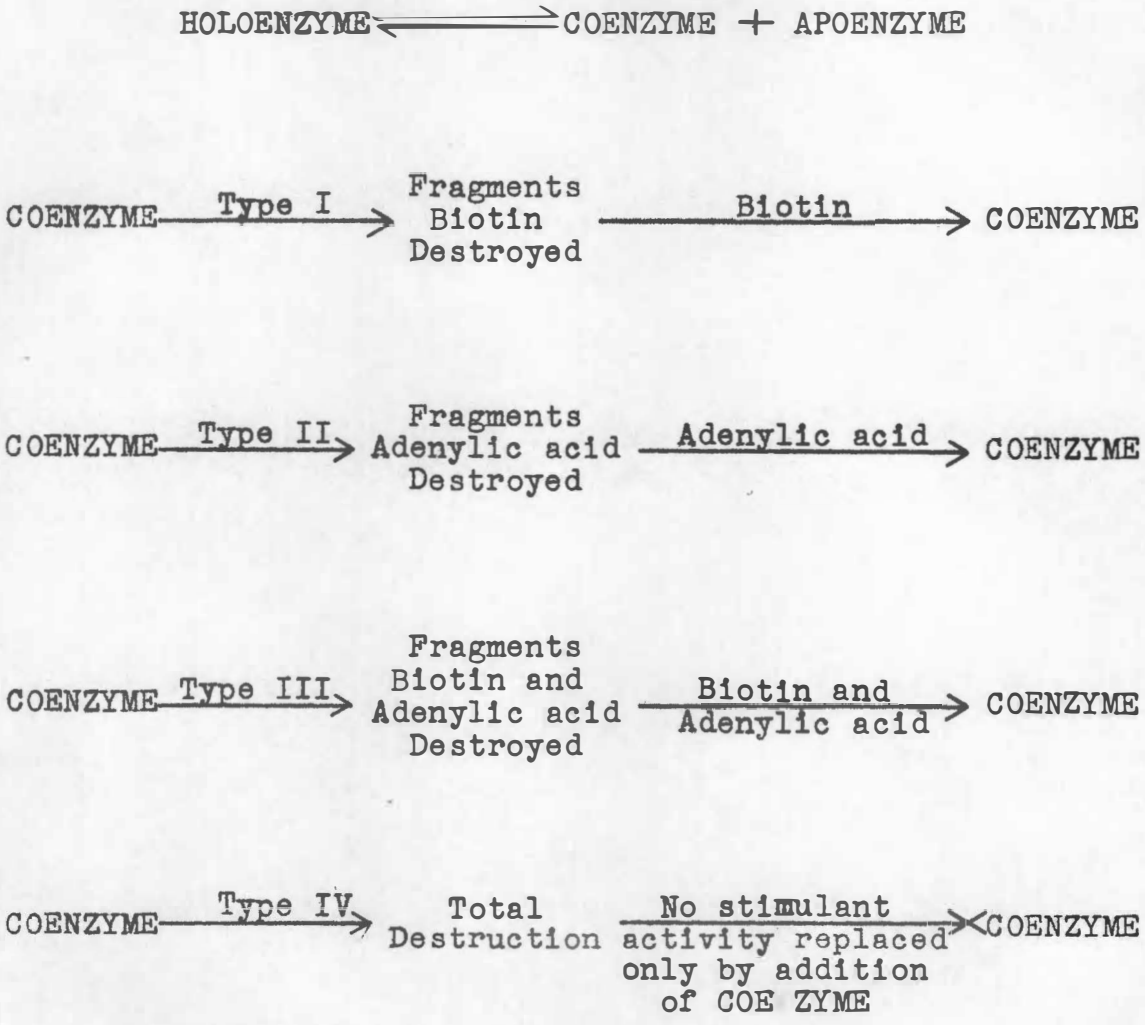


Figure 27: Hypothetical degradations of the coenzyme caused by aging

The active material in yeast or liver extract, which either is or closely resembles the coenzyme, is organic in nature; it is very stable (at least in impure form) to most physical and chemical manipulations including mild acid or alkaline hydrolysis. Strong acid hydrolysis of this material, after it has been freed of D-biotinic acid, does destroy the coenzymatic activity simultaneously liberating a substance which can replace D-biotinic acid as a growth requirement for S. cerevisiae (139). These observations suggest strongly that a component of the coenzyme can act as a substitute for biotin, and further, that biotin is chemically converted into the coenzyme. This is schematically presented in Figure 28.

The actual role of biotin is apparently placed in the formation of this coenzyme. The acid-stable intermediate has never been reported, but its existence is evident from the data presented here, and may be inferred from data of Bowden and Peterson (1949) who studied acid degradation of bound forms of biotin.

The role of adenylic acid remains unsolved; it is entirely possible that adenylic acid can serve either as part of the coenzyme moiety (in which case it would be involved in the conversion of the acid-stable form of biotin into the coenzyme), or as a specific phosphorylating agent in either of the proposed reactions.

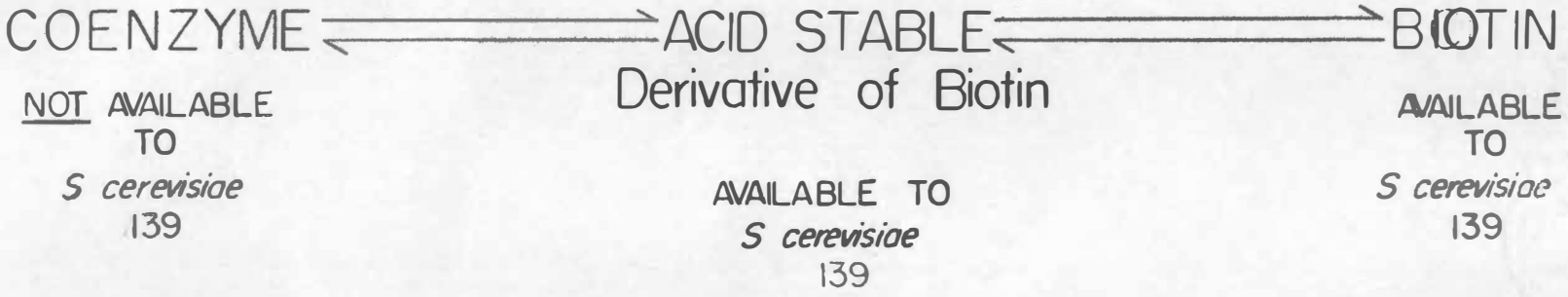


Figure 28: Hypothetical formation of the coenzyme of aspartic acid deaminase from biotin

CHAPTER V

SUMMARY

Data are presented which characterize the coenzyme of aspartic acid, serine and threonine deaminases. This coenzyme, found both in liver and yeast extracts, is organic in nature, and quite stable to most physical and chemical manipulations. Techniques for the isolation of this coenzyme from natural sources and its separation from free biotin are presented. It is destroyed on strong acid hydrolysis; simultaneous with the loss of coenzyme activity, the acid hydrolysate will support the growth of S. cerevisiae (139) which does not respond to the unhydrolysed material. The substance liberated from the coenzyme fraction is not D-biotinic acid, as is evidenced by the differences in acid stability. It is proposed that this substance is a chemical intermediate in the enzymatic synthesis of the coenzyme from biotin.

Hypothetical mechanisms for the aging process and the coenzyme formation from biotin are proposed and discussed.

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A. Biography

John Francis Christman was born in Terre Haute, Indiana, on February 17, 1924. He was educated in the elementary schools of this city and was graduated from high school in June, 1941. The following September he matriculated at the University of Notre Dame, and was graduated with a B.S. in Chemistry degree in June 1944. He then entered the Graduate School of Indiana University in September 1944 and was graduated with an M.A. degree with a major in Chemistry in August 1946. He entered the Graduate School of The University of Tennessee in September 1946, and received the M.S. degree with a major in Bacteriology in June 1948.

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