INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame. If copyrighted materials were deleted you will find a target note listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.

University
Microfilms
International

300 N. ZEEB RD., ANN ARBOR, MI 48106

Gupta, Narendra K.

ISOLATION AND CHARACTERIZATION OF HUMAN LIVER GUANINE DEAMINASE

City University of New York PH.D. 1982

ISOLATION AND CHARACTERIZATION OF

HUMAN LIVER GUANINE DEAMINASE

by

Narendra K. Gupta

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1982

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirements for the degree of Doctor of Philosophy.

January 18, 1982
Date

Morton D. Glantz
Professor Morton D. Glantz

Date

Aaron Lukton
Professor Aaron Lukton

Professor Diana Beattie

Professor William Berkowitz

Professor Irwin Oreskes

Professor Thomas Strekas

Supervisory Committee

The City University of New York

Abstract

Isolation and Characterization of

Human Liver Guanine Deaminase

by

Narendra K. Gupta

Advisor: Professor Morton D. Glantz

Guanine deaminase (E.C.3.5.4.3, guanine aminohydrolase [GAH]) was purified 3248 fold from human liver by a combination of ammonium sulfate fractionation, DEAE-cellulose, hydroxylapatite and affinity chromatography to the homogeneity. The enzyme is a dimer protein of the molecular weight of 120,000 with each subunit of 59,000 as determined by gel filtration and SDS-gel electrophoresis. GAH is an acidic protein as evidenced by the amino acid analysis and isoelectric focusing with a pI of 4.76, and is enriched with glutamic acid, aspartate, alanine and glycine. It shows a sharp pH-optimum of 8.0 and was very sensitive to p - hydroxymercuribenzoate inhibition. A Ki of 5×10^{-5}M and 1.53×10^{-5}M was obtained for 5 - aminoimidazole - 4 - carboxamide and p - hydroxymercuribenzoate respectively demonstrating strong inhibition. The inhibition with idoacetic acid showed only a 7% loss in the activity at 1×10^{-4}M and 24% loss at 1×10^{-3}M concentration after 30 minutes of incubation. Guanine was the substrate for GAH in all the inhibition studies. p - Hydroxymercuribenzoate incubation for 30 minutes, however, resulted in a loss of 91% of

the activity at a concentration of 1×10^{-4}M. Enzyme was found to be stable up to 40°C but lost almost all of its activity at 65°C at 30 minutes incubation. Km value of 2×10^{-4}M for 8 - azaguanine was obtained at pH 6.0, while a Km of 1.538×10^{-5}M was obtained for guanine as substrate at pH 7.0. The two pKa values obtained in the pH studies were 5.85 and 8.0. The plots of log Vmax/Km vs. pH, Vmax vs. pH and pK_m vs. pH were made. The N-terminal amino acid was found to be valine while the C-terminal residue was alanine.

Acknowledgements

I am highly indebted to Dr. Morton D. Glantz, for his expert guidance, excellent valuable suggestions, support and understanding during the course of this study.

I sincerely thank the members of my advisory committee, Dr. Diana Beattie, Dr. William Berkowitz, Dr. Irwin Oreskes and Dr. Thomas Strekas for their cooperation and encouragement.

I would like to thank all my family members for the patience, support and encouragement shown during the course of my graduate studies.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Km	Michaelis constant
PHMB	p - hydroxymercuribenzoate
BIS	N,N' - methylenebisacrylamide
DEAE	Diethylaminoethyl cellulose
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
DTT	Dithiothreitol
SDS	Sodium dodecyl sulfate
TEMED	N,N, N,' N' - tetramethylenediamine
Tris	Tris hydroxymethyl aminoethane
GMP	Guanosine monophosphate
IMP	Inosine monophosphate
PRPP	5 phosphoribosyl 1 pyrophosphate
Pi	Isoelectric point
PPi	Pyrophosphate
HG-PRTase	Hypoxanthine guanine phosphoribosyl transferase
A-PRT	Adenosine phosphoribosyl transferase
TLC	Thin layer chromatography
Ovalb	Ovalbumin
Chymo	Chymotrypsinogen

Introduction

A. Biological Significance

Purines are intricately interwoven into human intermediary metabolism as substrates, cofactors and regulatory molecules. Specialized functions of these compounds include a role in cell energy transport, nucleic acid synthesis, vasodilation, neurotransmission and platelet aggregation.

Purine metabolism refers to a complicated series of enzyme reactions which synthesize, catabolize and transform purine compounds. These pathways have attracted great interest in clinical medicine as a result of their relevance to a number of disease states. Hyperuricemia and gout, which afflict as many as 1 - 2% of the Normth American population, are most likely related to disorders of purine metabolism in a large proportion of cases. The description and elucidation of inborn arrors (Table 1) of purine metabolism have added a wealth of information concerning the function and regulation of these pathways in man. Drugs used for cancer chemotherapy or immunosuppression such as azathiopurine, 6 - mercapto-purine and methotrexate, inhibit one or more reactions of purine bio-synthesis. Guanine deaminase (Guanine Aminohydrolase or GAH or guanase EC 3.5.4.3) catalyzes the conversion of guanine to xanthine. Free guanine comes wither from the diet or from the breakdown of guanine monophosphate (GMP) and consequently either it is recovered by the salvage pathway enzymes, or eliminated as uric acid by catabolism to xanthine with guanine deaminase.

Fig 1. Purine catabolism (86). Three major pathways lead from nucleotides to uric acid: (A) inosine is converted to hypoxanthine, xanthine and uric acid; (B) xanthosine is converted to xanthine and uric acid; (C) guanosine is converted to guanine, xanthine and uric acid. (1) dephosphorylation reaction by 5'-nucleotidase or nonspecific phosphatase; (2) AMP deaminase; (3) adenosine deaminase; (4) purine nucleoside phosphorylase; (5) guanine deaminase; (6) xanthine oxidase.

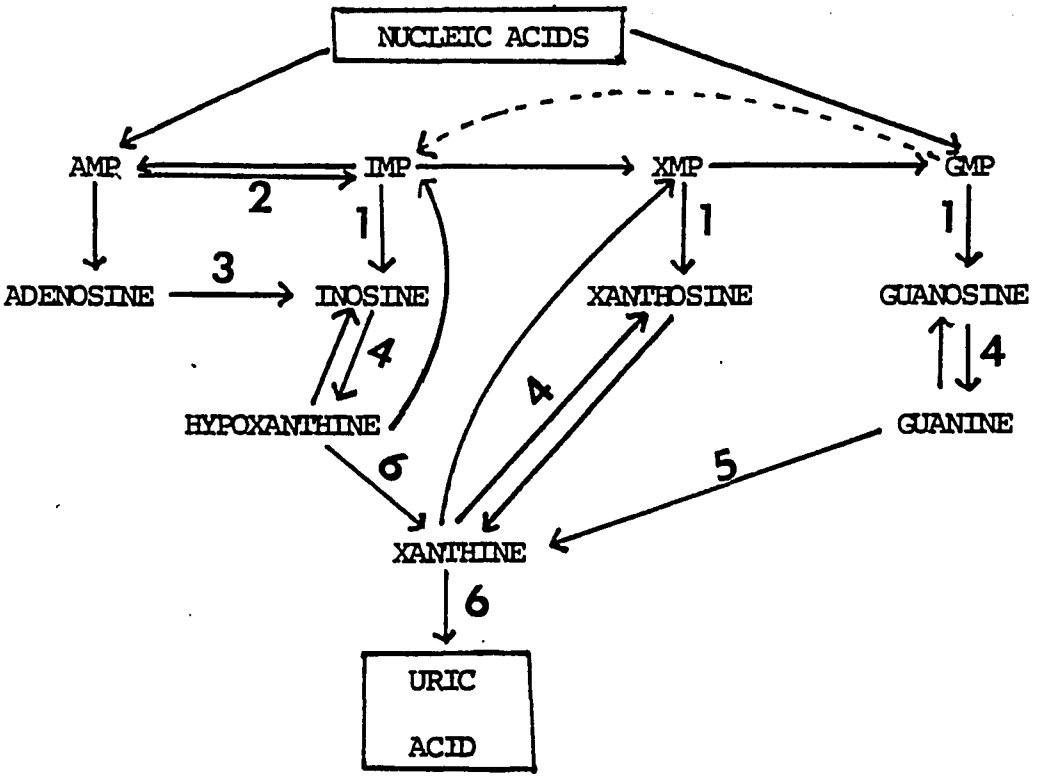

NUCLEIC ACIDS
AMP
IMP
XMP
GMP
2
1
1
1
ADENOSINE
3
INOSINE
XANTHOSINE
GUANOSINE
4
4
4
HYPOXANTHINE
GUANINE
6
5
XANTHINE
6
URIC
ACID

TABLE 1

Inborn Errors of Human Purine Metabolism (86)

Components of Purine Metabolism	Inborn Errors
1. Purine biosynthesis de novo	a) PRPP synthetase mutant
	b) Glutamine PRPP amidotransferase mutant
2. Nucleotide interconversions	None known
3. Salvage pathways	a) Lesch-Nyhan syndrome
	b) Partial HG-PRT deficiency
	c) Partial A-PRT deficiency
4. Purine catabolism	a) Adenosine deaminase deficiency in combined immuno deficiency disease
	b) Xanthinuria
	c) Elevated xanthine oxidase

This network of reaction is of interest because of the chemotherapeutic activity of 6 - thioguanine and 8 - azaguanine (1). When 6 - thioguanine is administered, it is incorporated in DNA and the result is a cell that is viable but cannot replicate DNA. When 8 - azaguanine is administered, it is incorporated into RNA, which causes errors in protein synthesis. However, the effectiveness of both drugs is limited because guanine deaminase converts 6 - thioguanine to 6 - thioxanthine and 8 - azaguanine to 8 - azaxanthine, biologically inert compounds which have no growth inhibiting effect. Kanzawa (2) has shown that by inhibiting guanine deaminase with 4 - amino - 5 - imidazole carboxamide, the antitumor activity of 8 - azaguanine was enhanced, therefore, the importance of the deaminating enzymes of the nucleic acid system cannot be underestimated. The abnormal cell growth of cancer tissue may be atributed to the absence or possible destruction of one or more of these enzymes in the cancerous tissue of the animals. It has been postulated that because of the absence or low level of these deaminating enzymes and of the xanthine oxidase and uricase in cancer tissue, there is a continuous recycling of uncatabolized purine fragments into the pool of synthetic substances of nucleic acids (3).

Guanine deaminase (GAH) appears to play a role in the normal metabolism of guanine. According to Brown, et al. (4), when guanine (^{14}C-8) is given orally or intraperitoneally to Sherman strain white rats, there is recovery of over 90% of the radioactivity in the urine, plus high activity of the urinary allantoin which could be at least partially attributed to the abundance of GAH in the tissue. Balis (5)

however does not consider this as enough evidence for the GAH playing a rate determining step in the utilization of exogenous guanine. However, 4 - amino 5 - imidazole carboxamide, an inhibitor of GAH, increases the incorporation of guanine into nucleic acid.

Since the neoplastic tissues are reported to have decreased levels of catabolic enzymes, the disorder of the guanine catabolism in tumor cells which are able to retain their metabolites in a utilizable form, promotes a recycling of these metabolites, and causes an increased synthesis of nucleic acids due to a larger pool of the precursors. The studies of De Lamirande (3) with rat liver bearing hepatoma revealed that the catabolism of purines is completely blocked at the level of xanthine and hypoxanthine suggesting their recycling. The activities of guanine deaminase are greatly reduced. Kidder (6) noticed a difference in the utilization of guanine between normal and tumor tissues, indicating that the tumor cells might require a purine for nucleic acid synthesis. Mandel and Carlo (7) found insignificant incorporation of guanine (^{14}C-4) into the tumor nucleic acid of CAF mice bearing sarcoma 37 while incorporating significant amounts into the liver which has a rapid turnover of nucleic acid. Tumor cells have a rapid cell division too, but his results showed a very poor incorporation of guanine in the RNA and DNA fraction of the tumor. Balis (8), however, concluded from his studies that the tumors do possess the enzymatic machinery for the incorporation of exogenous guanine into nucleic acid. The difference between normal and tumor tissue lies in the concentration of guanine. Tumors in the host utilize exogenous guanine at a much lower fraction of their potential than do normal tissues.

Moore and Le Page (9) demonstrated that thioguanine in normal tissues shows a parallel between incorporation into nucleic acids and catabolism to thiouric acid while in tumor cells, it primarily goes into nucleic acid synthesis.

The level of catabolic activities in tumors was indirectly demonstrated by Bennett et al (10), by studying the rate of disappearance of labelled guanine in actively growing tumors suggesting further a recycling hypothesis. There was no significant loss of administered labelled nucleic acid precursor. This was further supported by the studies of De Lamirande, et al (3), thus suggesting that the balance between anabolism and catabolism in tumor cells was oriented towards anabolism and that the increased synthesis of nucleic acid in tumors might be due to the large pool of the precursors. In the case of 8 - azaguanine, its carcinostatic action is reversed by administration of guanine (11). The action of the drug does not destroy the tumor, but suppresses the nucleic acid synthesis and hence cell proliferation is inhibited (12).

In the Lesch - Nyhan Syndrome (13), the salvage pathway enzyme HGPRTase is missing or not functioning, which results in severe mental retardation, muscular spasm and excessive build up of uric acid. This can very well demonstrate that the irregularities at this point of guanine metabolism can greatly effect the organism. Hence because of the key role of GAH in the guanine metabolism, investigation into the biochemical nature of the enzyme are of great interest.

B. Historical Background

In 1904 Walter Jones (11) observed that autolyzed thymus or kidneys form xanthine and hypoxanthine, while acid hydrolysis produces adenine and guanine. Shortly afterwards, Jones and Partridge (12) noted that pancreas contains guanine deaminase enzyme, which converts guanine to xanthine. They named it "guanase". Although Schittenhelm (14) took up further studies a few years later, a brief period of inactivity followed because of the lack of specific methodology for characterization.

With the work of Jorpes and Myrback (15), a revival of interest in the purines occurred as they became interested in the identification of the individual components of nucleic acid systems. Their method was based on the determination of the nitrogen content in mixtures of silver and copper precipitated purines. Micro-procedures based on the principle of Jorpes were later developed by Graff and Maculla (16) for the determination of guanine and adenine in nucleic acids. The work of Schmidt (17) in 1933 introducing enzyme specificity as an analytical tool, laid the foundation of the modern methods in the identification of nucleic acid constituents. He used the rabbit liver as a source of guanine deaminase, estimating ammonia release by deamination. He further showed that guanosine or GMP could not act as substrate for guanine deaminase and that it was different from guanylic acid deaminase. However, Hitchings and Falco (18) in 1944 tried to purify the enzyme from rabbit liver acetone powder extract by dialyzing and using alumina absorption column. However, they did not achieve the purification successfully.

In 1947 Kalckar (19, 20) succeeded in combining the enzymatic method with the spectrophotometric method for the micro estimation of individual purines. This was a tremendous advance for it was then possible for one to follow the course of enzyme activity. Kalckar estimated the concentration of various purines with considerable specificity by measuring spectral changes in the UV region caused by specific enzymes on purine substrates. He determined the activities of guanine deaminase in preparations from rat liver and muscle by recording spectral changes in time caused by transformation of guanine as substrate to xanthine as product. He was also able to partially purify the enzyme by ammonium sulfate fractionation and cold ethanol precipitation.

In 1948 Vischer and Chargaff (21) combined paper chromatographic separation of purines with spectrophotometric analysis. A mixture of butanol: ethylene glycol: water in 40:40:20 ratio was used as solvent and the purines were eluted with the aid of guide stips on the basis of their Rf values, and subjected to ultraviolet spectroscopy. Their success in this preliminary experimentation led to the application of the method to the quantitative estimation of the composition of nucleic acids both in animal tissues and yeast hydrolysates.

In 1948 Kream and Chargaff (23) continued the study of the metabolic inter-conversions of purines on the basis of enzyme specificity. At the same time Chargaff and Vischer (21) worked on the isolation, purification and properties of the deaminating enzymes. In 1950, Rousch and Norris (22) spectrophotometrically assayed guanine deaminase at 245nm, differentiating between guanine and xanthine absorption.

8 - azaguanine also was shown to be a substrate for the enzyme. In 1952, an extensive study of the deaminating enzymes was made by Kream (23) in collaboration with Chargaff. Extracts of acetone powder preparations of rabbit liver were utilized as the enzyme source. In 1955 Rakosky, et al. (24) reported the isolation of an enzyme similar in several respects to guanine deaminase from Clostridum acidiurici, showing xanthine as an isolable intermediate, in the anaerobic degradation of guanine. The studies of De Lamirande and Allard (3), of the purine metabolizing enzymes in normal and tumor of rat liver tissue brought much information in cancer research. Their studies on the increased incorporation of guanine or its derivatives in tumor RNA revealed an impairment in the catabolism of guanine or its derivatives promoting a recycling of these metabolites into nucleic acids. They found that unlike normal rat liver tissue, tumor is either deficient or devoid of xanthine oxidase and uricase activity, indicating that purine catabolism is blocked at the stage of xanthine and hypoxanthine.

C. Guanine Deaminase in Brain Tumors and Various Human Organs

Comparative activity of the guanine deaminase was studied by Norstrand (55) in human viscera using autopsy material in spinal fluid, blood, in various levels of spinal cord, normal brain and in two malignant brain tumors.

The report (55) suggested that the values for the liver were the highest of all organs, followed closely by the brain. In three out of four autopsies, guanine deaminase was highest in liver. The one case in which

liver was exceeded by brain was that of a Laennec's cirrhosis, where part of the liver was replaced by fibrous tissue. Even then, the value for guanine deaminase was high.

Guanine deaminase activity in organs of experimental animals.

In spite of the fact that guanine deaminase activity of experimental animals has been investigated, the existing data are conflicting and do not as yet provide a clear picture as to the distribution and the role of this ferment in metabolism.

Dixon and Webb (77) listed determinations which had been performed on six organs of the rat and mouse in the following order of decreasing activity: Rat: = brain, spleen, liver, kidney, pancreas, skeletal muscle. Mouse: = thymus, spleen, small intestine, kidney, liver and brain. Although the findings in the rat were somewhat similar to this report the high activity in the spleen does not compare with the low activity seen in these cases.

The values given for the normal mouse by Hirschberg, Kream and Gelhorn (78) for three organs (brain-4.18; liver-2.42; and kidney-0.56) are the reverse of those given by Dixon and Webb. In the tumor-bearing mice of Hirschberg et al. there was extraordinarily high activity in the intestine (greater than 16). These results are not in agreement with findings in the human, where no activity was seen in the large intestine, and only rather low activity (45.2uM) found in the small intestine. The latter authors found significant deaminase activity of mouse blood, with activity only associated with erythrocytes.

Greenstein (79) et al. found no guanine deaminase in mouse pancreas but considerable amounts in rat pancreas. There was, however, no

outstanding difference between the appreciable guanine deaminase content of other tissues of the two species.

Korotkoruchko (80) et al. studied deaminase activity in tissues of normal mice, rats, rabbits, dogs, cattle, horses and clots of human blood, and of mice with Ehrlich's carcinoma (ascitic and hard tumor forms). Data showed that guanine deaminase activity is present in brain, liver and muscles of healthy and carcinomatous mice, with highest activity found in brain tissues, and lower in muscle tissue. These authors also found the activity of guanine deaminase to be higher in extract of hard tumors than in the muscles of healthy and of carcinomatous mice, and in ascitic tumors, in which the ferment is located in cells. Block and Johnson (81) found that rat skin homogenates converted guanine deaminase to xanthine. Among rabbit tissues investigated by Hirschberg, Kream and Gelhorn (78) only liver and intestinal mucosa exhibited measurable activity. There was no demonstrable activity in rabbit blood, serum, kidney, testis or spleen.

Guanine deaminase activity in human viscera and body fluids.

1. Normal human viscera:

As mentioned previously, data on the activity of the enzyme guanine deaminase in human organs are scarce and fragmentary. Gellhorn (82) who determined the concentration of guanine deaminase in two biopsy specimens of human liver obtained at laparotomy from patients without malignant or liver diesase found the enzyme to be abundant. (Two and a half times that of rabbit liver, and four times that of mouse liver.)

Another human tissue assayed for its 8 - azaguanine deaminase content is skin, which, unlike rat skin has none. Enzyme assays of split

thickness grafts of normal human skin removed at the time of plastic surgery revealed no guanine deaminase activity (81, 82). This is of interest since the major toxicity of 8 - azaguanine is manifested by skin rashes due to the fact that the tissue, unprotected by the detoxifying agent guanine deaminase is unable to metabolize 8 - azaguanine to the biologically inactive 8 - azaxanthine.

According to Hirschberg et al. (78) homogenates of adult human brain exhibit the highest deaminase activity of any animal tissue which has been studied. This was not the case, however, in this report where the enzyme was highest in liver in all cases but one.

2. Human body fluids:

a. Spinal fluid

In the sixteen spinal fluids taken at random from patients with various disease of the nervous system or with no disease, the values were all negligible, and far below the limits of sensitivity of the method, ranging from 0.072uM in a patient with multiple sclerosis and cerebral metastases due to a bronchogenic carcinoma, to 0.996uM in a patient with amyotrophic lateral sclerosis. The average value of the four normal patients was 0.207uM.

b. Blood

Plasma: - No detectable guanine deaminase was found in the plasma of 12 cases studied (4 normals, 4 individuals with far-advanced multiple sclerosis, one case of multiple cerebral infarctions due to cerebrovascular disease, and three cases of far-advanced Laennec's cirrhosis).

Red cells: - In the red cells from the above cases, however, the values were hardly detectable (average 25.1 uM/ml of red

The above findings are in agreement with those of Thomas Passananti (83) who observed no guanine deaminase activity in sera of 55 normal individuals, and in 230 patients with various nonhepatic disease. In contrast to leucine amino peptidase, guanine deaminase activity is not elevated in any stage of pregnancy. Nor is it elevated in cases of salicylate intoxication, acute infectious mononucleosis, with hepatomegaly, muscular dystrophy, gout, pancreatitis, or infiltration of the liver caused by carcinoma, leukemia or lymphoma.

Failure to find any guanine deaminase in the plasma of three cases of far-advanced Laennec's cirrhosis is likewise in agreement with the results of Passananti (83) who found that the sera from 90 patients with cirrhosis or obstructive jaundice displayed very little or no guanine deaminase activity. On the other hand, sera of 30 patients with infectious hepatitis or homologous serum jaundice has 100% conversion. To date no false positive or false negative tests have been reported in the cases of infectious hepatitis studied by Passananti. It is conceivable that the acute necrosis of liver cells that occurs with infectious hepatitis, with release of enzyme into the blood stream is an explanation for the high serum values in this condition. In cases of far-advanced Laennec's cirrhosis, on the other hand, where a considerable portion of the liver has been replaced by fibrous tissue one would expect to find the enzyme in the blood. As mentioned previously, Hirschberg et al. found significant deaminase activity in mouse blood, with activity only associated with erythrocytes, but no demonstrable activity in rabbit blood.

3. Human brain tumors:

In contrast to the high values in normal brain, homogenates of

glioblastoma multiforme, a highly malignant brain tumor have been found to be devoid of measurable azaguanine deaminase by Hirschberg (78) et al. The two cases studied (55) were both malignant infiltrating tumors; - one, an astrocytoma, Grade III, of the left occipital lobe, and the other, a glioblastoma multiforme of the right cerebral hemisphere. In the astrocytoma Grade III, an autopsy case, there was considerable guanine deaminase activity (105uM), possible due to admixture with adjacent normal brain, with deaminase values, however, only one third those in the normal right occipital lobe. Similarly, homogenates of portions of a glioblastoma multiforme removed at operation also contained a fair amount of enzyme (54.7uM), about 64% as much as portions of the surrounding normal brain tissue. (Because of the infiltrating nature of the tumor, it is very difficult to obtain a pure specimen of the neoplasm.)

Gelhorn (82) et al. have shown that, although human malignant gliomas can be destroyed in vitro by 8 - azaguanine, no apparent therapeutic benefit has been observed in vivo in humans given 8 - azaguanine intravenously at New York Neurological Institute by Pool et al. (84). They suggested that this was due to the rapid metabolism of the drug which prevents maintenance of effective blood and tissue concentration of the compound. These observations are strong indications that the deaminating enzyme must be abundant in normal tissues in addition to the brain.

D. Distribution of the Enzyme

Guanine deaminase is abundantly found in various animal organs, particularly in the liver, kidneys, spleen and pancreas. Enzyme has been found in the liver of mouse (25, 26, 27), guinea pig (25), and rabbit (28 - 35), brain of mouse (29 - 33), rat (29, 33, 34, 36), hamster (33), guinea pig (29), rabbit (33), sheep (36), dog (38), pig (39), and man (30, 33, 40, 41).

The enzyme has been found in lupine seedlings (43). It has been found in bacteria (15, 44), insects (44, 46), fish (47, 49) and molluscs (50). The level of enzyme in fish muscle is high in ling cod muscle. Low level of the enzymes was found in the serum of rat (29, 30), hamster (30), guinea pig (29, 30) and mouse (29, 30, 33), and absent from the serum of rabbit (30, 51), sheep (51) and man (33, 51). Elevated levels of enzyme were present in hepatitis (29, 30). It is absent from the human and rabbit heart (30). Low levels of enzyme were detected in the human skin (52), intestine (30), lung of rat (34), and pig (39). A study of the distribution of the enzyme in various parts of the central and peripheral nervous system of monkeys and rats (53), indicated that guanine deaminase activity was highest in the thalamus, and fairly abundant in most of the cerebral cortex regions. No detectable activity was observed in the cerebellum, the optic nerve, medulla and spinal column.

Studies of the distribution of guanine deaminase within the subcellular region have been conducted (3). Soluble fractions seem to have the highest concentration but reports on subcellular fractions are conflicting. De Lamirande (3) et al. reported no activity present in the nuclear, mitochondrial or microsomal fractions, with all the activity associated with the soluble fractions. Jordan (37) found more than 60% of the enzyme activity of the rabbit brain in the soluble fraction and about 20% in the microsomes with very little in nuclei and mitochondria. Kumar, et al. (34) have reported the enzyme activity in rat brain and liver distributed among all subcellular fractions and greater part of the activity in the soluble fractions. Triton X-100 released enzyme activity from subcellular particles. They also noticed an inhibitor of guanine deaminase in the heavy mitochondria of rat liver and brain which seemed to be a

protein. A comparative study of the distribution and level of guanine deaminase in human organs and tissues after post mortem was conducted by Norstrand (55), showing a similar pattern to that in the other mammalian systems studied.

E. Isolation and Properties of the Enzyme

Mansoor, et al. (53), reported an 80 - 100 fold purification of rat and sheep brain guanine deaminase using ammonium sulfate $(NH4)_2SO_4$ fractionation, calcium phosphate gel treatment and DEAE chromatography. 8 - azaguanine and 6 - thioguanine were other substrates for the enzyme. A broad optimum pH was determined as 6.5 - 9.0. Lewis (56) purified the rabbit liver enzyme 15 fold by DEAE chromatography with acetone powder extract of rabbit liver. Kumar, et al. (54), reported residual activity of the rat liver and brain enzyme in the particulate fractions from differential centrifugations of the homogenate, suggesting its occurrence in more than one intracellular site. In the same year Roy (47, 48), purified guanine deaminase from ling cod muscle 200 fold by ammonium sulfate fractionation and DEAE chromatography plus a heat denaturation step. Two guanine deaminases in ling cod muscle were suggested, because of the difference in the pH activity profile for different preparations.

Currie (29) purified rabbit liver enzyme 200 fold with 20% yield using ammonium sulfate fractionation, DEAE sephadex G-200, and hydroxylapatite column. The Km for guanine was 1.05×10^{-4}M and pH optimum 5.9 with molecular weights of 170,000 and 525,000. Kumar, et al. (54), reported a 600 fold purification of the rat brain enzyme. A 360 fold purification of the soluble fraction of rat liver was resolved into two isozymes. However, there was a variation in the properties of the enzyme depending upon the tissue and fractions. Panzica (57), also was able to purify the rat liver

enzyme to 20 fold, but the yield was very low, 5%, an optimum pH determined at 8 and Km at this pH of 4.9×10^{-6}M.

Baker and Siebeneick (59), utilized affinity chromatography using 9 - p - aminoethoxyphenyl guanine and obtained a 200 fold purification of the rat liver enzyme. Hertig (63) also purified human brain enzyme 45 fold using ammonium sulfate fractionation, dialysis with RNase and DEAE column chromatography. pH optimum was obtained at 8.5, with a molecular weight of 200,000.

In 1972 Kumar, et al. (58), reported a 360 fold purification of rat liver from soluble fraction, a 182 fold purification of mouse liver enzyme and a 15 fold purification of mouse brain enzyme.

Lewis and Glantz (31 - 33), for the first time reported the purification of guanine deaminase from rabbit liver to homogeneity with a 9000 fold purification and 10% yield, using repeated ammonium sulfate fractionations and DEAE chromatography. The pH optimum was found to be 6.8 with a Km for guanine 1.25×10^{-5}M at pH 7 and 3.33×10^{-4}M at pH 6 for 8 - azaguanine. The enzyme was found to be a single polypeptide chain with a 55,000 molecular weight. They also studied the deuterium isotopic effect with a $^{K}D_2O/^{K}H_2O = .58$, at saturating substrate concentration, while at nonsaturating concentration of guanine, ratio was 1.46, at 20% D_2O. A mechanism was proposed showing a proton shuttling through histidine and cysteine for protonating the amino group of guanine which upon deamination gives rise to a covalent intermediate. Parachloromercuri-benzoate inhibited the enzyme and the kinetic parameters were studied.

Kanzawa (2) studied the inhibition of guanine deaminase with the derivatives of 5 amino - 4 - imidazole carboxamide and found these to be potent inhibitors. Prodarnov (60) found changes in the guanine

deaminase activity in lungs and serum of guinea pigs with anaphylactic shock.

Fogel and Bieber (34, 35) used affinity chromatography on 9 - (p - aminoethoxyphenyl) guanine-sepharose-4B to purify rabbit liver GAH 1250 fold, with 50% recovery of activity. After homogenization and one ammonium sulfate fractionation, they performed affinity chromatography. Molecular weight of 100,000 was obtained. SDS studies revealed two bands of 48,500 and 26,000 molecular weight. The enzyme showed activity over a wide pH range with an optimum at 8.00 Km for guanine was 5.6×10^{-6}M at pH 8.0. Day (61) also used the affinity chromatography on 9 -(p - aminoethoxyphenyl) guanine-sepharose. Day purified the enzyme 136 fold with 10% yield and obtained a molecular weight of 102,000 and Km of 6.06×10^{-6}M. In 1979 Bergstrom and Bieber (62) reported the purification of rabbit liver enzyme using the 9 - (p - aminoethoxyphenyl) guanine-sepharose affinity ligand. A molecular weight of 110,000 was obtained for the enzyme. Fluoride ion inhibited the enzyme in a noncompetitive manner. The p - chloromercuribenzoate and rose bengal inactivated the enzyme completely. They also proposed two plausible mechanisms for the reaction catalyzed by GAH.

Currently, a very limited depth of knowledge and information is present on a highly purified guanine deaminase. Lewis and Glantz (31, 33) were the first to report to purify the enzyme to homogenity, although they used a tedious purification scheme with repeated ammonium sulfate fractionations and ion exchange and hydroxylapatite chromatography. The yield was also low. Besides most of the studies have been made with crude homogenates or partially purified preparations. Fogel and Bieger and Bergstrom, et al. (62) were the next group to report studies on a purified enzyme.

However, a great deal of variation in the physical and chemical properties exists in the literature with very little information on the

characteristics of the enzyme. Particularly the information on Km, sub unit molecular weight, and the mechanism of the catalyzed reaction have conflicting reports.

Because of the key position of guanine deaminase in the purine metabolism, and its relations to the cancer etiology and because the project involved a human enzyme, this work was undertaken to purify the guanine deaminase and study some of its properties, to gain a better understanding of this highly important purine metabolic enzyme.

F. Survey of Assay Methods

The methods utilized for determination of the catalytic activity of guanine deaminase involve the hydrolytic cleavage of the primary amino group of the purine substrate molecule by the enzyme, with subsequent formation of the hydroxyl derivative of the substrate (xanthine), with release of free ammonia into the medium. A quantitative estimation of catalytic activity of the enzyme may then be made by directly determining: (a) The disappearance in absorbance of the substrate at 245nm, or, (b) The increase in absorbance of the product at the wavelength of their maximal absorbance in the ultraviolet region at 270nm.

Indirectly, catalytic activity may be estimated by estimation of ammonia released as a result of deamination of the substrate.

The following methods, in original form, were used by various investigators of guanine deaminase activity:

(1) Titrimetric Method - This method was devised by Schmidt (17) and is still utilized in conjunction with more classical methods. By this method the activity of guanine deaminase was indirectly determined by the amount of ammonia liberated from an aliquot of excess substrate, by a

known amount of enzyme protein, within a certain reaction period. The author reacted guanine deaminase with a colloidal suspension of guanine gelatin, at 40°C for 30 minutes, at pH 8.7. The ammonia formed was liberated with concentrated base, and aerated into a known volume of standard acid, which was then back-titrated to determine the equivalents of neutralization by ammonia. The guanine deaminase unit of the author was the amount of the enzyme protein required to form 0.1 mg of ammonia in 30 minutes at pH 8.7.

(2) Nesslerization Method - This was another assay method of Schmidt (17). The procedures are the same as for the titrimetric method, except that the liberated ammonia was aerated into a known volume of Nessler's reagent for determination of ammonia nitrogen. Standard ammonium sulfate solution was utilized as standard.

(3) Spectrophotometric Method - The spectrophotometric method was introduced by Kalckar (19, 20). He introduced the micro-estimation of the enzymatic degradation of purines by spectrophotometric analysis. He measured the spectral changes in the ultraviolet region, induced by specific enzymes on purine substrates. This method allowed for estimation with a high degree of specificity.

Kalckar measured the absorbance of 54 gamma of guanine substrate in solution, on the Beckman DU model Spectrophotometer at 270nm. He then added 0.4 gamma of guanine deaminase protein preparation and 0.04 gamma of xanthine oxidase preparation, and measured both the decrease in absorbance at 270 nm and the increase in absorbance at 290 nm, with respect to the reaction:

$$\text{Guanine} \xrightarrow{\text{guanine deaminase}} \underset{(270\text{nm})}{\text{xanthine}} \xrightarrow[\text{oxidase}]{\text{xanthine}} \underset{(290\text{nm})}{\text{uric acid}}$$

(4) Chromatography - Spectrophotometry - The chromatographic method was developed by Kream and Chargaff (23). This methods consisted of incubating enzyme and substrate on moist filter paper, followed by chromatography with a butanol-ethylene glycol-water solvent. Purine spots were then located by ultraviolet radiation, and their nature and quantity were then determined by elution and spectrophotometry at 245nm.

(5) Pre-incubation, Dilution and Spectrophotometry - This method of analysis was utilized by Mitchell and McElroy (76). It consisted of incubating an aliquot of enzyme solution containing 0.2 mg protein per ml. with an equal volume of M/15 phosphate buffer (pH 7.0) containing an excess of substrate (1.98mg/ml.) at 40°C. At intervals of 0, 10, 20 and 120 minutes, samples were removed and diluted 1:100 with phosphate buffer for analysis at 245nm.

(6) Direct Spectrophotometry - Rouch and Norris (22) took advantage of the large difference in absorbance of guanine and xanthine and of 8 - azaxanthine at 245 nm to make direct observations of changes in absorbance produced by enzymatic activity. 0.1 ml. of enzyme solution was mixed with 3.0 ml of substrate in 0.05 M phosphate buffer at pH 7.0. The decrease in substrate concentration in time, was observed at 245 nm, as a change in optical density. This method has been adopted, sometimes with minor modifications, as the routine method for estimation of the catalytic activity of guanine deaminase.

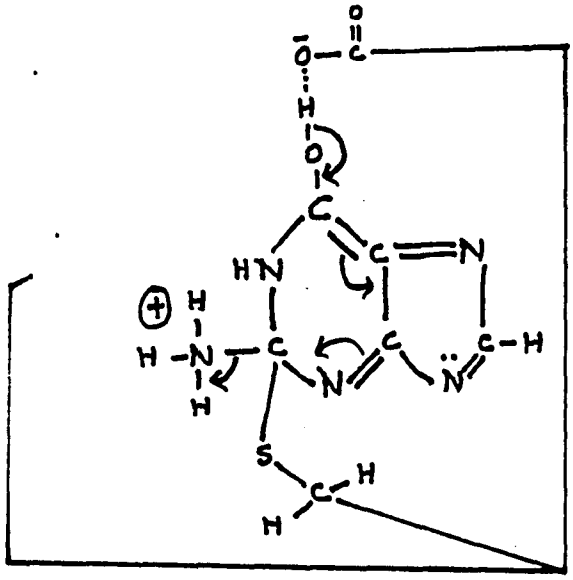
G. Mechanistic Analysis

Lewis and Glantz (31) proposed a mechanism for catalysis by

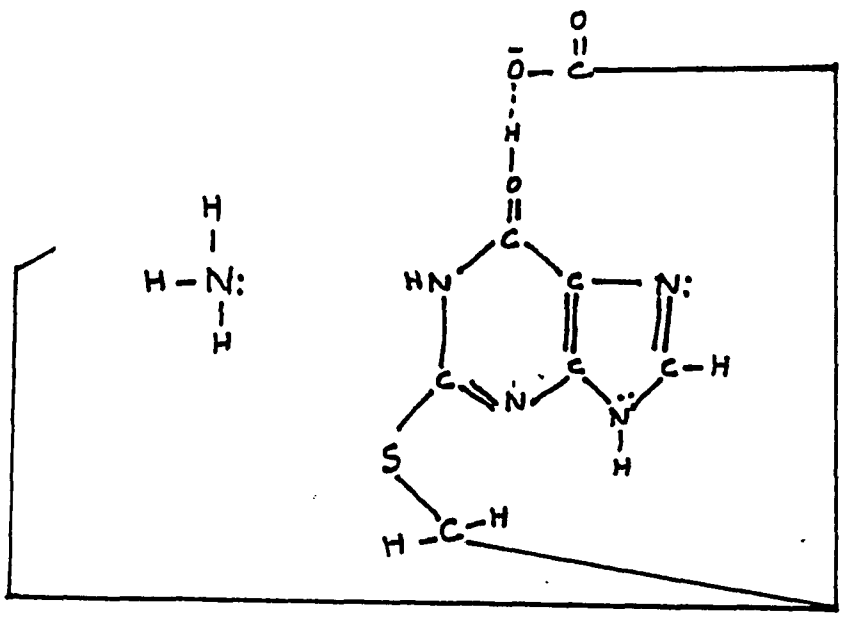
guanine deaminase which involved general acid base catalysis by histidine and cysteine. Cysteine in its normal state at the active site of the enzyme, as proposed by them, shares the acidic hydrogen of histidine. When the enzyme is activated in the presence of substrate, the acidic proton is abstracted by or released to cysteine to form free sulfhydryl. Protonation of substrate is achieved by free sulfhydryl with subsequent alkylation of the substrate by sulfide at carbon atom 2. The transient tetrahedral ammonium intermediate I, dissociates with the release of ammonia, with formation of the purinyl-enzyme intermediate II.

The transition of histidine from base to acid by abstraction of protons from the solvent releases hydroxyls for nucleophilic displacement of sulfur is the rate determining step. The association of the basic sulfide of cysteine, with the acidic hydrogen of histidine is resumed, and the catalytic process is repeated. The role of hydroxyl as nucleophile and of proton transfer from solvent were deduced by them from deuterium isotope studies. They concluded that the functional hydroxyl at C_6 of the substrate may serve the role of anchorage of the substrate through hydrogen bonding with the enzymes. Their proposed mechanism is shown here.

Fig. 2 Proposed intermediates in the catalysis of guanine deaminase. Intermediate I is a short-lived intermediate resulting from concerted protonation and aklylation of the substrate by cysteine in initiating the deamination of the substrate.
Intermediate II is the enzyme purinyl intermediate formed upon the deamination of the substrate which is subject to nucleophilic attack by the hydroxyl in the rate determining step of catalysis.
Reproduced from Lewis and Glantz (31).



I



II

Bergstrom and Bieber (62) recently proposed the mechanism for guanine deaminase, which is being reproduced as follows. They proposed that the reaction catalyzed by guanine deaminase is a substitution reaction where an hydroxyl group replaces an amino group. The reaction being taken place on an aromatic group and species removed and substituted are nucleophiles with the presence of cysteine as nucleophilic. Thus they proposed a nucleophilic aromatic substitution reaction. The nucleophile attacks at the substitution site with formation of a tetrahedral intermediate that can be stabilized by resonance. In their report (62) they indicated that two nucleophilic groups, an enzyme sulfhydryl and hydroxide ion, may participate in the enzymatic reaction. They presented two plausible mechanisms. In the first one, where they showed the formation of a covalent enzyme intermediate, guanine is converted at N-3 and the sulfhydryl group serves as the nucleophile that attaches C-2 by the second nucleophilic reagent, hydroxide ion. Cleavage of the bond between the purine ring and the sulfur atom leads to formation of xanthine and subsequent regeneration of enzyme by addition of a proton. In the other mechanism hydroxide ion was shown as the initial nucleophile to attach the C-2 of the enzyme-bound guanine moiety to form a tetravalent intermediate that is not covalently attached to the protein. The sulfhydryl group was shown as the acceptor of a proton from N-3, allowing the unpaired electrons of N-3 to be utilized to displace the amino group from C-2. This results in the formation of the 2-enol tautomer of xanthine, which can be released at the second product. The common

features of both proposed mechanisms were (1) protonation of N-3, (2) displacement of the amino group by hydroxide ion and (3) participation of an enzyme sulfhydryl group in the reaction. They also showed some resonance forms that could serve to stabilize the proposed tetrahedral intermediates by delocalization of electrons throughout the purine ring.

Based upon the possibilities of the involvement of aspartate, histidine, and cysteine, as evidenced by the pKa values obtained in the pH studies in my thesis, Professor William F. Berkowitz, member of my Supervisory Committe, proposes a mechanism very similar to the mechanism of chymotrypsin. The mechanism suggests the involvement of histidine residue as a free base (pKa 6), and is being produced here by his kind permission (Figure 4a).

Fig. 3. Proposed mechanism for catalysis by guanine deaminase with the formation of a covalent enzyme intermediate.
Reproduced from Bergstrom and Bieber (62).

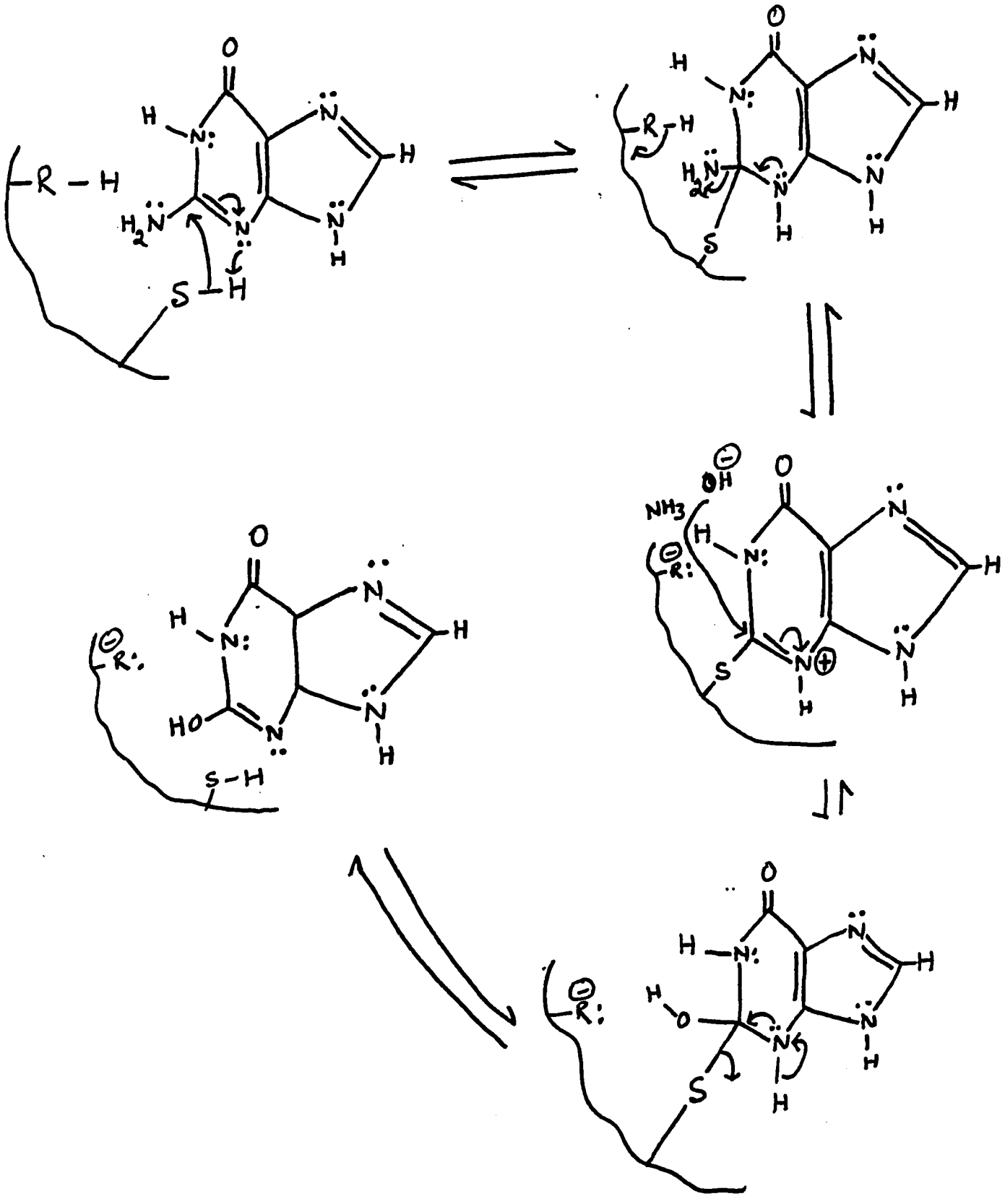
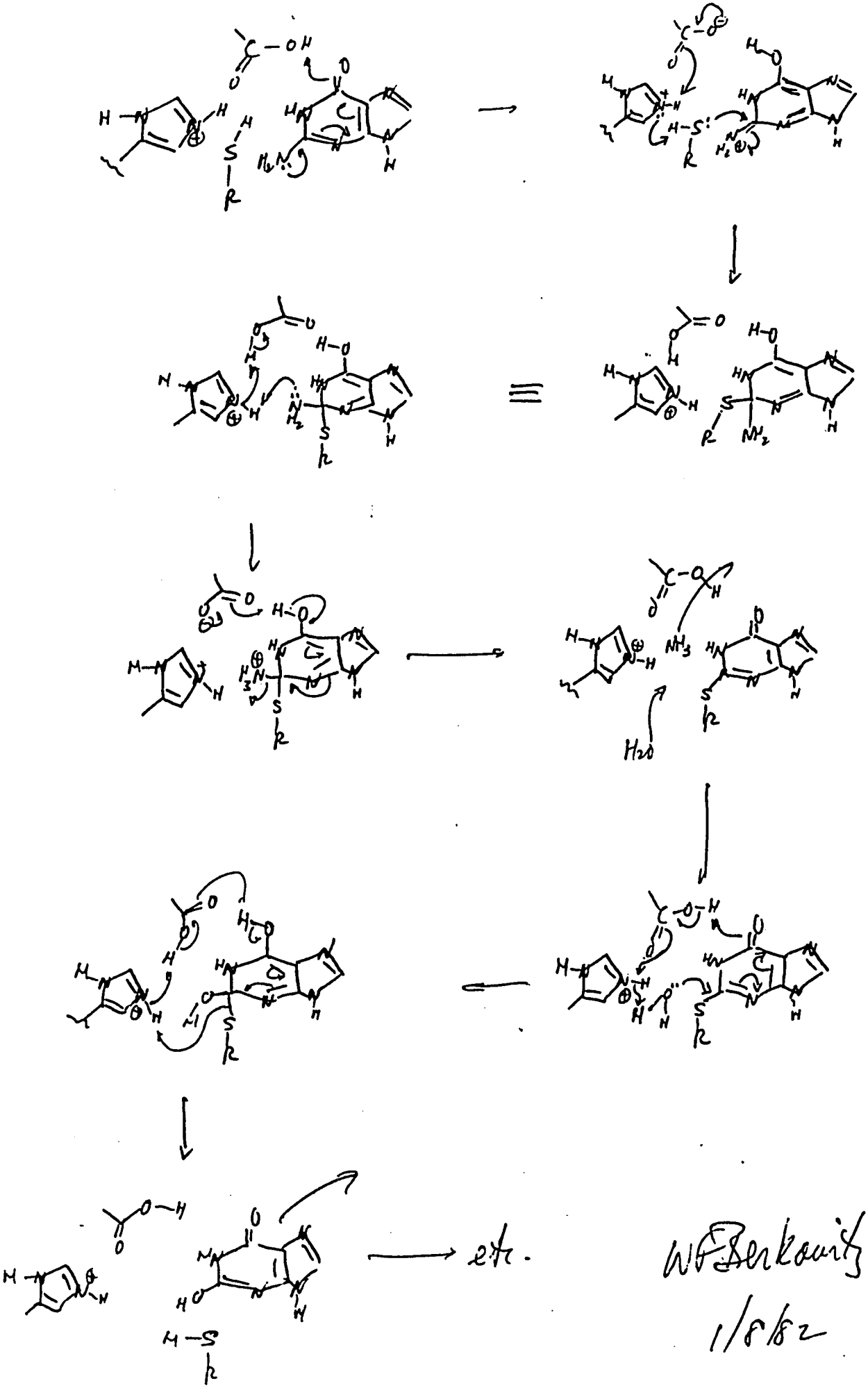
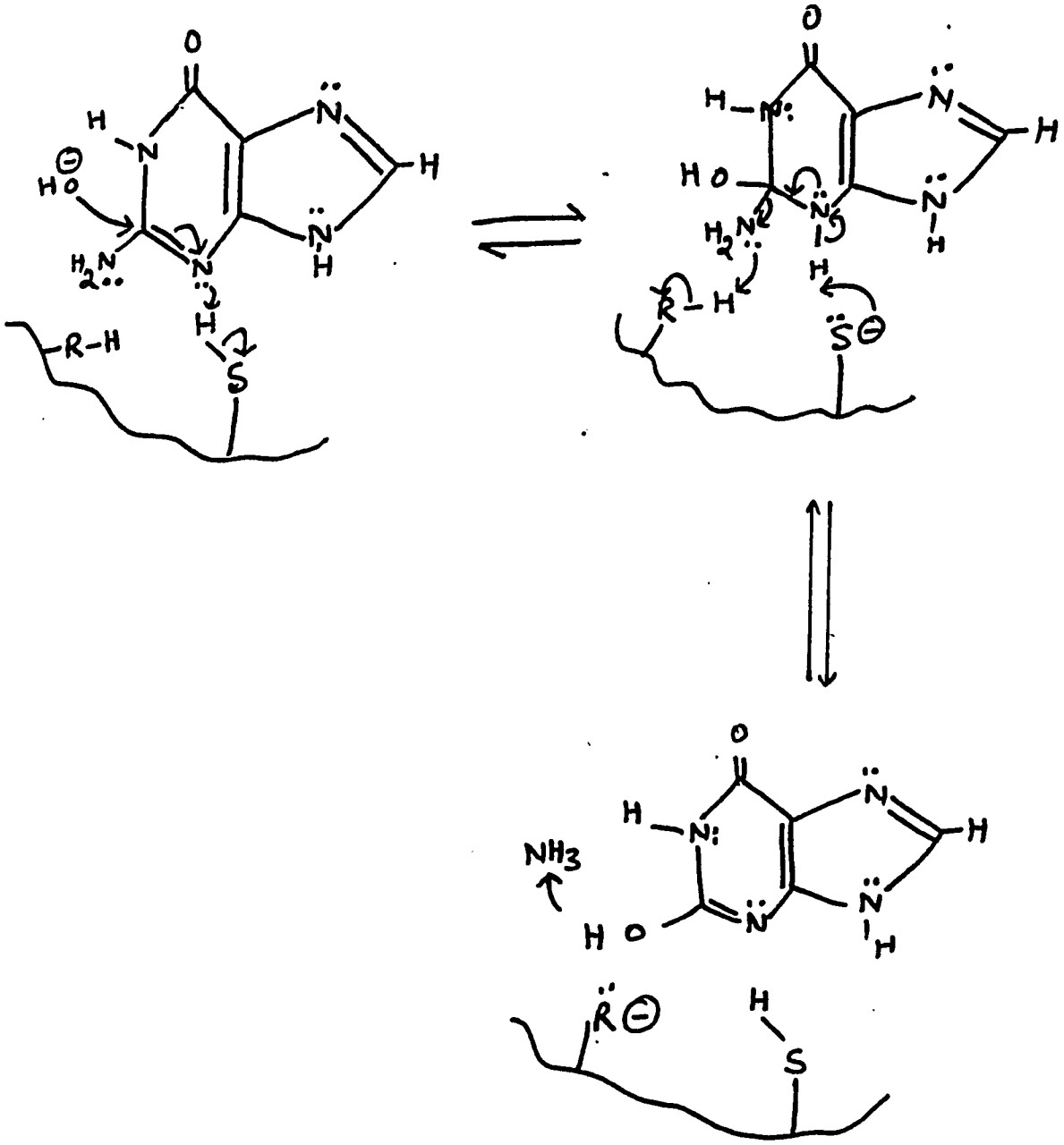


Fig. 4. Second mechanism proposed by Bergstrom and Bieber (62) without formation of a covalent enzyme intermediate.
Reproduced from Bergstrom and Bieber (62).

Fig. 4a. Mechanism proposed by Professor William Berkowitz.



W. F. Benkovic
1/8/82



Materials and Methods

Source of Materials and Equipment:

Human liver tissue was provided very kindly by Dr. Iris Norstrand, M.D., Ph.D, of the Neurology Department of Veterans Administration Hospital, Brooklyn, New York. The tissue was from normal human liver, quickly removed and frozen. Chromatography columns were obtained from Pharmacia Chemical Company, New Jersey. DEAE cellulose was purchased from Bio Rad Laboratories, Richmond, California. Protein standards, ovalbumin, aldolase, RNase, chymotrypsinogen, Sephadex and blue dextran were also purchased from Pharmacia, while Soyabean trypsin inhibitor, bovine serum albumin and RNase were obtained from Pierce Chemicals, Rockford, Illinois. Guanine and carboxypeptidase A and B were obtained from Sigma Chemicals, St. Louis, Missouri. PTH amino acid standard kit was obtained from Pierce Chemical Company.

LKB Constant Power Supply #2103 was used for isoelectric focusing. Electrophoresis Cell was a Model 150A of the Bio Rad Laboratories, and Model 1200 of Canalco. Disc gel electrophoresis power supply was from Savant Instruments, New York, Model 420, with a Canalco safety interlock. Amino acid analyzer used was Model 116, Serial #202, of the Beckman Instruments Company. Agarose hexane guanine triphosphate was bought from P.L. Biochemicals, Milwaukee, Wisconsin. Phosphate buffer was prepared from di and mono basic sodium phosphate. Tris buffer was made from Tris base and hydrochloric acid. Deionized distilled water was used in preparations of all buffers. All buffers contained 2-mercaptoethanol unless otherwise

stated. Enzyme assays were determined with a Gilford UV-Visible Spectrophotometer Model 240. Iodoacetate was purchased from Mann Research Laboratory, New York and para hydroxymercuribenzoate, 5 - aminoimidazole - 4 - carboxamide and 8 - azaguanine were bought from Pierce Chemical and citrate buffer for the amino acid analyzer was bought from Beckman Instruments Company, Inc., Palo Alto, California. Ampholines were from LKB, Rockville, Maryland. Acrylamide, bis - acrylamide, riboflavin, ammonium per sulfate, Coomassie blue were from Bio Rad. Sodium mono and di phosphate were from J.T. Baker Company, New Jersey. A Sorvall Model RC-22B refrigerated centrifuge was used. Rainin Repipet System of Rainin Instrument Company, Inc., Woburn, Massachusetts was used for all the assays and operations. A Waring blender was used for homogenization. All other chemicals were of reagent grade supplied either by Baker or Fisher Chemical Company of New Jersey. Fraction collector used was Model 2112, LKB Instruments, Inc., Rockville, Maryland. Lyophilization was done on automatic freeze-dryer of Virtis Company, New York, Model #10-010. Ultrafilatration Cells of 90 mm and 25 mm were of Millipore, Bedford, Massachusetts.

Enzyme Assay

Activity measurements were performed using the modified method of Rousch and Norris (22). One unit of enzyme activity is defined as the amount of the enzyme that hydrolyzes one micromole of guanine in one minute at 30°C. The assay mixture contained 2 x 10^{-4}M guanine, 0.01M Tris-HCl, pH 8.0, in a final volume of 3.0 ml. The reaction was initiated by the addition of enzyme solution (20 - 100 microliters) and was followed by the decrease in absorbance at 245 nm. The difference in molar absorptivity between guanine and xanthine was 3.70 x $10^{3}Cm^{-1}M^{-1}$. Guanine at 2 x 10^{-4}M concentration gave an absorbance of 2.260 which after the addition of 20 - 100 ul. of the enzyme solution decreased from 0.025 - 0.140 spectrophotometric units.

Protein Assay

A modified microbiuret method of Zamenhof was used for protein estimation with bovine serum albumin as the standard (64). Biuret reagent was prepared with dropwise addition of 80 ml. of 1% copper sulfate to 300 ml of 40% sodium hydroxide. A standard curve was constructed with the known amount of bovine serum albumin and the absorbance was measured at 310 nm (Figure 5).

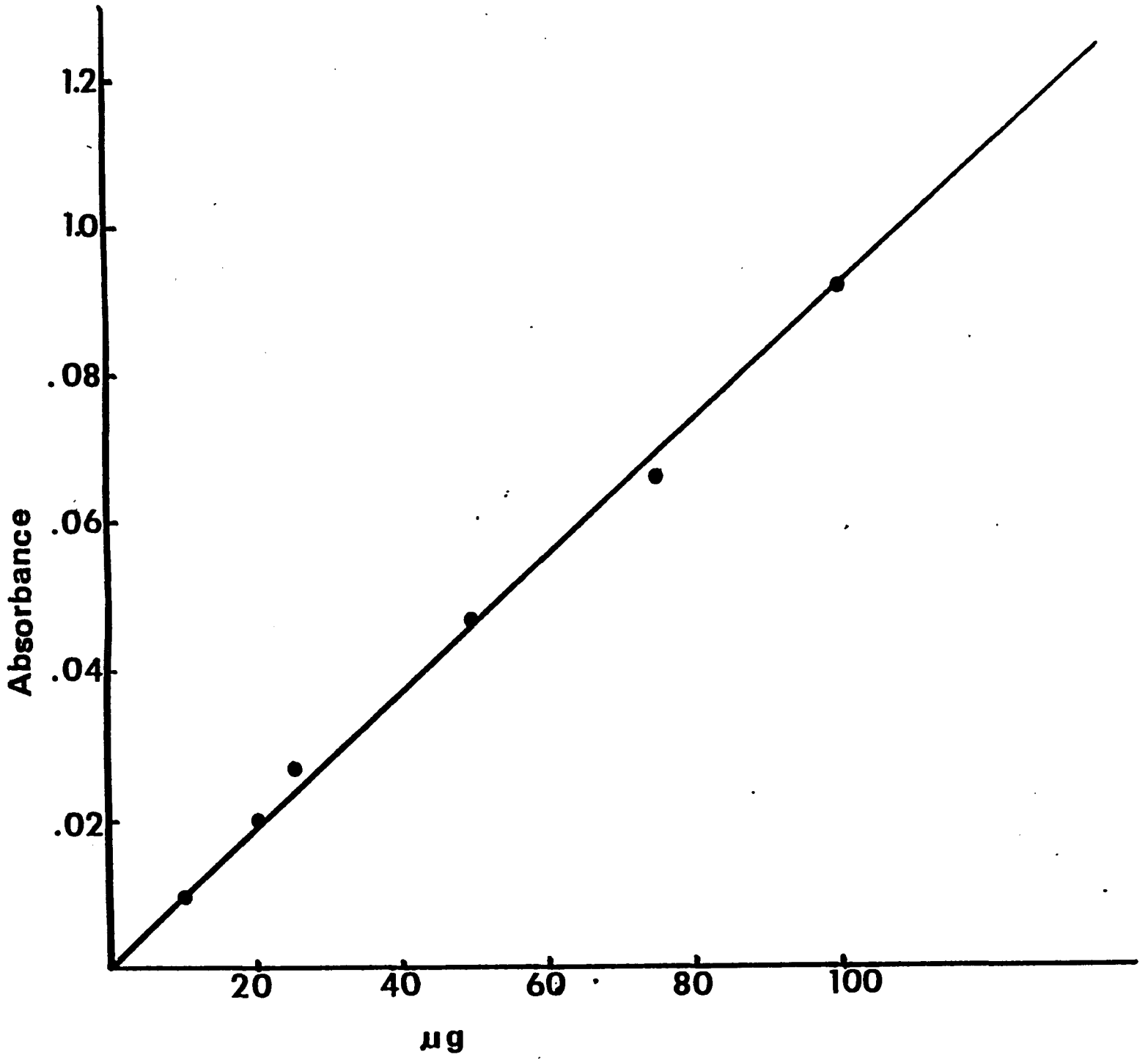
Purification of Guanine Deaminase

All procedures were carried out at 0 - 4°C unless described otherwise.

Preparation of DEAE-Cellulose Column

10 - 20 gm of Cellex-D was suspended in 500 ml of 0.05M phosphate buffer at pH 7.0 with gentle stirring. It was allowed to settle for 15 minutes, then decanted and the coudy supernatant was discarded. An additional 400 ml of the buffer was added to the precipitate, stirred

Fig. 5. Protein standard curve. Bovine serum albumin was used as the standard protein and the absorbance was measured at 310nm.


1.2
1.0
.08
.06
.04
.02
Absorbance
20
40
60
80
100
µg

and allowed to settle again for 15 minutes. The supernatant was again discarded. The settled Cellex was resuspended in a small volume of buffer and the column was poured all at once. Packed column was then equilibrated against several column volumes of 0.05M phosphate buffer, pH 7.0 containing 5 x 10^{-3}M dithiothreitol. A small amount of Sephadex G-10 was poured on the Cellex bed surface to avoid the bed surface disturbance.

Method

Guanine deaminase was purified by using a combination of ammonium sulfate fractionation, DEAE-cellulose chromatography, hydroxylapatite chromatography and affinity chromatography on agarose hexane guanine triphosphate. Frozen liver tissue was thawed, cut into pieces and homogenized in a Waring blender with two volumes of sodium phosphate buffer 0.05M, pH 5.0 containing 0.005% phenylmethylsulfonyl fluoride and 5mM dithiothreitol. The homogenate was stirred for one and a half hour and then centrifuged for 40 minutes at 20,000xg in a Sorvall refrigerated centrifuge at 0°C. The clear supernatant was adjusted to pH 6.5 and this was filtered through a cheese cloth to remove fat and other floating particles. The precipitate was discarded. The supernatant was brought to 40% saturation by adding solid ammonium sulfate slowly and with constant stirring along with the adjustment of pH with 1N NaOH. This was again subjected to centrifugation for 40 minutes and the supernatant was taken to 80% saturation with ammonium sulfate with the maintenance of the pH, and vigorous stirring. The precipitated proteins, containing most of the enzyme activity, were recovered after two hours of stirring, followed by centrifugation. The

Fig. 6. DEAE-cellulose elution profile. The column was pre-equilibrated with 0.02M phosphate buffer, pH 7.5, containing 5×10^{-3}M dithiothreitol and was eluted with 0.04 - 0.3M phosphate buffer gradient, pH 7.5. Protein absorbance is shown as (▬▬) and enzyme activity is represented with (▬ ▬).

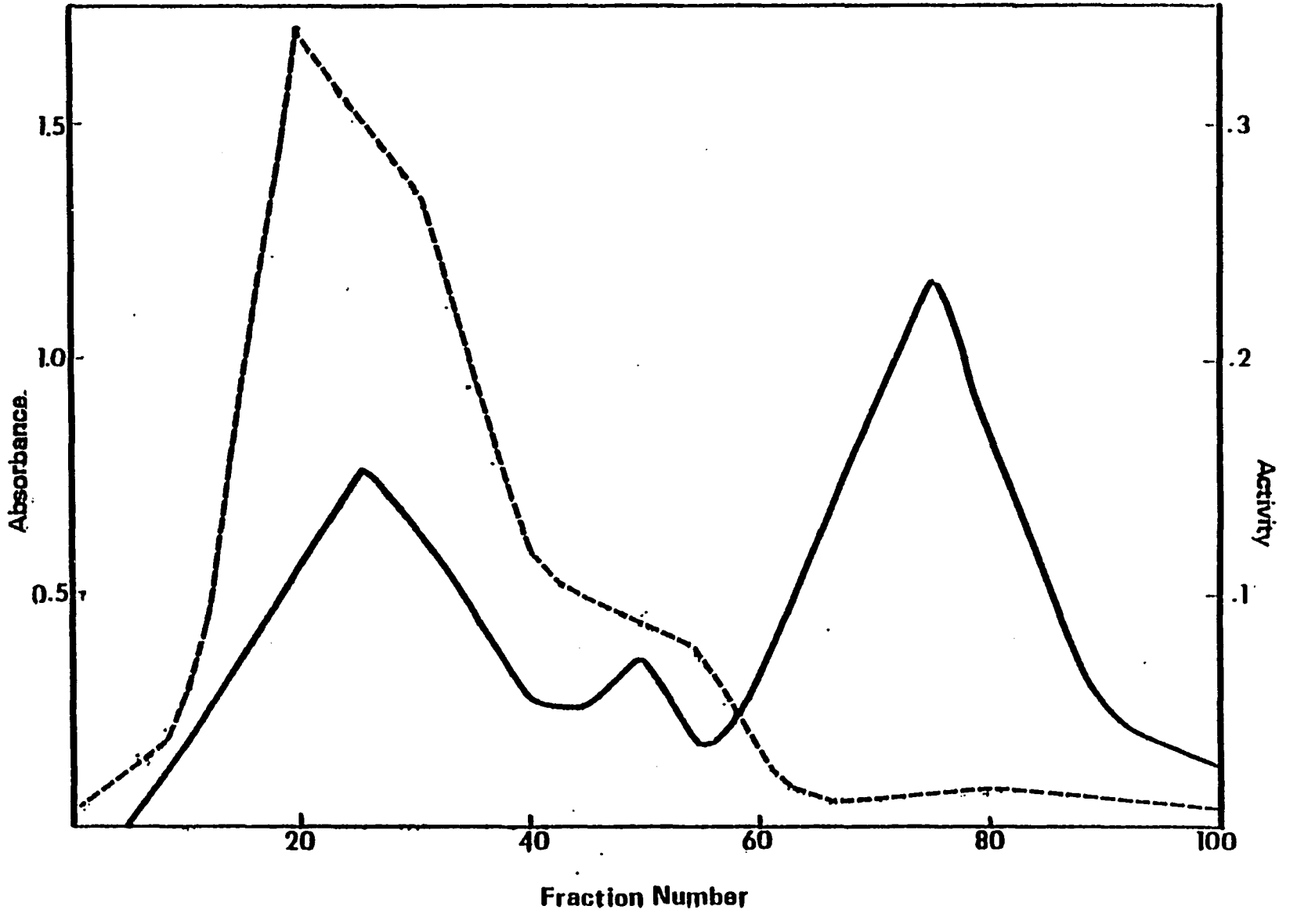
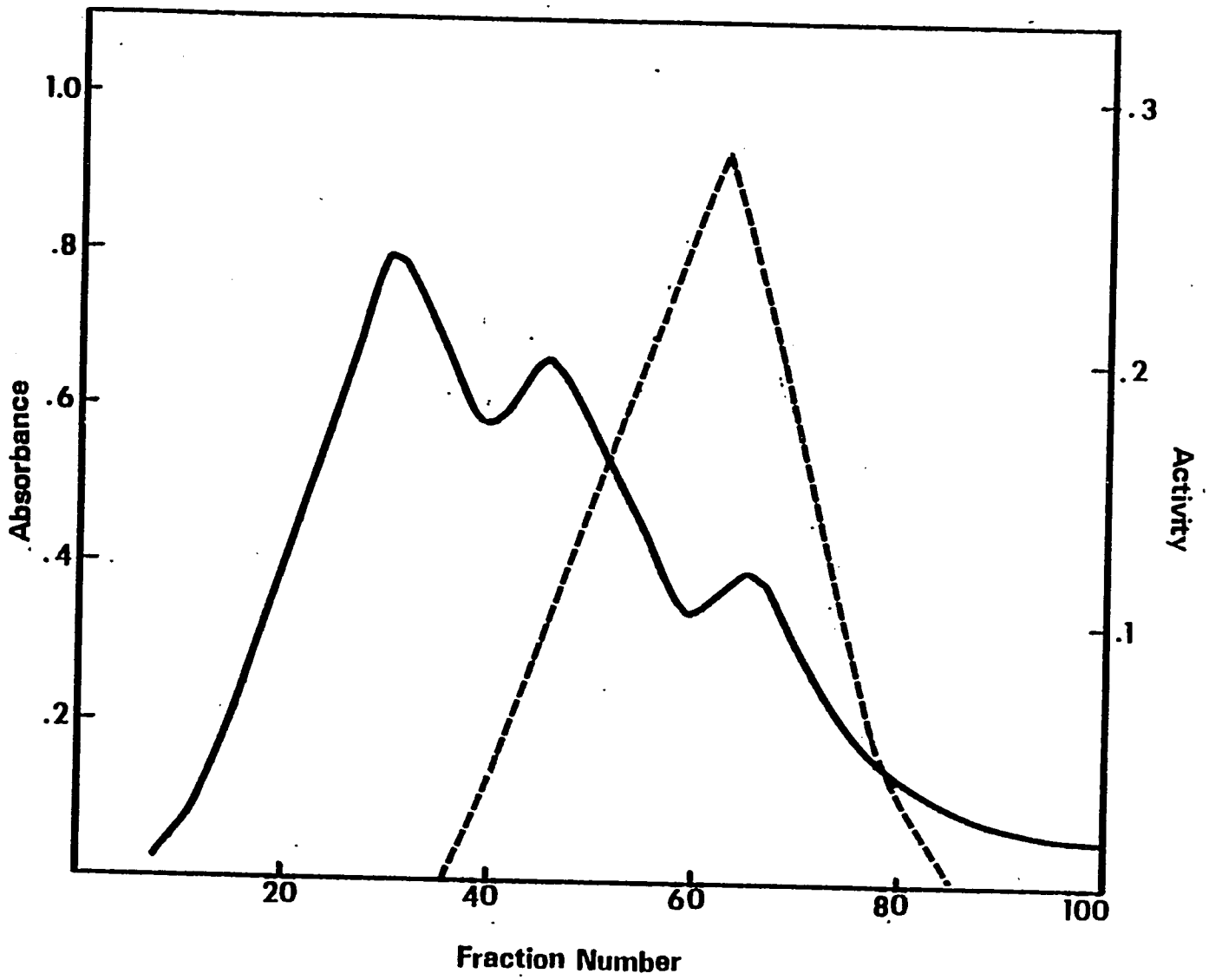
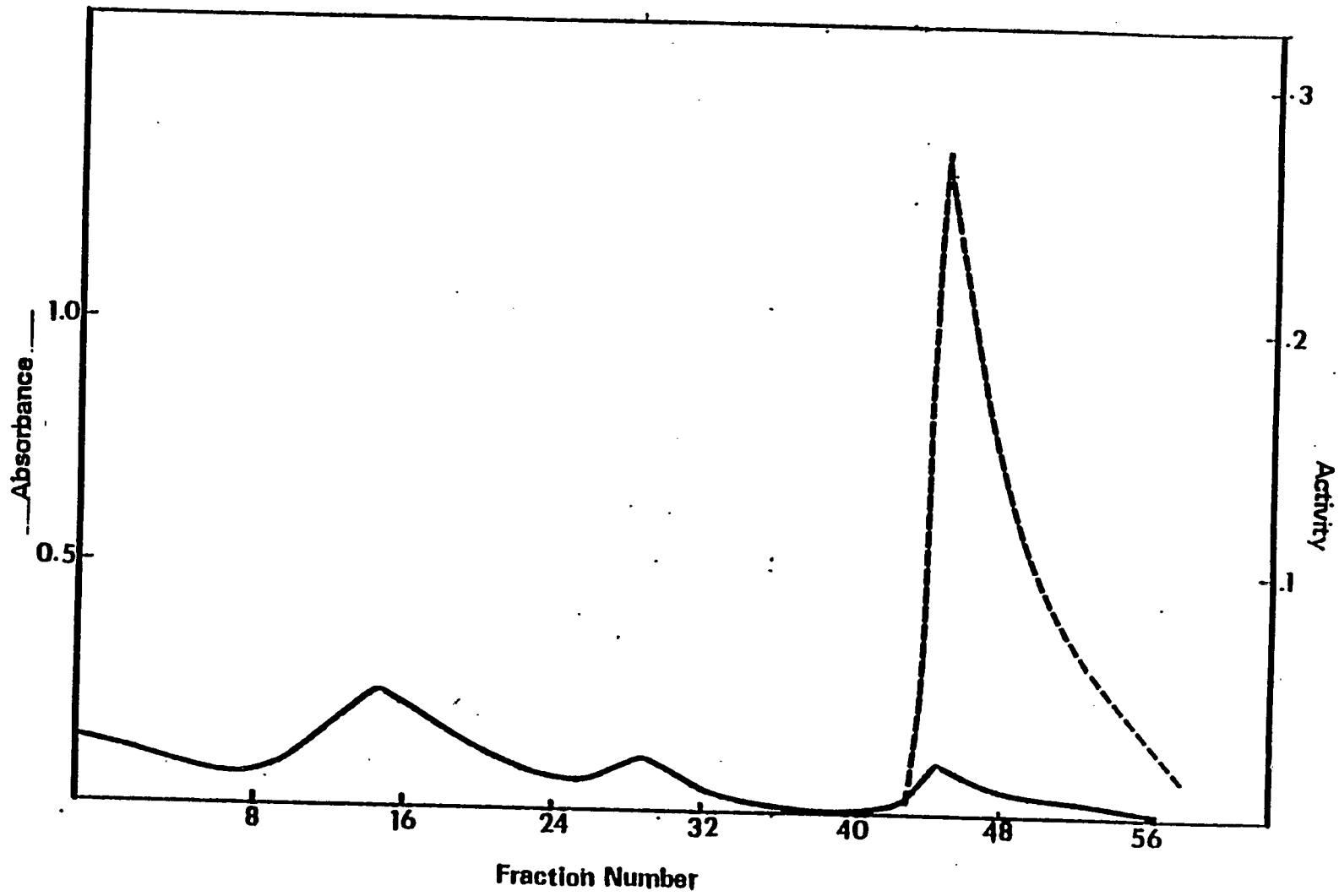

1.5
1.0
0.5
Absorbance
.3
.2
.1
Activity
20
40
60
80
100
Fraction Number

Fig. 7. Hydroxylapatite chromatography. The column was pre-equilibrated with 0.02M phosphate buffer, pH 7.0, and eluted with phosphate buffer 0.045 - 0.15M gradient, pH 7.0. Protein absorbance is shown as (▬▬) and enzyme activity is represented with (▬ ▬).


1.0
.8
.6
.4
.2
Absorbance
.3
.2
.1
Activity
20
40
60
80
100
Fraction Number

precipitate obtained was redissolved in a minimal volume of 0.02M sodium phosphate buffer, pH 7.8, containing 5×10^{-4}M allopurinol and 5×10^{-3}M dithiothreitol. This was dialyzed against the same buffer containing dithiothreitol and allopurinol until all the ammonium ions were removed (approximately 6 changes of 4 liters of buffer). The pH of the solution was brought to 6.5 and the precipitate formed during the dialysis was removed by the centrifugation. A DEAE-cellulose column was prepared as described earlier and equilibrated with 0.02M phosphate buffer pH 7.5 and 5×10^{-3}M dithiothreitol. The enzyme solution was applied to the column (42 x 3.8cm) and the column was eluted with three column-volumes of 0.02M phosphate buffer, pH 7.5, followed by two column-volumes of 0.04M phosphate buffer pH 7.5. The enzyme protein was removed by gradient elution with 0.04M - 0.3M phosphate buffer pH 7.5. Fractions of high specific activity were pooled and were brought to 45% saturation with solid ammonium sulfate pH 7.0 and subjected to centrifugation for 45 minutes at 20,000xg. The clear supernantant was brought to 75% saturation with the addition of solid ammonium sulfate at pH 7.0 and was subjected to centrifugation at 20,000xg. The precipitate containing the active enzyme was dissolved in a minimal volume of 0.02M sodium phosphate buffer pH 7.0 containing 5×10^{-4}M allopurinol and 5×10^{-3}M dithiothreitol. This was dialyzed against the same buffer with several changes and applied to a previously equilibrated hydroxylapatite column 42 x 3.8cm., prepared with the same method as DEAE-cellulose, with 0.02M phosphate buffer pH 7.0. The column was washed with three column-volumes of 0.35M phosphate buffer, pH 7.0 containing 5×10^{-3}M dithiothreitol. The column was finally eluted at a low rate with 0.1M phosphate buffer pH 7.0. The fractions showing most of the enzyme activity were pooled, concentrated with 80% saturation of ammonium sulfate and dialyzed. The enzyme solution was

Fig. 8. Elution profile of affinity chromatography on agarose hexane guanine triphosphate. The enzyme was eluted with a sodium glycine buffer 0.008 - 0.1M gradient at pH 9.0 containing dithiothreitol (5×10^{-3}M) allopurinol (5×10^{-4}M). Protein absorbance is shown as (▬) and the enzyme activity is represented with ▬▬.


Absorbance
1.0
0.5
.3
.2
.1
Activity
8
16
24
32
40
48
56
Fraction Number

then applied to the same column and eluted with a 0.045 - 0.15M phosphate gradient at pH 7.0. Active fractions of the enzyme were pooled and concentrated in a Millipore ultrafiltration cell.

An agarose hexane guanine triphosphate column (29 x 1.7cm), containing 50 ml of this affinity material, was used for the affinity chromatography. The column material was washed with the distilled water and the column was pre-equilibrated with two colum volumes of 0.02M sodium phosphate buffer pH 7.8 containing 5×10^{-4}M allopurinol and 5×10^{-3}M dithiothreitol. The enzyme solution was applied to the column and the column was washed with four column volumes of the same buffer followed by three column volumes of 0.05M sodium pyrophosphate pH 9.0. The column was then eluted with 0.008M - 0.1M glycine - NaOH buffer pH 9.0 containing 5×10^{-3}M dithiothreitol and 5×10^{-4}M allopurinol. Fractions eluted from the column showing high enzyme activity were pooled, concentrated by 85% ammonium sulfate and again passed through the column. Pure enzyme fractions after elution from the column were then concentrated and stored. Freezing of the pure enzyme was found to be a more satisfactory and preferred method of storing the enzyme than refrigerating.

Criteria of Purity

Disc gel electrophoresis, SDS polyacrylamide gel electrophoresis and isoelectric focusing were used as the criteria of the purity of the enzyme.

Purification - Flow Sheet

All the buffers had 5×10^{-3}M dithiothreitol.

Tissue.

↓

Homogenize in 2 volume of sodium phosphate buffer 0.05M pH 5.0 containing 0.005% phenylmethylsulfonyl fluoride and 5×10^{-3}M dithiothreitol.

↓

Stir for one and a half hour.

↓

Centrifuge 40 minutes at 20,000xg.

↓

Supernatant filter through cheese cloth and pH adjusted to 6.5. Discard the precipitate.

↓

40% ammonium sulfate saturation (maintain pH).

↓

Centrifuge 40 minutes. Discard precipitate. (20,000xg)

↓

Supernatant to 80% saturation with ammonium sulfate. Stir two hours. Maintain pH.

↓

Centrifuge (20,000xg).

↓

Precipitate dissolve in a minimal volume of 0.02M phosphate buffer pH 7.8 (5×10^{-4}M allopurinol, 5×10^{-3} dithiothreitol).

↓

Dialyze against buffer, adjust pH to 6.5, remove precipitate by centrifugation (if any).

↓

Apply to DEAE-cellulose column (pre-equilibrated with 0.02M phosphate buffer pH 7.5 and 5×10^{-3}M dithiothreitol).

↓

Wash with 3 column-volumes of 0.02M phosphate buffer pH 7.5 followed by 2 column-volumes of 0.04M buffer pH 7.5.

↓

Elute with 0.04 - 0.3M phosphate buffer pH 7.5 gradient.

↓

Only high specific activity fractions collected, brought to 45% saturation with ammonium sulfate pH 7.0.

↓

Centrifuge 45 minutes (20,000xg).

↓

Supernatant to 75% saturation pH 7.0.

↓

Precipitate, dissolved and dialyzed as earlier.

Apply to a pre-equilibrated hydroxylapatite column (0.02M pH 7.0).

↓

Wash column with 3 column-volumes of 0.035M buffer pH 7.0 (DTT).

↓

Slow elution with 0.1M phosphate buffer pH 7.0.

↓

Only highly active fractions pooled, concentrated by 80% saturation, and dialyzed as above. Applied to the same column, eluted with phosphate buffer gradient (0.045 - 0.15M pH 7.0) and concentrated.

↓

Put on agarose hexane guanine triphosphate affinity column (pre-equilibrated with 2 column-volumes of buffer 0.02M pH 7.8 containing 5×10^{-3}M DTT and 5×10^{-4}M allopurinol).

↓

Wash with 4 column-volumes of same buffer and 3 column-volumes of 0.05M sodium pyrophosphate pH 9.0.

↓

Elute with 0.008 - 0.1M sodium glycine buffer pH 9.0 (DTT and allopurinol).

↓

Collect only high activity fractions, concentrate with 85% ammonium sulfate and reload on the same affinity column and elute.

↓

Pure enzyme, Concentrate with Millipore ultrafiltration device and store in freezer.

Polyacrylamide Disc Gel Electrophoresis

A modified version of the disc gel electrophoresis method as outlined by Canalco (65) was followed. 24ml of 1NHCl, 18.1 gm of Tris and 0.12 ml of TEMED were mixed in a total volume of 100ml using distilled water. This was Part A. Part B was prepared by mixing 28 gm of acrylamide, 0.735 gm of Bis and diluting to volume of 100ml with distilled water. Both Part A and Part B were kept in dark-colored bottles. Acrylamide is a neurotoxin and the possible contact with the skin must be avoided. Ammonium per sulfate (3mg/ml) was prepared in distilled water. All the solutions were filtered. Ammonium per sulfate was prepared fresh. One part of A and one part of B were mixed with two parts of ammonium per sulfate which acts as a catalyst, quickly deaerated and poured in the glass tubes (9 x 0.5cm) and set in the preparation racks. Glass tubes were washed thoroughly after soaking in cleansing solution for a few hours or preferably overnight and dried in the oven at 110°C. A stock solution of glycine buffer was used for the electrophoresis which was diluted out 1:10. Stock solution contained 6gm Tris and 28.8gm glycine per liter at pH 8.6 and was stored at 0 - 4°C.

Preparation of the Gels and Sample Application

Stock solutions of Part A and B were allowed to cool at room temperature and mixed with the ammonium per sulfate as described above. The glass tubes were sealed at the bottom with parafilm and were very carefully filled up to the desired height with the above solution (usually

3/4 of the height of the tube) and distilled water was gently overlayed with the aid of a microliter pipet. The tubes were allowed to stand for 30 to 60 minutes for polymerization and the water from the top was removed with a disposable pipet. A stock solution of 40% sucrose and 0.1% bromophenol blue was prepared in the glycine buffer (prepared as above). This was mixed with equal volume of enzyme solution (50 - 80 ugm) in a small test tube and it was gently overlayed on the gel top after removing the previously overlayed water layer. The tubes were filled to the top with electrophoresis buffer after the sample was applied. The gels were placed in the electrophoresis buffer after the sample was applied. The gels were placed in the electrophoresis apparatus and a current of 2.5mA/gel tube was applied until the dye (bromophenol blue) appeared at the end of the tube.

Protein Staining

Gels were stained with Coomassie blue (2gm/liter in 7.5% acetic acid and in 5% methanol). The gels were removed from the glass tubes with the aid of a syringe and stained for 30 to 60 minutes in the above staining solution.

Protein Destaining

A destaining solution of 7.5% acetic acid in 5% methanol was prepared and an electrolytic destaining cell was used to destain the gels requiring from one to one and a half hour.

SDS Gel Electrophoresis

The subunit molecular weight was determined by the method of Weber and Osborn (66). The standard proteins used were soyabean tyrpsin inhibitor (22,000), bovine serum albumin (68,000) and RNase with three

different molecular weight subunits (α subunit 39,000, β subunit 155,000 and β' subunit 165,000). All the proteins were preincubated in a solution containing 1% SDS and 1% mercaptoethanol in 0.2M phosphate buffer. A current of 8mA/tube was applied until the dye reached the end of the tubes. Gels were stained and destained as described above.

Determination of N-Terminal Amino Acid

A modified procedure of Edman degradation (67) was followed to determine the N-terminal amino acid. 0.2mg of enzyme was dissolved in 200 microliters of the coupling buffer (dimethylallylamine) to which 10 microliters of the concentrated PITC (phenylisothiocyanate) was added. After flushing with nitrogen for 30 seconds, it was stoppered immediately and the solution was mixed thoroughly for 30 seconds on a Vortex. The tube was placed in 50°C water bath and it was removed at intervals of 5 minutes and mixed on the Vortex for 15 seconds and returned to the water bath. This process was continued for 30 minutes. At the completion of coupling, thetube was removed from the water bath and 0.6ml of benzene was added. This was placed for 30 seconds on a Vortex mixer. The tube was placed in a table top centrifuge and the components were separated. The upper organic layer was discarded. This extraction procedure was repeated three more times. The traces of the benzene were removed with a gentle stream of nitrogen blown over the surface of the aqueous (lower) layer. This layer was frozen by immersing into an acetone dry ice mixture. The solution was spread over the lowest quarter of the tube by rotating and tilting the tube as it froze. This reaction tube was covered with parafilm in which several small holes were made with a needle. The tube was placed in a Virtis

flask attached to the lyophilizing apparatus until the sample was dry. Anhydrous trifluoroacetic acid (0.1ml) was added to this dried phenylthiocarbanyl peptide. This was flushed with nitrogen for 30 seconds, directing the nitrogen stream onto the wall of the tube and not onto the surface of the acid to avoid evaporation. The tube was stoppered immediately and mixed on the Vortex for 15 seconds and then placed in a water bath at 50°C for 7 minutes. Trifluoroacetic acid was dried with a gentle stream of nitrogen making sure that the sample dries as a thin film on the lower quarter of the tube wall.

To the dried sample, 0.6ml of butyl chloride was added. It was mixed gently and the butyl chloride removed and transfered to another tube. This contained the thiazolinone derivative of the cleaved protein. This extraction was repeated and both extracts combined. This extract was dried with a stream of nitrogen. 1NHCl (0.2ml) was added to the dried butyl chloride extract which was then flushed with nitrogen for 30 seconds and stoppered immediately. It was mixed on the Vortex for 15 seconds and placed in a water bath at 80°C for 10 minutes. The aqueous HCl solution was extracted twice with 0.7ml of ethyl acetate by mixing the phases thoroughly for 30 seconds. The layers were separated by centrifugation. The upper organic layer was extracted and dried with nitrogen stream. 50 microliters of ethyl acetate was added to this dried layer and mixed on the Vortex. This extract was used for the thin layer chromatography identification. The solvent system used for TLC was 90ml of chloroform and 10ml of methanol of which lower phase was used. The PTH amino acid spots were identified under UV light.

Determination of C-Terminal Amino Acid

The method of Ambler (68) to determine C-terminal amino acid was used. The method was modified as follows:

The enzyme was denatured by performic acid oxidation as outlined by Hirs(87). A mixture of 5 volumesof 30% hydrogen peroxide and 95 volumes of 99% formic acid was permitted to stand in a closed bottle at 25°C for two hours. The titer of per acid reached a maximum at about this time and decreased slowly thereafter. Only freshly prepared reagent was used. 1mg of the enzyme protein in sodium phosphate buffer (0.1M) was incubated with 1ml of the performic acid reagent, 0.1ml of methanol and 0.5ml of 99% formic acid. All the solutions were cooled in a bath at 0 - 5°C for 30 minutes before adding together. The reaction was permitted to proceed at the same temperature for 150 minutes. Methanol prevents freezing when the formic acid is almost anhydrous. This solution was diluted with 50ml of prechilled distilled water and subjected to lyophilization. Performic acid condensates are corrosive and the coils of lyophilizers were carefully washed with the distilled water after the lyophilization was complete. The process of lyophilization was repeated twice on the sample after addition of another 50ml of cold distilled water. The denatured protein was dissolved in 5ml of 0.2M N-ethylmorpholine-acetate pH 8.5 and the solution was divided in five test tubes equally. DFP-treated carboxypeptidase A and B (0.4mg each) were added to these tubes. A blank was prepared containing all the constituents except the enzyme protein. The mixtures were incubated at 37°C and the reaction was terminated by the addition of sufficient acetic acid to bring the pH lower to 2.5 - 3. This was mixed for 15 minutes, centrifuged, and the supernatant was applied on Silica TLC plates for

the identification of the cleaved amino acid together with standard amino acids. Samples at the intervals of 5, 10, 15, 20 and 25 minutes were withdrawn and chromatographed on TLC plates.

Amino Acid Analysis

Amino acid analysis was performed by the method of Moore and Stein (69). 4mg of pure enzyme was lyophilized and was then treated with 6 ml of constant boiling HCl along with 1% mercaptoethanol. The solution was transferred to combustion tubes which were connected to a vacuum pump and frozen by immersing into a liquid nitrogen bath and subjected to air evacuation. It was repeated five times and the tubes were sealed by a flame torch at the constriction point. The tubes were then placed in an oven for 24 hours at 120°C ± 2°C. After the digestion, tubes were broken at the tip and the HCl was dried at 40°C by a gentle nitrogen stream. 4ml of sample citrate buffer (Beckman Company) pH 2.2 was used to dissolve the digested peptide. The samples were kindly injected and analyzed by Miss Joanne Waldstreicher under Dr. Glantz's supervision on Beckman 116 amino acid analyzer which was operated according to the instructions of the Beckman instruments. Peaks were integrated by an Infotronics integrator. Standards were run on the machine a few times before injecting the sample.

Native Molecular Weight Determination by Gel Filtration

The general method of Andrews (70) was followed to determine the molecular weight of the guanine deaminase. Sephadex G-100 was swollen for five hours at 90°C in 0.05M sodium phosphate buffer pH 7.0 containing 5×10^{-3}M mercaptoethanol. A 60cm x 1cm column was then packed and

equilibrated with the same buffer. The void volume of the column was determined by blue dextran. Protein standards used were bovine serum albumin (68,000), chymotrypsin (25,000), RNase (13,700), Ovalbumin (45,000) and aldolase (158,000) each 3mg/ml in a total volume of 2 ml. The emergence of the peaks was measured at 280nm. Guanine deaminase was followed by assaying the enzyme activity.

Isoelectric Focusing

Isoelectric focusing was performed in polyacrylamide gels. A modified version of the Vesterberg procedure (71, 72) was followed which is described below:

0.41ml Bis acrylamide (30gm acrylamide and 1gm Bis per 100ml)

0.41ml TEMED (0.2ml TEMED/100ml)

50 microliters ampholines (pH 4 - 6)

0.688ml protein (50 - 100mg) in 20% glycerol

0.1ml riboflavin (15mg/100ml), prepared freshly, stirred for 45 minutes and filtered.

This composition is for 1 gel tube (9 x 0.5 cm ID) and was multiplied by the number of the tubes used. The glass tubes were soaked overnight in cleaning solution and were rinsed thoroughly with distilled water then oven dried. Ampholines (pH 4 - 6) were cooled at $5^{o}C$ before using. Riboflavin was added in the last after mixing the rest of the solutions described above. The solutions, after mixing, were added to the gel tubes gently with a Pasteur pipet at the same mark height and gently overlayed with distilled water. The gels were allowed to stand for 15 minutes before putting them under fluoroscent light for polymerization. All the solutions were deaerated to prevent cracking in

the gels. The polymerization was allowed to continue for two hours after which the distilled water from the top of the gels was removed with a Pasteur pipet and the top of the gels were overlayed with the buffer. Top of the gel was anode while bottom was made cathode. Anodic solution was sulfuric acid (0.018moles/liter) and the cathodic solution was monoethanolamine (0.033moles/liter). The tubes were set at the same height in the bath. A constant power supply of 0.5W/tube was used with a constant voltage maximum of 500. The current cut off was set at the maximum. After four hours of voltage maximum, the tubes were removed and stained to check for the completion of focusing. This was done at one half, three quarters, one and two hour intervals. The staining was done by the LKB instruments method by dissolving 0.7gm of Coomassie stain (G-250) in 59 ml of concentrated perchloric acid and was diluted to one liter (filtered). The gels were incubated at 37°C in the staining solution and bands were located. This required one to one and a half hours. The gels were then transferred to the destaining solution. Isoelectric point was determined by slicing the gels and checking the pH on pH meter.

Determination of Km

The Km was determined by varying the concentration of guanine and by following a decrease in absorbance at 245nm. The absorbance values were converted to the micromoles guanine hydrolyzed per minute. Double reciprocal plot (73) was constructed to determine the value of Km.

Effect of pH on the Kinetics of the Enzyme

Effect of pH was studied on the kinetic parameters of the enzyme. Km and Vmax were measured from pH 5.5 to pH 9.0 at each 0.5 pH increments. Eight varying guanine concentrations were used. Buffers used at different pH were pH 5.5 - 6.5 sodium citrate, pH 7 - 7.5 sodium phosphate and pH 8 - 9.0 Tris buffer. From the data obtained, double reciprocal plots were constructed and Km and Vmax values were calculated. The relationship of - log Km(pKm) to the pH changes was plotted (74). The log of the ratio of the maximal velocity (Vmax) to the binding constant (Km or Michaelis constant) as a function of hydrogen ion concentration was also plotted. To determine the optimum pH, maximum velocity vs. different pH was plotted.

Heat Inactivation of the Enzyme

Temperature stability of the enzyme was studied by incubating the enzyme at 45°C, 55°C and 65°C. The enzyme was incubated at these temperatures and at the intervals of five minutes an aliquot was removed from the incubation mixture and assayed. The percentage of the remaining activity was plotted against the time.

8 - Azaguanine Effect Study

Effect of 8 - azaguanine was studied on the enzyme at pH 6.0 , the optimum pH for 8 - azaguanine, at varying concentrations of 8 - azaguanine. Michaelis constant (Km) and maximum velocity were determined from the double reciprocal plots. Km and Vmax were also measured with guanine at pH 6.0 and pH 8.0 for the comparison with 8 - azaguanine.

Inhibition by Para Hydroxymercuribenzoate

The effect of para hydroxymercuribenzoate on the guanine deaminase was studied at pH 8.0. The enzyme was dialyzed again 0.02M sodium phosphate buffer at pH 8.0 to remove dithiothreitol. The inhibitor concentrations used were x 10^{-4}M and 5 x 10^{-4}M. Varying guanine concentrations were used to determine the inhibition constant.

The time inactivation of the p - hydroxymercuribenzoate was also studied at 1 x 10^{-4}M concentration with guanine (2 x 10^{-4}M) at pH 8.0. The enzyme was incubated with para hydroxymercuribenzoate and the aliquots were withdrawn at the intervals of five minutes and assayed for the enzyme activity. Enzyme activity at zero time was teken as the initial velocity of the enzyme in the absence of inhibitor. This value of the initial velocity was set at 100% activity. The percent remaining activity was plotted against time.

Inhibition by Iodoacetic Acid

Effect of iodoacetate was studied at 1 x 10^{-3}M and 1 x 10^{-4}M concentration. The enzyme was incubated with iodoacetate and the samples were withdrawn at the intervals of five minutes and assayed for the enzyme activity. The remaining percent activity was plotted against the time intervals as described for the para hydroxymercuribenzoate.

Inhibition by 5 - Aminoimidazole 4 - Carboxamide

The effect of the purine precursor 5 - aminoimidazole 4 - carboxamide was studied. Concentrations of 5 - aminoimidazole 4 - carboxamide used were 1 x 10^{-3}M and 1 x 10^{-4}M. Double reciprocal plots were constructed to determine the pattern and the degree of the inhibition.

Results and Discussion

Purification of the Enzyme

This is the first attempt to purify the guanine deaminase using agarose hexane guanine triphosphate as an affinity material. Several problems were encountered during the course of this investigation but very satisfactory results were obtained in terms of obtaining a pure enzyme preparation. Use of phenylmethylsulfonyl fluoride by Bergstrom and Bieber (62) inhibited chymotrypsin like proteases and thus increased their yield by 33%. During the ammonium sulfate fractionation, maintenance of pH at 6.5 was important, because pH keeps falling back to 5, below which enzyme is not stable. This results in a good yield.

After one or two uses, the affinity column develops some fine particles in it and the fractions have to be recycled a few times to obtain a pure enzyme preparation. Also care must be taken in collecting the active enzyme fractions and only the best fractions should be pooled, which otherwise will result in a preparation with traces of undesired proteins. The recycling of such impure preparations on the columns a few times and subjecting them to the gradient elution helps in getting pure enzyme. Sometimes not all the xanthine oxidase is bound to allopurinol, and so inclusion of this in the final glycine buffer helps in removing this contaminant. The first few steps of ammonium sulfate fractionation and DEAE-cellulose and hydroxylapatite chromatography are quite helpful in removing the bulk of the undesirable protein and helps in getting a preparation for applying on the affinity column which is very rich in the enzyme protein. This results in a good yield of the enzyme.

During the ammonium sulfate fractionation, care should be taken in adding it very slowly with constant stirring and with the maintenance of the pH. In a previous report from this laboratory by Lewis and Glantz (31, 32) a long and laborious scheme of the conventional chromatography was used to purify this enzyme. However, this method saves considerable time and a number of steps and results in a homogeneous preparation. A long and extensive chromatographic method used by them might have resulted in the structural changes of the enzyme protein, as indicated by different results I obtained on the molecular weight of the enzyme. Nonetheless, their work still remains helpful in that it was the first published report on the methodology of the purification of the guanine deaminase to homogenity.

During protein determination, dithiothreitol was also present in the blanks. Although Bieber et al (62) used 5×10^{-3}M mercaptoethanol, I preferred to use dithiothreitol, at the same concentration, which has two sulfydryl groups, compared to one in mercaptoethanol. Lewis and Glantz used 20 times higher concentration of mercaptoethanol. The reason I preferred to use dithiothreitol, although it is quite expensive compared to mercaptoethanol, is its low volatility and hence low toxicity. While obtaining affinity material, it is advisable to ask for the supply from the same batch of the preparation, since every batch behaved in a different manner for eluting the enzyme. This might result in the inconsistency of the results. Fogle and Bieber (35) report that the rabbit liver guanine deaminase and rat liver guanine deaminse can be eluted with 0.05M glycine at pH 9.0. However, Bergstrom and Bieber (62) report that the rat liver guanine deaminase could not be eluted with 0.1M

glycine buffer at pH 9.0 nor with 0.05M glycine that contained 0.1M NaCl. The reasons for the different behavior were not clear. They further reported that the glycine did not affect the activity of the guanine deaminase, hence the elution by glycine was not a competitive effect. Day (61) showed that increasing the ionic strength by the addition of neutral salt prevented the elution of guanine deaminase at 0.05M glycine, pH 9.0. Bergstrom and Bieber (62) reported different solutions that eluted guanine deaminase at pH 9.0. These were 0.05M glycine, 0.05M beta alanine, 0.002M ammonium chloride and 0.01M glycine. They further reported that the glass distilled water removed guanine deaminase from the column in a very sharp peak, and the solutions with the ionic strength greater than 7×10^{-3} such as 0.1M glycine pH 9.0 and 0.01M ammonium chloride pH 9.0 did not remove guanine deaminase from the column. However, I used a gradient elution which removed the guanine deaminase slowly and produced satisfactory results. The results obtained at the various steps of purification are shown in Table 2.

Disc Gel Polyacrylamide Electrophoresis

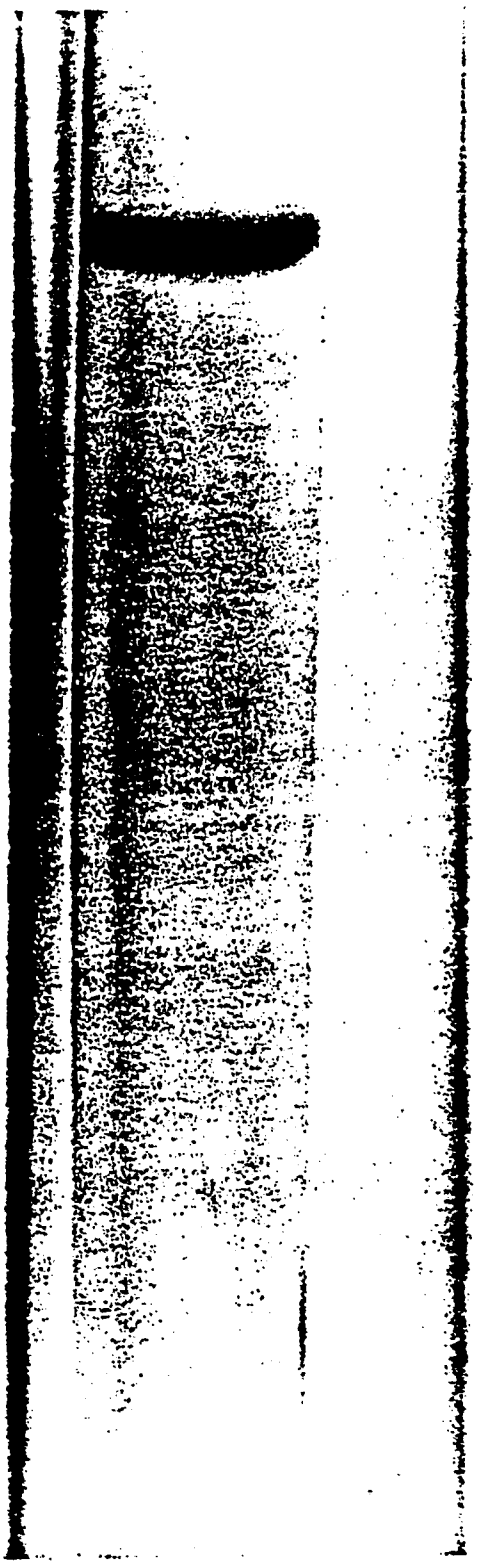
The homogenity of the enzyme was determined by the disc gel polyacrylamide electrophoresis as described earlier. The pure enzyme showed a clear single band indicating its homogenity. The electrophoretic pattern is shown in Figure 9. The results are in agreement with the reports on rabbit, pig and rat guanine deaminase (31, 32, 63, 54, 62, 75).

Table 2

Purification Scheme for 100gm of Human Liver

Purification Step	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Fold Purification
1. Crude extract	8750	58	0.00662	100	1
2. First ammonium sulfate fractionation (40 - 80%)	5833	48.5	0.0083	83	1.25
3. DEAE-cellulose chromatography	530	43	0.081	74	12.3
4. Second ammonium sulfate fractionation (75%)	353	41	0.116	70	17.6
5. Hydroxylapatite chromatography	15	26	1.73	44	261.4
6. Affinity chromatography	0.9	19.3	21.5	33	3248

Fig. 9. Disc gel electrophoresis. The enzyme (80ugm) was subjected to electrophoresis, after the final purification, in 7.5% gel, and the gels were stained with Coomassie blue.



SDS Gel Polyacrylamide Electrophoresis

The subunit molecular weight of the enzyme was determined by SDS gel electrophoresis. A single band appeared on the SDS gel electrophoresis with the molecular weight of 59,000. This was in agreement with the reports of Lewis and Glantz and Rossi, et al. (31, 32, 75). Figures 10,11.

Sephadex Gel Filtration

The molecular weight of the native enzyme was obtained as described in the materials and methods. A molecular weight of 120,000 was obtained. SDS gel electrophoresis gave a molecular weight of 59,000 for the subunit, hence the native enzyme must be a dimer of approximately 60,000 molecular weight subunits. This is in close agreement with the reports on this enzyme in rabbit liver (34, 35, 62, 75) except of Lewis. Bergstrom and Bieber (62) reported a molecular weight of 110,000 while Fogle and Bieber (35) reported a 100,000 molecular weight. Lewis reported a native molecular weight of 56,000. The results of this study are shown in Figure 13.

Isoelectrofocusing

The isoelectric point of the enzyme was determined by the method described earlier. The enzyme showed two major bands and a very faint band. The third very faint band might have been a diffused form of one of these two subunits. The two bands had a Pi of 4.76 and 4.68. The results are shown in Figure 12. Guanine deaminase, therefore, is an acidic protein. Lewis (33), however, reported a single band on isoelectrofocusing while Bieber, et al. (62) have reported two bands on isoelectrofocusing on rabbit liver enzyme.

Fig. 10. SDS gel electrophoresis. Weber and Osborn (66) method for SDS gel electrophoresis was followed. Lane "A" contains soyabean trypsin inhibitor (STI) M.W. 22,000, Lane "B" contains bovine serum albumin (BSA) M.W. 68,000 showing one major band (lower) and a minor band (upper). Lane "C" contains guanine deaminase, while Lane "D" contains ribonuclease (RNase). Two major units of RNase are β subunit (155,000, top) and α subunit (39,000, lower). Center of Lane "D" shows impurities in the sample. Lane "E" is blank. 60 mgm. enzyme was applied to the gels.

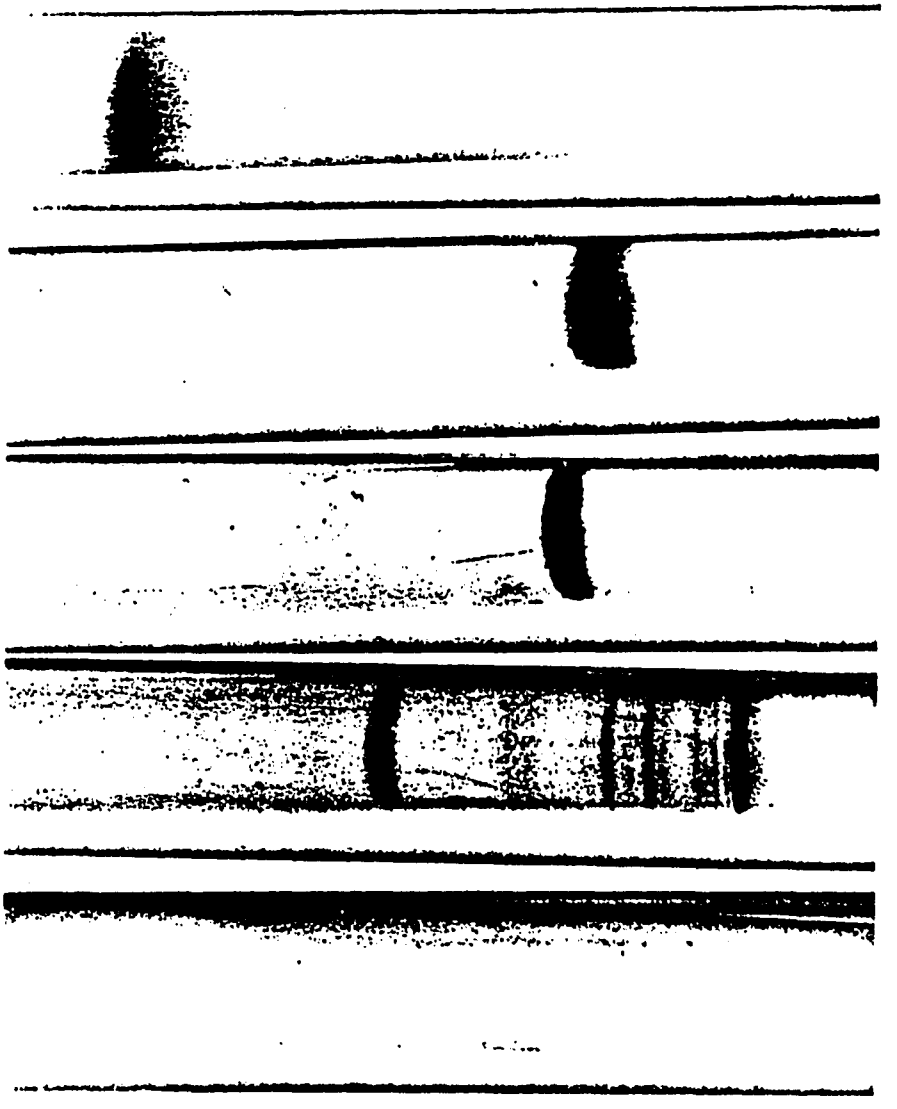

A
B
C
D
E

Fig. 11. Subunit molecular weight determination by SDS gel electrophoresis. The molecular weight of subunits was determined by plotting the log of molecular weight vs. the electrophoretic mobilities (Rf). Standards used were bovine serum albumin (BSA, 68,000), ribonuclease (RNase, 39,000) and soyabean trypsin inhibitor (STI, 22,000).

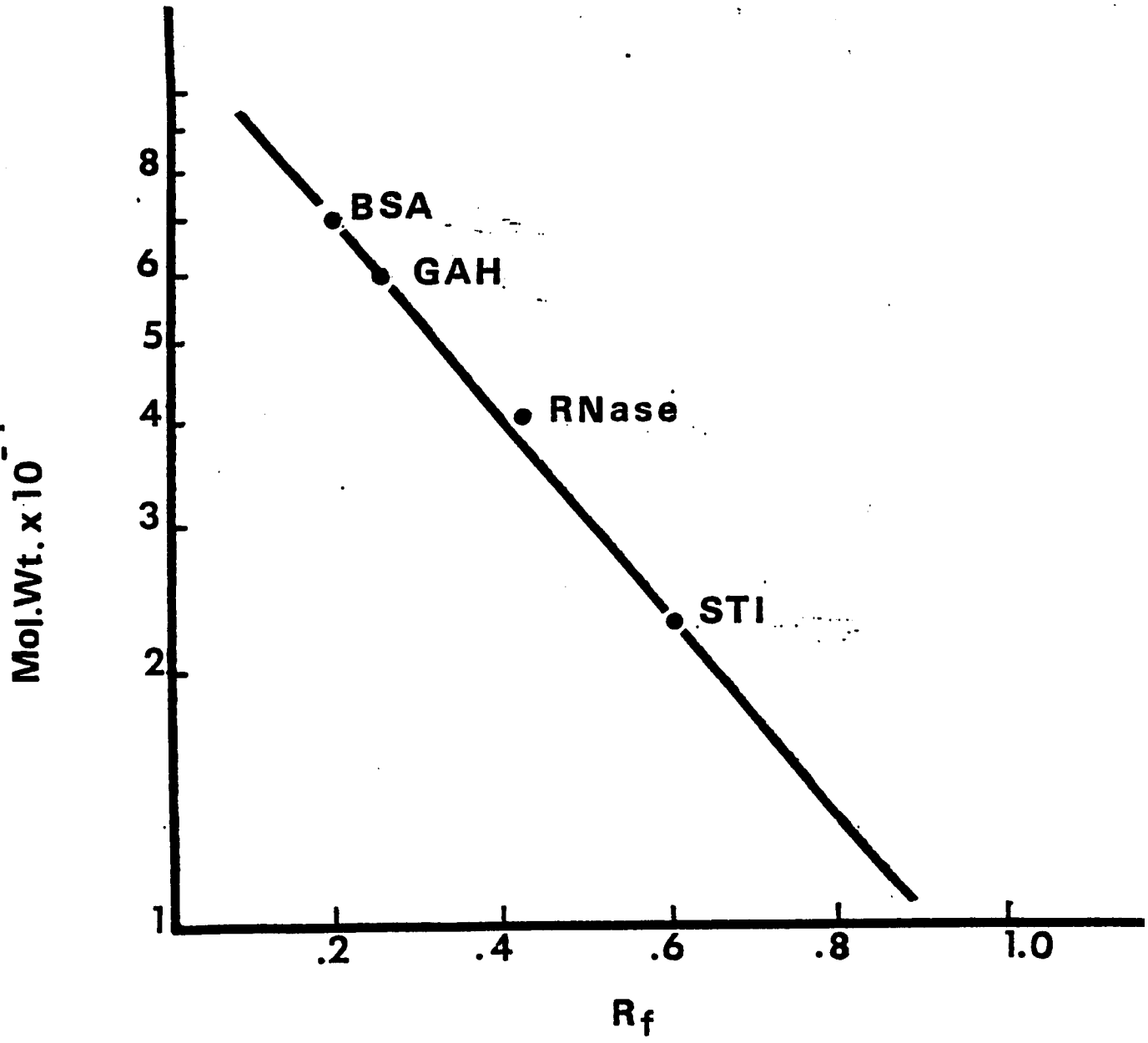

Mol.Wt. x 10^-4
8
6
5
4
3
2
1
BSA
GAH
RNase
STI
.2
.4
.6
.8
1.0
Rf

Fig. 13. Determination of native molecular weight of the enzyme by gel filtration on sephadex G-100. The molecular weight of the native enzyme was obtained by plotting the log of molecular weight vs. the ratio of elution volume to void volume. The standards used were aldolase (158,000), bovine serum albumin (68,000), Ovalbumin (45,000), chymotrypsinogen (25,000) and RNase A (13,700).

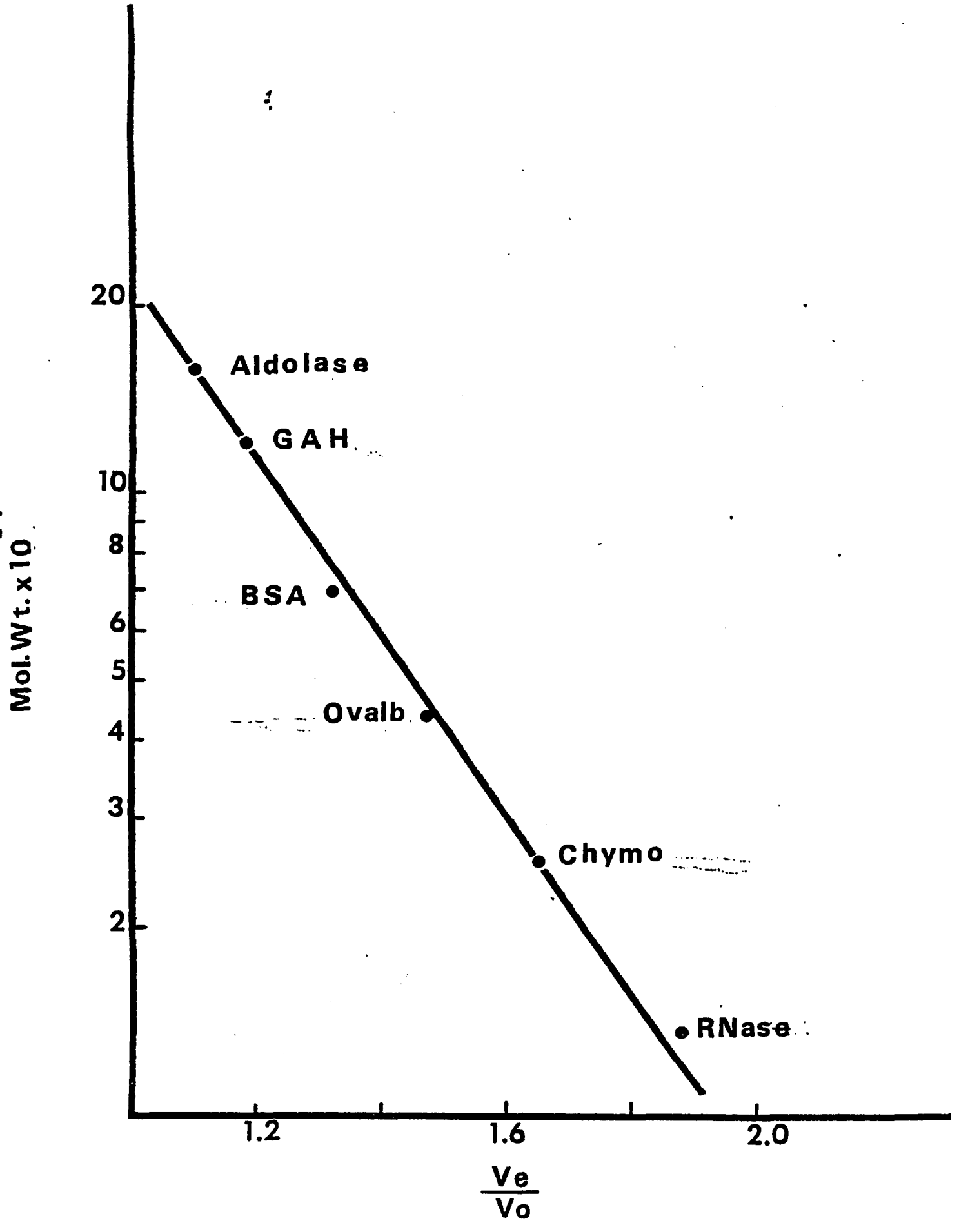

20
10
8
6
5
4
3
2
Mol.Wt. x 10^-4
Aldolase
GAH
BSA
Ovalb
Chymo
RNase
1.2
1.6
2.0
Ve/Vo

Fig. 12. Isoelectric focusing. The photograph shows isoelectric focusing of guanine deaminase in polyacrylamide gels, containing 60 micrograms of the enzyme. The isoelectric point (Pi) was determined by slicing the gels and checking the respective pH. Lane "A" represents blank, while Lane "B" contains guanine deaminase. The two bands indicate the presence of two subunits.

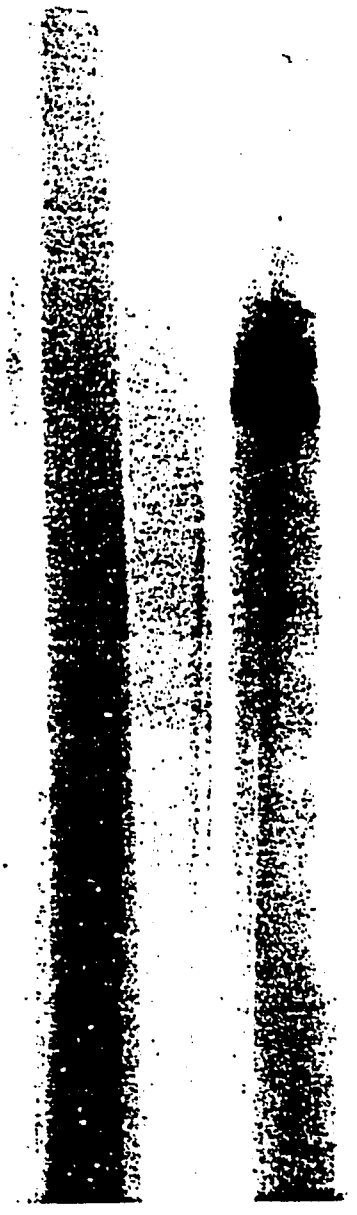
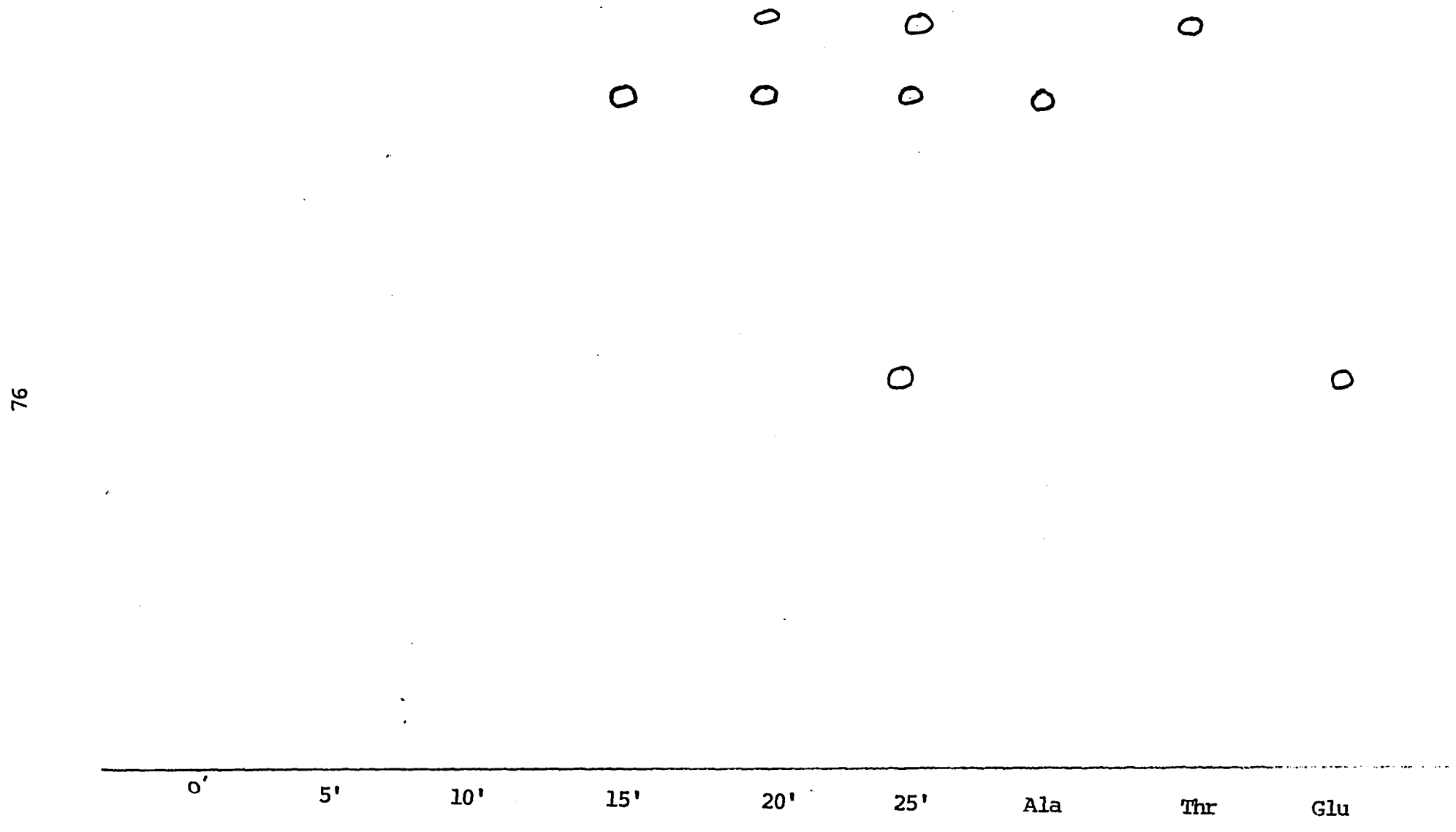

A
B

Fig. 14. Chromatography for C-terminal amino acid. Samples at 0, 5 and 10 minutes did not show any amino acid released.


76
0'
5'
10'
15'
20'
25'
Ala
Thr
Glu

N-Terminal Amino Acid Determination

The enzyme was subjected to N-terminal amino acid determination as described in the methods. Valine was found to be N-terminal amino acid.

C-Terminal Amino Acid Determination

The procedure to determine the C-terminal end of the enzyme protein was followed as described earlier. Alanine was found to be the C-terminal amino acid. (Figure 14)

Since this is the first report on the N and C-terminal studies of this enzyme, no other data were available for comparison.

Amino Acid Analysis

Amino acid analysis was performed on a 24 hour digestion of the enzyme protein. Minimal amino acid present was cysteine while a dominant amount of aspartate was observed from the analysis. The data are presented in Table 3. The enzyme was high in glutamic acid, alanine, aspartate, glycine and leucine and was found low in cystein and tyrosine. The large number of acidic residues are expected since the nature of the enzyme protein is acidic, as evidenced by acidic pI value of the guanine deaminase. Since there are two subunits in the enzyme, the amino acid composition represents a combination of the subunits. The two subunits could be different in amino acid composition. However, they were found very similar in molecular weight and pI values. This is in good agreement with the reports of Bergstrom and Bieber (62).

Determination of Km

Km was determined at varying concentration of guanine at pH 7.0.

Table 3

Amino Acid Analysis of Guanine Deaminase
Based Upon MW of 120,000

Amino Acid	Empirical Residue	Residues/ mole	Integer Empirical Residue
Asp	11.3	88	11
Thr	6.0	48	6
Ser	6.66	56	7
Cys	1.0	8	1
Glu	10.78	88	11
Prol	6.8	56	7
Gly	9.6	80	10
Ala	13.2	104	13
Arg	7.24	56	7
His	1.7	16	2
Lys	6.636	56	7
Val	10.0	80	10
Met	2.26	16	2
Ileu	8.0	64	8
Leu	9.6	80	10
Tyr	1.4	16	2
Phe	4.4	40	5
Trp	N.D.		N.D.

N.D. = Not determined

Fig. 15. Double reciprocal plot (Lineweaver Burk plot) for guanine as substrate. Velocity is measured at 245nm and is expressed as micromoles of guanine hydrolyzed per minute. The plot shows velocity of the enzyme catalyzed reaction as a function of guanine concentration.

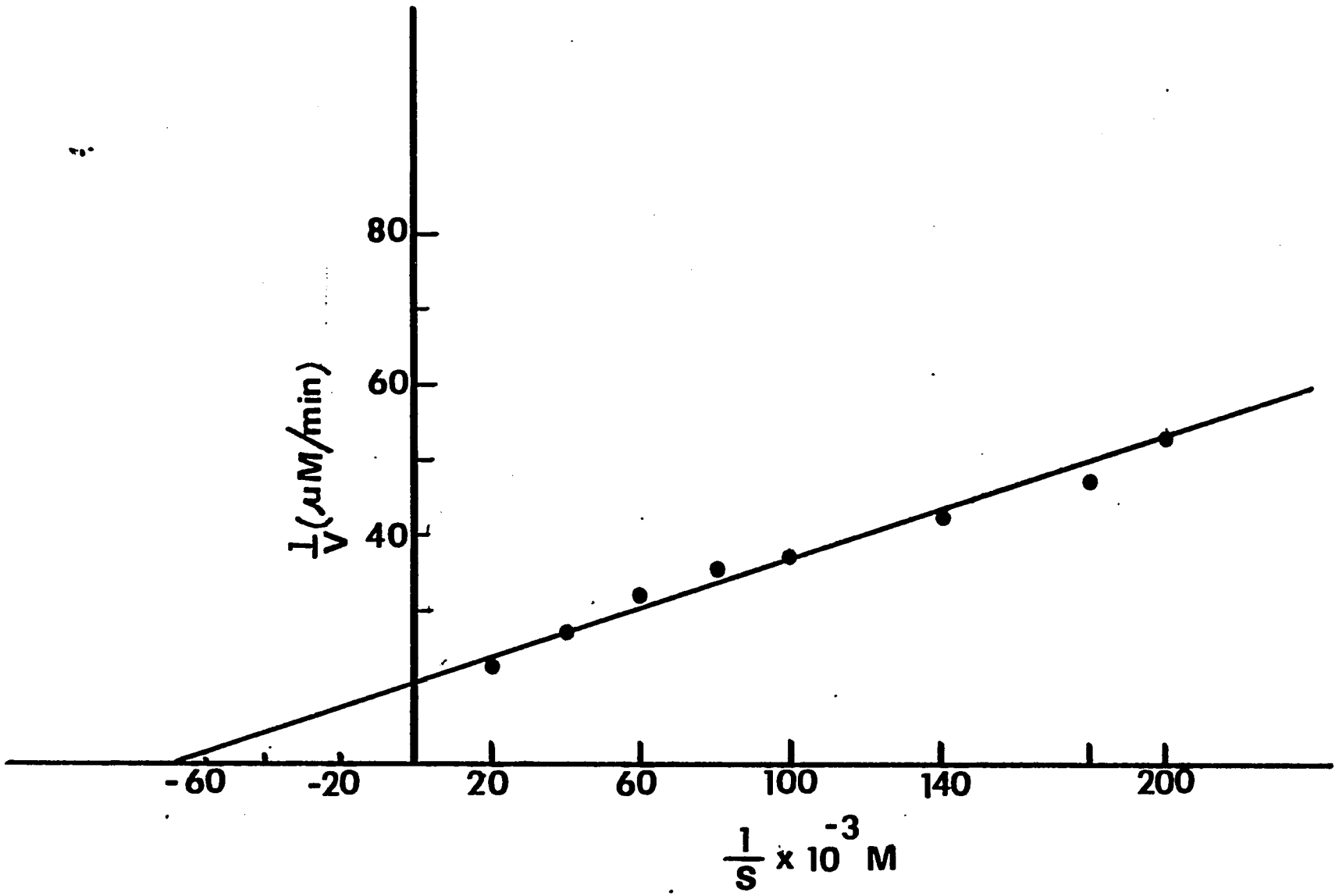

80
60
40
$\frac{1}{V}$ (μM/min)
-60
-20
20
60
100
140
200
$\frac{1}{S}$ x 10^{-3} M

Fig. 16. Double reciprocal plot at varying pH. The plot shows effect of pH on the guanine deaminase catalyzed reaction with guanine as substrate. The velocity is expressed as micromoles of guanine hydrolyzed per minute. The plots for each pH are: pH 5.5(•••••), pH 6.0 (·····), pH 6.5 (+++++), pH 7.0 (▬▬), pH 7.5 (▬), pH 8.0 (——), pH 8.5 (••••), pH 9.0 (----).

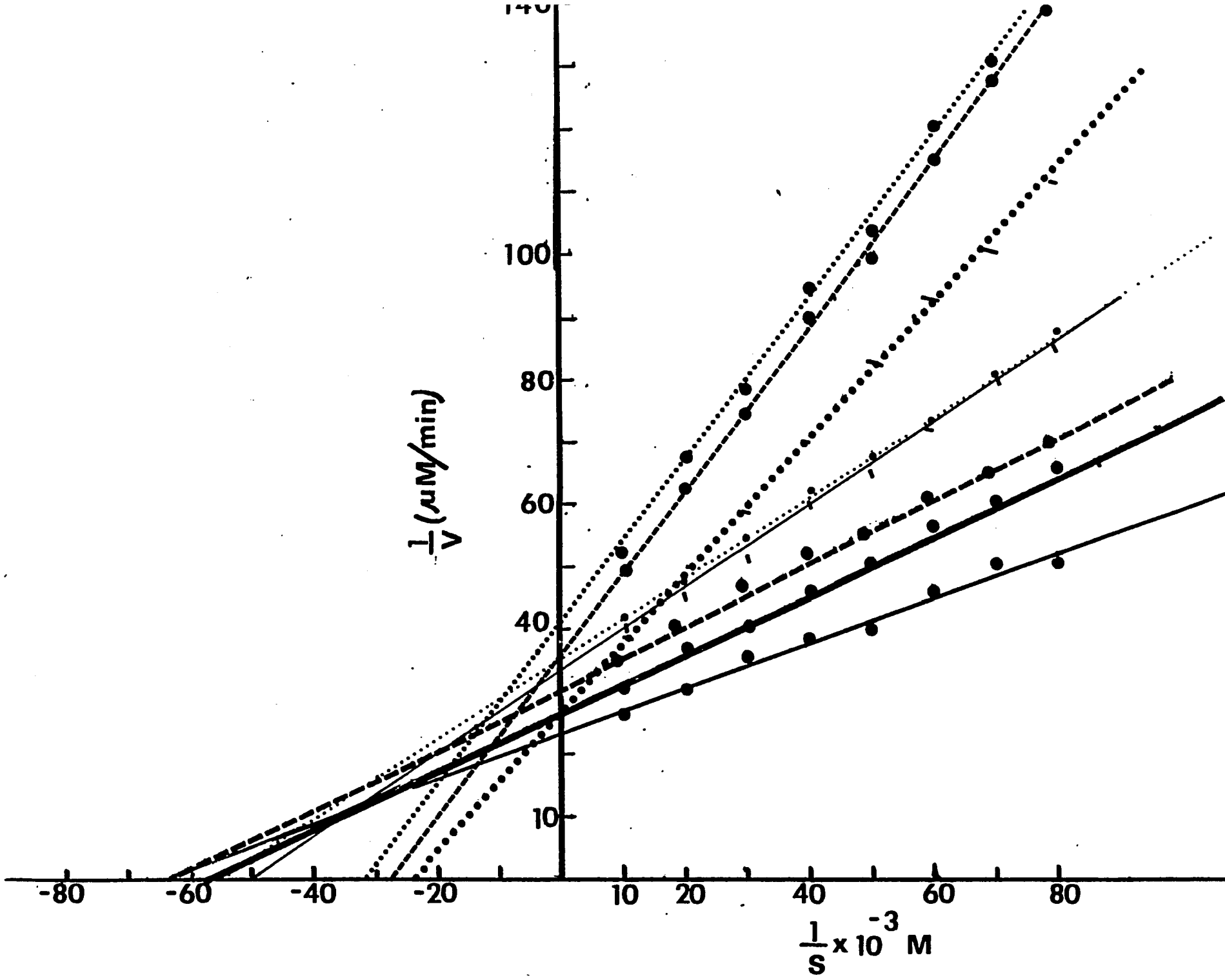

140
100
80
60
40
10
$\frac{1}{V}$ (μM/min)
-80
-60
-40
-20
10
20
40
60
80
$\frac{1}{S} \times 10^{-3}$ M

Table 4

Kinetic Parameters from Double Reciprocal Plots of Guanine Deaminase at Different pH Values

pH	Vmax x 10^{-2}	Km x 10^{-5}	pKm	Log Vmax/Km
5.5	2.50	3.13	4.50	2.90
6.0	2.85	1.82	4.74	3.20
6.5	3.03	2.00	4.71	3.18
7.0	3.33	1.66	4.77	3.30
7.5	3.70	1.78	4.74	3.31
8.0	4.34	1.56	4.80	3.44
8.5	3.84	4.17	4.38	2.96
9.0	2.80	3.70	4.43	2.87

Fig. 17. Plot of log Vmax/Km vs. pH. The Km and Vmax values as obtained from Figure 15, are plotted against pH. The pka values obtained are at pH 5.8 and at pH 8.0.

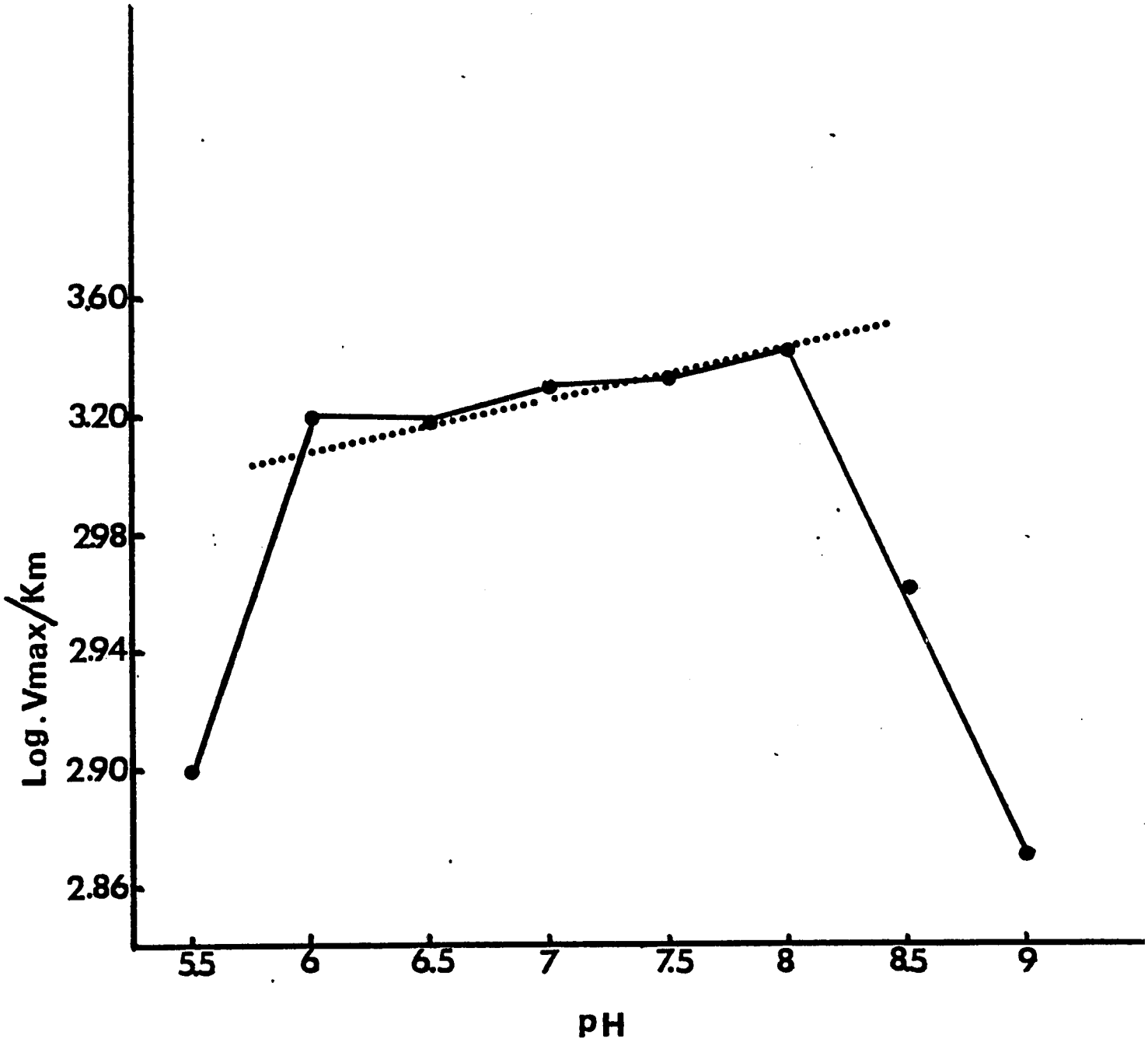

3.60
3.20
2.98
2.94
2.90
2.86
Log. Vmax/Km
5.5
6
6.5
7
7.5
8
8.5
9
pH

Fig. 18. Plot of pKm (-log Km) vs. pH. Km values were obtained from Figure 16.

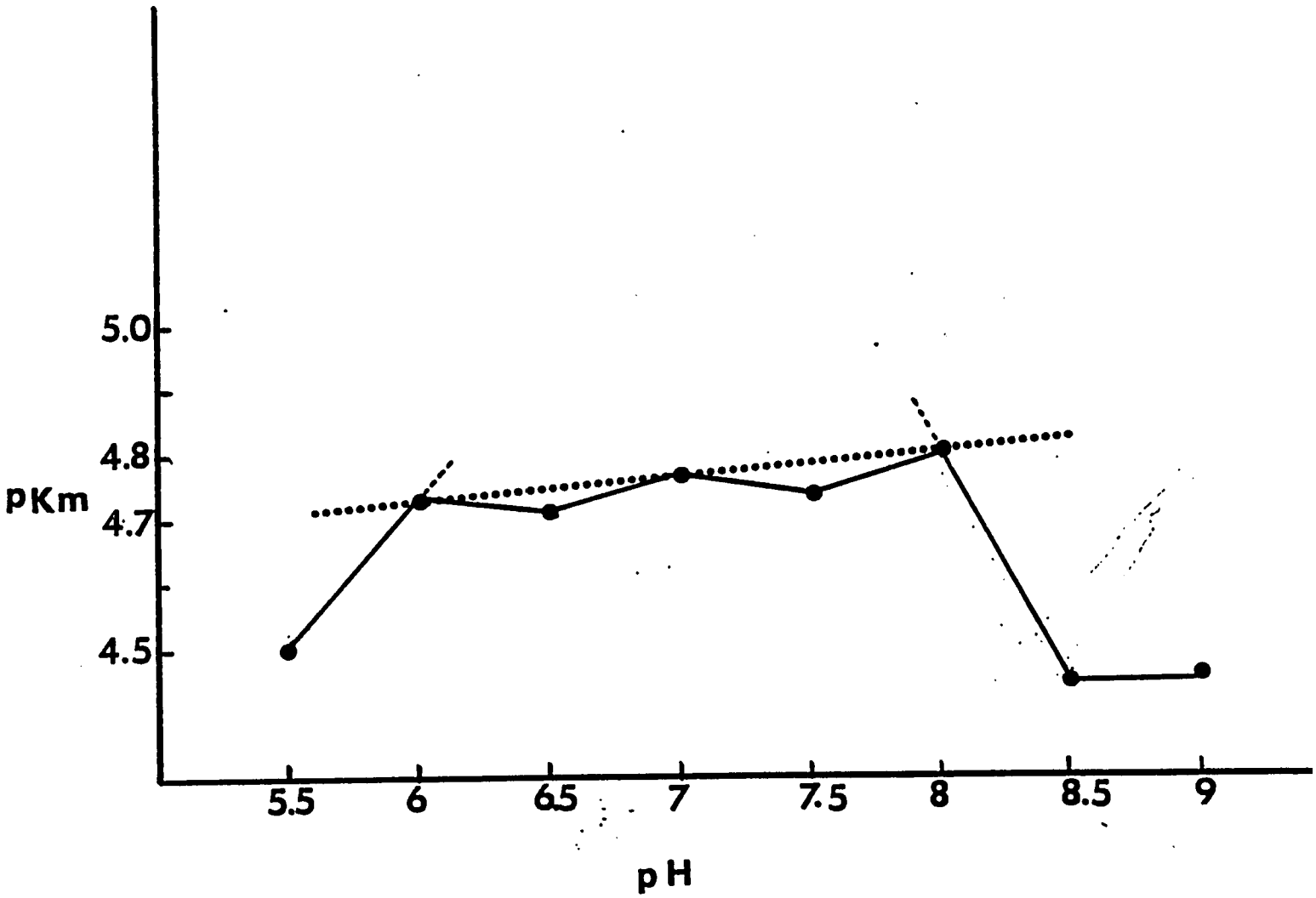
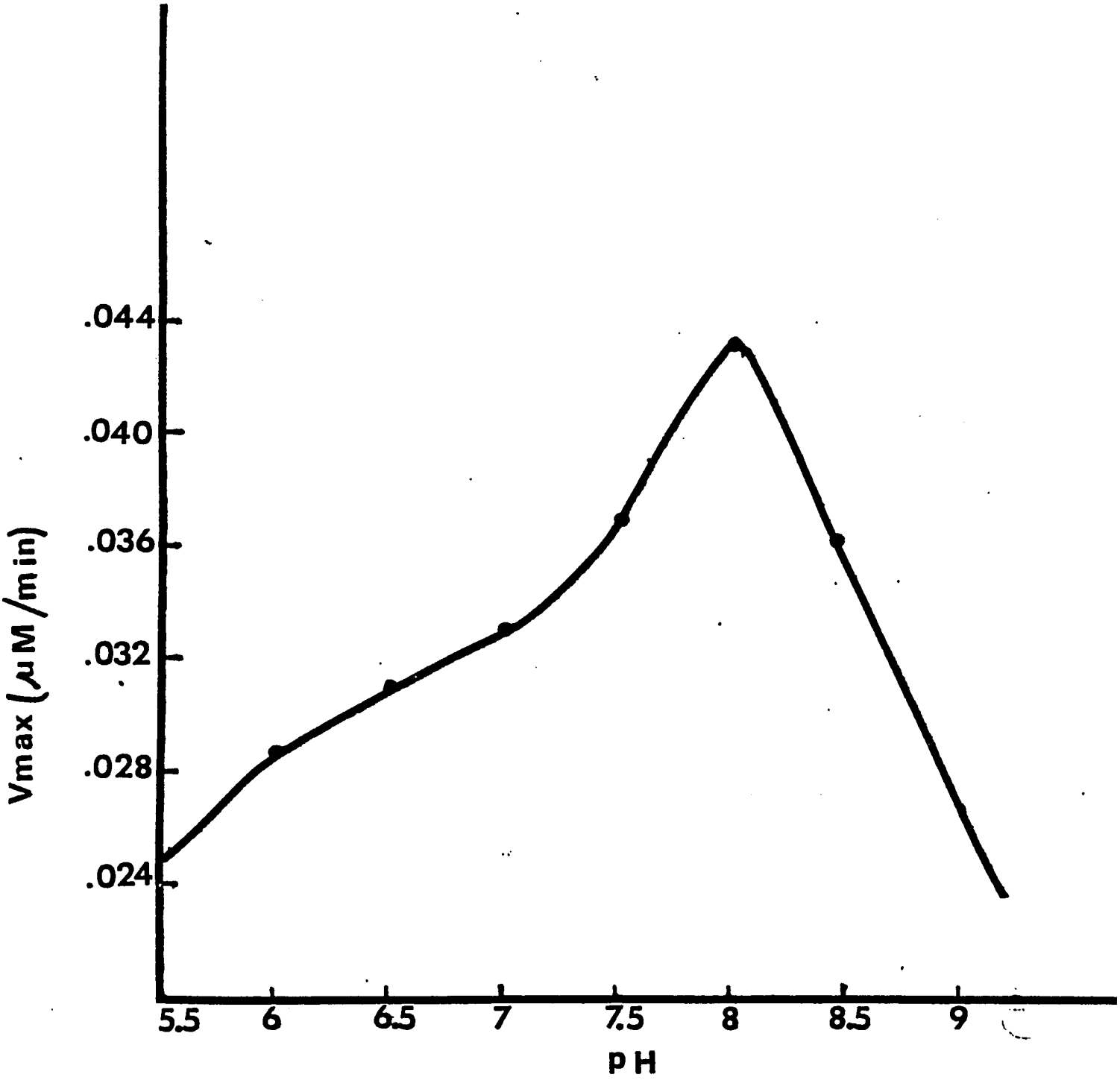

5.0
4.8
4.7
4.5
pKm
5.5
6
6.5
7
7.5
8
8.5
9
pH

Fig. 19. Plot of maximum velocity vs. pH. A sharp optimum pH value was obtained at pH 8.0.


.044
.040
.036
.032
.028
.024
Vmax (μM/min)
5.5
6
6.5
7
7.5
8
8.5
9
pH

A Km of 1.538 x 10^{-5}M was obtained in this study for the human liver enzyme. The results are expressed in Figure 15. The reported values of Km of the rabbit liver guanine deaminase vary from 1 x 10^{-5}M to 6 x 10^{-6}M (31, 32, 34, 35, 53, 54, 61, 62, 63, 75).

pH Effects on the Kinetic Properties

pH effect on the enzyme was studied by varying the pH from 5.5 to 9.0, in the increments of 0.5 pH units. The kM and the Vmax were calculated at different pH. The plots of pKm (-log Km) vs. pH, log Vmax/Km vs. pH and the Vmax vs. pH were constructed from these data, which are shown in Figures 16, 17, 18 and 19 and Table 4. The optimum pH for the guanine deaminase was found to be 8.0. The kinetic parameters reported by Bieber, et al. (62) and Lewis (33) are mostly in good agreement with my observations, except at the extremities of the pH. In the plot of Vmax vs. pH (Figure 19) Vmax constantly increased up to pH 8.0. Since Vmax is increasing with pH until pH 8.0 (or with hydroxideion concentration) hydroxide ion might be truly involved in the reaction mechanism, particularly during complex formation between the enzyme and the substrate, abstracting the amino group from the guanine by the addition of a hydrogen ion and replacing it with a hydroxide. Also the pKa of 5.8, as obtained in this study indicates the possibility of a functional group that has a pKa in this range. The pKa value reported by Bieber, et al. (62) is 5.6 and 6.0 by Lewis. This indicates the possible involvement of a carboxyl group of aspartate or glutamate or the dissociation of a protonated imidazole. Bieber, et al. (62) have also indicated the same possibility, as well as the involvement of the hydroxide ion in the rate limiting step and in the release of ammonia as discussed above. The optimum

pH value reported by Lewis (33) was 6.5 and 8.0 by Bieber, et al. (62).

Effect of 8 - Azaguanine

8 - Azaguanine effects as a substrate were studied. 8 - Azaguanine has an optimum pH of 6.0 as reported by Lewis and Glantz (32). However, Rousch and Norris (22) reported a pH of 6.3 for 8 - azaguanine. Varying concentrations of 8 - azaguanine were taken and the activity of the enzyme measured. Measurements with guanine were also performed at pH 6.0 and at pH 8.0 to compare the pattern of the kinetics of guanine and 8 - azaguanine. The Km obtained for 8 - azaguanine is 2×10^{-4}M. Km was higher for 8 - azaguanine which indicates that it is weakly bound to the enzyme as compared to guanine whose Km is lower. The results are expressed in Figure 20. Bieber, et al. (62) have reported a Km value of 2.3×10^{-4}M for 8 - azaguanine. However, Lewis (33) reported a Km value for 8 - azaguanine at 3.33×10^{-4}M.

Inhibition by Iodoacetate

Effects of iodoacetate were studied at a concentration of 1×10^{-4}M and 1×10^{-3}M at pH of 8.0 with guanine as substrate. Enzyme was incubated with iodoacetate for 5 to 30 minutes and was removed and assayed for the activity at 5 minute intervals.

At 1×10^{-4}M concentration of the iodoacetate the enzyme showed very little inhibition with only a loss of 7% of its activity. At a concentration of 1×10^{-3}M of the iodoacetate, enzyme showed a slow decrease in the activity ranging from 5% at 5 minutes period to 24% at 30 minutes period. It, therefore, still retained most of its activity. Iodoacetate, therefore, is a poor inhibitor of guanine deaminase (Figure 21). Since iodoacetate is -SH group inhibitor, its COOH group might be involved in altering the enzyme environment making it a poor inhibitor. However, Lewis (33) reported a loss of about 10% in the activity of the enzyme at a

Fig. 20. 8 - azaguanine study. The figure shows the double reciprocal plot for 8 - azaguanine (- - - -) at pH 6.0 and guanine, as substrates for guanine deaminase. Guanine was used at pH 6.0 (•••••) and pH 8.0 (——) for comparison.

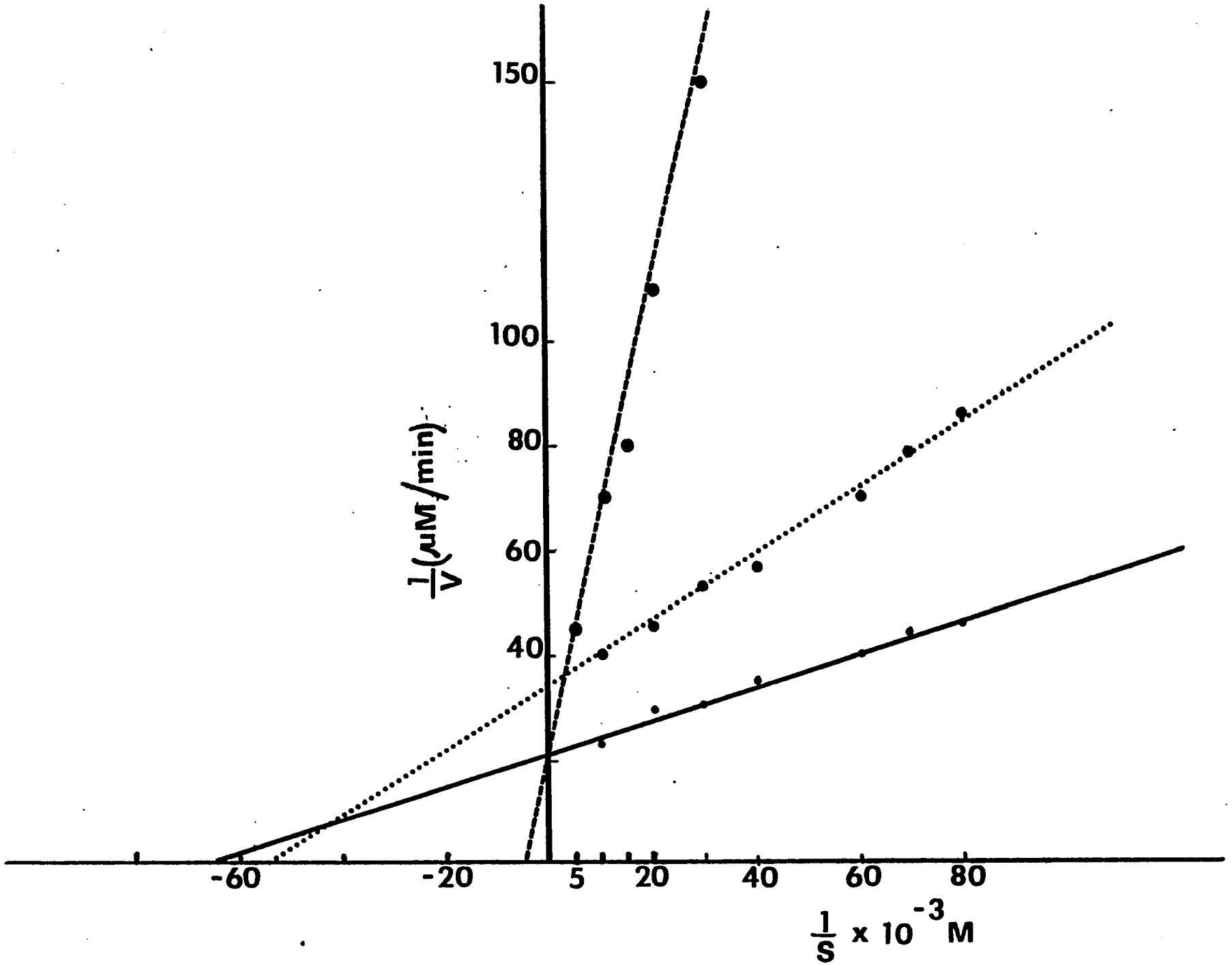

150
100
80
60
40
$\frac{1}{V}$(μM/min)
-60
-20
5
20
40
60
80
$\frac{1}{S}$ x 10^{-3} M

Fig. 21. Iodoacetate inhibition. Enzyme was incubated with iodoacetate at 1×10^{-3}M (A) and 1×10^{-4}M (B) concentration for the periods of time shown and the activity was measured at 245nm. Guanine was used as substrate at 2×10^{-4}M concentration.

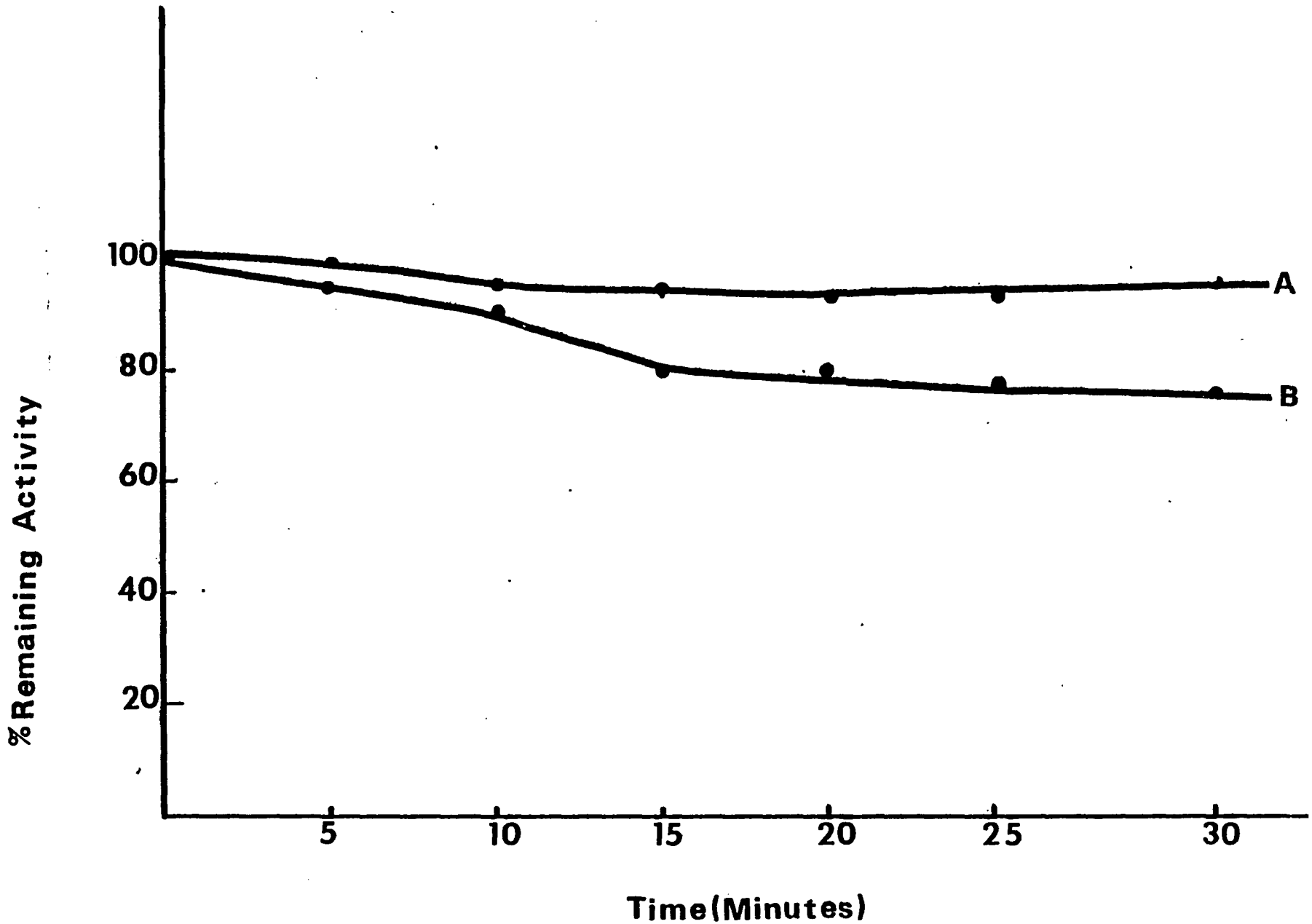

100
80
60
40
20
% Remaining Activity
5
10
15
20
25
30
Time (Minutes)
A
B

Fig. 22. Noncompetitive inhibition by p - hydroxymercuribenzoate p - Hydroxymercurobenzoate concentrations used were 1×10^{-4}M (----) and 5×10^{-4}M (.....). ———— represents control. Assays were done at pH 8.0 and the enzyme was dialyzed to remove dithiothreitol prior to assays. Guanine was used as substrate.

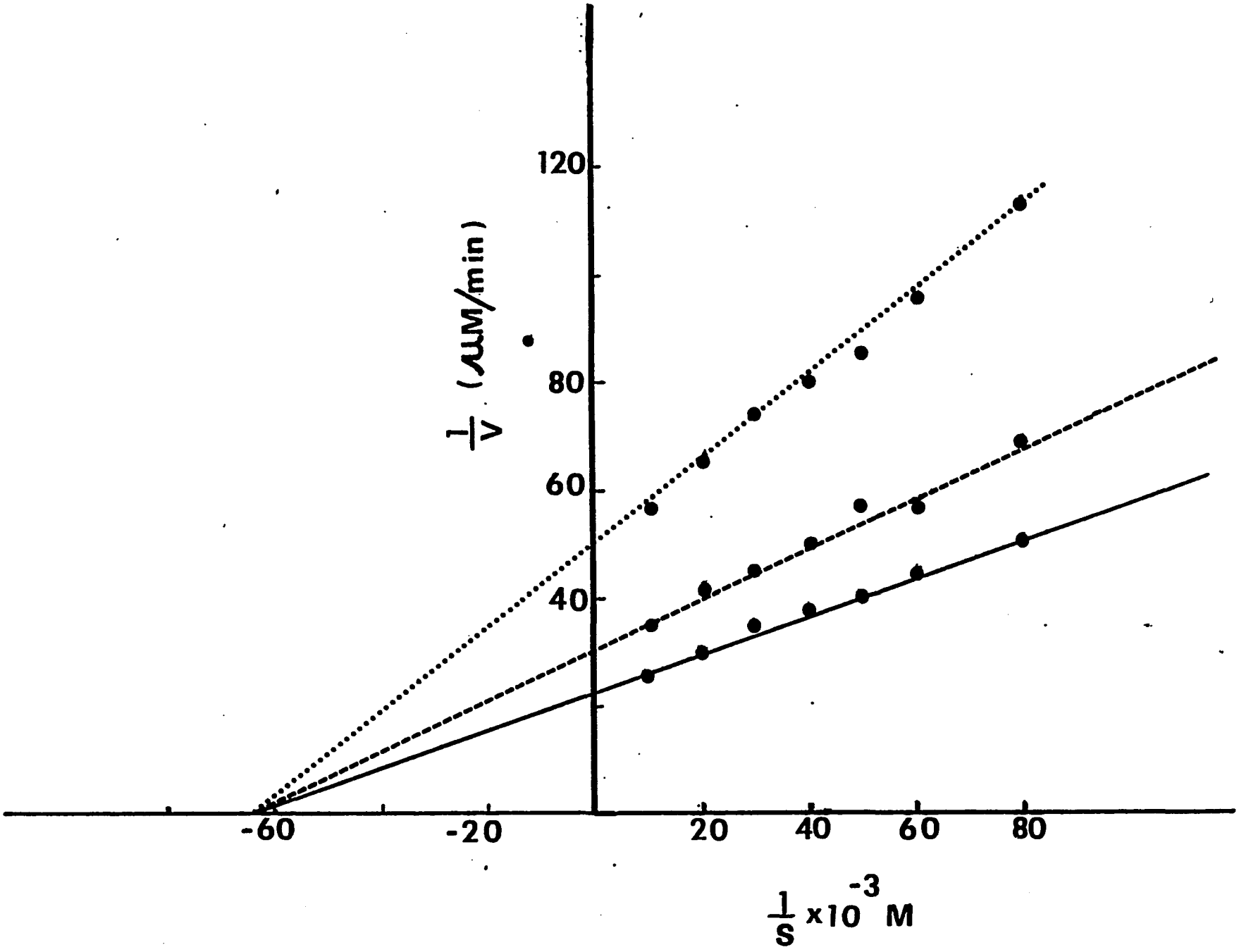
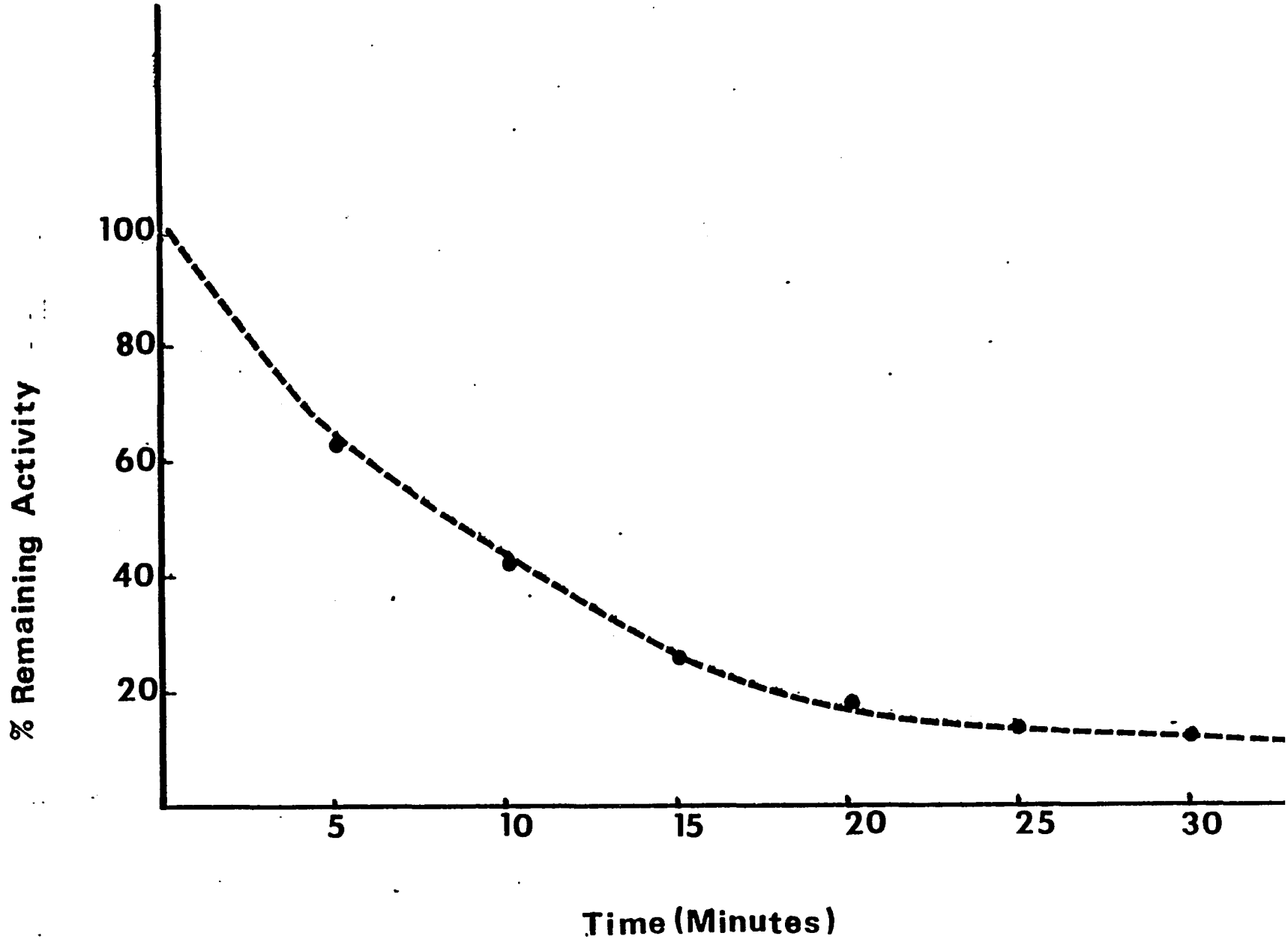

120
80
60
40
-60
-20
20
40
60
80
$\frac{1}{V}$ (μM/min)
$\frac{1}{S} \times 10^{-3}$ M

Fig. 23. Inhibition by PHMB. The time inactivation studies were done at 1×10^{-4}M PHMB concentration. An aliquot of the enzyme was removed at each time interval and assayed for its activity. Guanine was used as substrate at 2×10^{-4}M concentration.


100
80
60
40
20
% Remaining Activity
5
10
15
20
25
30
Time (Minutes)

concentration of 2 x 10^{-3}M of iodoacetate after 20 minutes.

Inhibition of p - Hydroxymercuribenzoate

The effect of PHMB was studied at pH 8.0 at the concentrations of 1 x 10^{-4}M and 5 x 10^{-4}M. It was observed that PHMB acts as a noncompetitive inhibitor, and it greatly reduces the activity of the enzyme. A Ki (inhibition constant) value of 1.53 x 10^{-5}M was obtained during this study. Lewis, et al. (31, 32) have reported a Ki of 4.56 x 10^{-4}M for the rabbit liver enzyme (Figure 22).

The time inactivation of the enzyme by PHMB was also studied. (Figure 23.) The enzyme was incubated with the PHMB (1 x 10^{-4}M) and an aliquot was withdrawn at 5 minute intervals and assayed for the enzyme activity. The enzyme showed a great degree of sensitivity to inhibition by p - hydroxymercuribenzoate with a 37% loss in the activity at the first 5 minutes and about 91% loss in the activity at 30 minutes. Since PHMB acts on the sulfhydryl group, the inhibition by PHMB suggests the involvement of -SH group at the active site of the enzyme.

Inhibition by 5 - Aminoimidazole - 4 - Carboxamide

The inhibitory effect of the purine precursor 5 - aminoimidazole - 4 - carboxamide was studied on the guanine deaminase at a concentration of 1 x 10^{-4}M and 1 x 10^{-3}M. It was observed to be a potent inhibitor of the guanine deaminase and the type of the inhibition observed was of a competitive nature. A Ki of 5 x 10^{-5}M was obtained at a concentration of 1 x 10^{-3}M for the inhibitor while a value of 3.33 x 10^{-5}M was calculated for the Ki at the concentration of 1 x 10^{-4}M. Mandel, et al. (7) had observed that the in vivo incorporation of guanine into the nucleic acid of mice was

potentiated by the simultaneous administration of 5 - aminoimidazole - 4 - carboxamide, and had concluded that the guanine deaminase was inhibited by 5 - aminoimidazole - 4 - carboxamide so that exogenously administered guanine was not catabolized. Lewis, et al. (31, 32) reported a Ki of 3.05×10^{-5}M for the inhibitor and observed value in my report is close to this value.

Fig. 24. Competitive inhibition by 5 - aminoimidazole - 4 - carboxamide. Control is represented as ---------- showing no inhibition. The concentrations of 5 - aminoimidazole - 4 - carboxamide used were 1×10^{-3}M (——) and 1×10^{-4}M (••••). Guanine was used as as substrate.

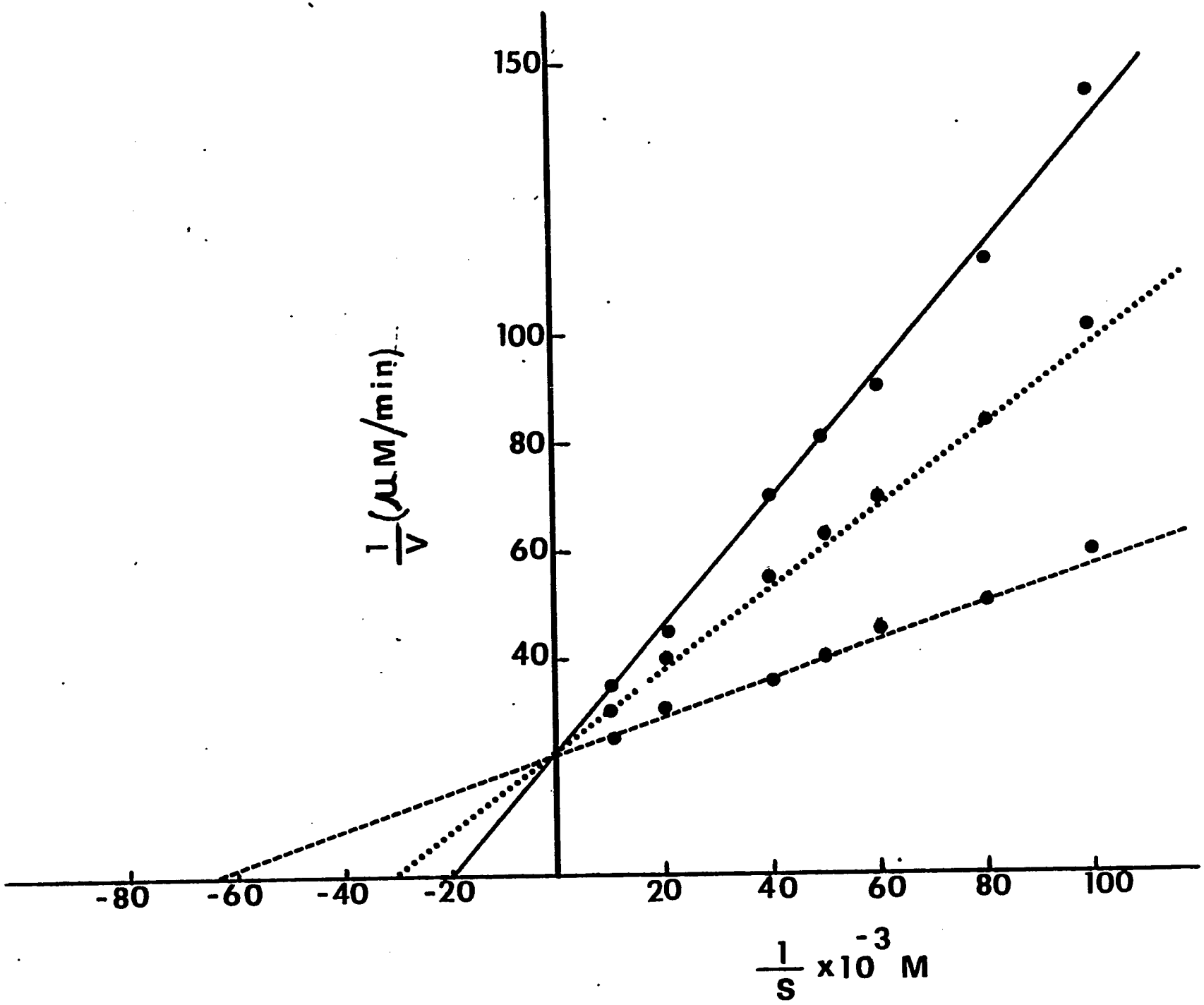
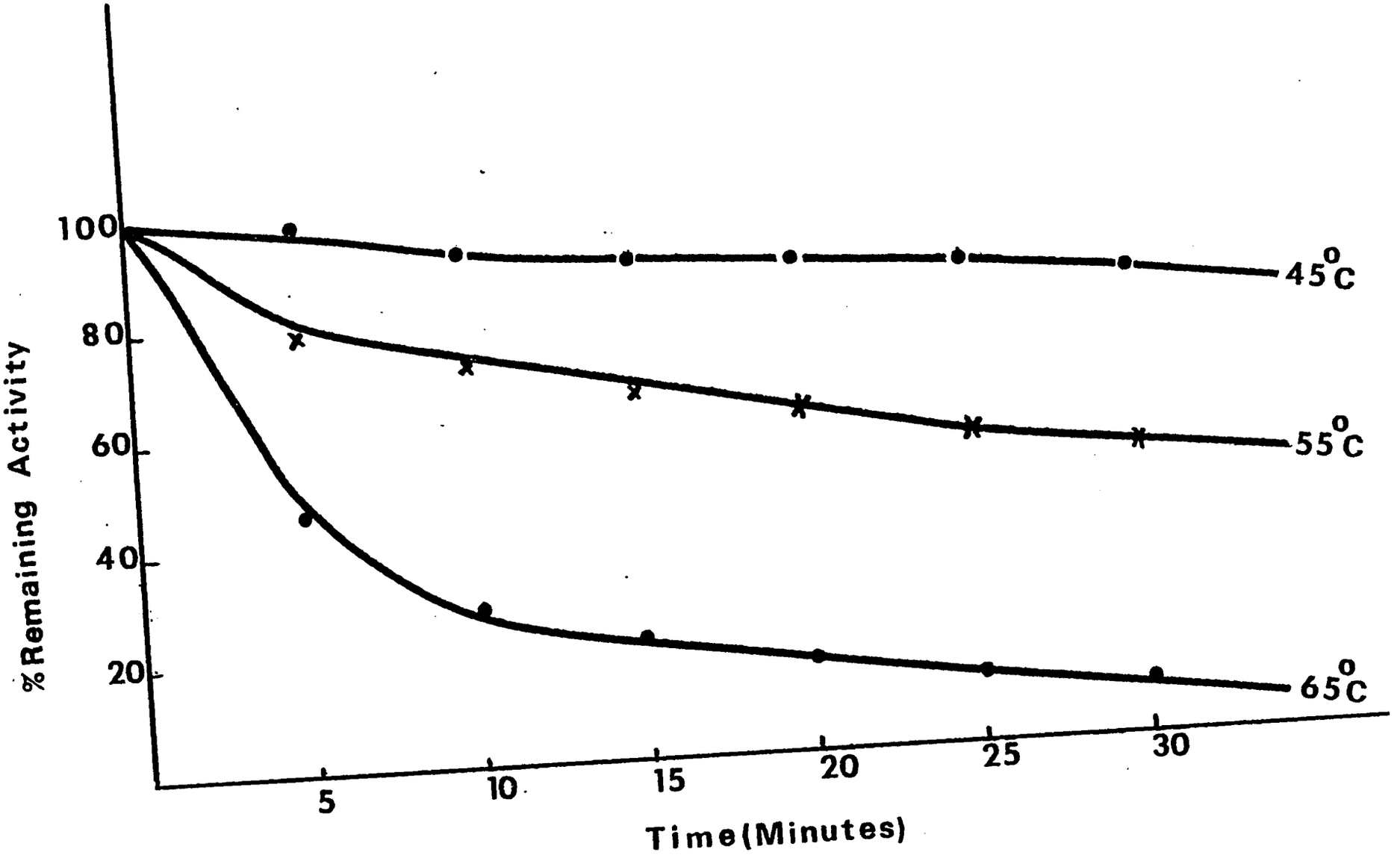

150
100
80
60
40
$\frac{1}{V}$ (μM/min)
-80
-60
-40
-20
20
40
60
80
100
$\frac{1}{S}$ x10^{-3} M

Fig. 25. Temperature inactivation studies. Guanine deaminase was incubated at different temperatures and an aliquot was removed to assay for the activity at different time intervals. 2×10^{-4}M guanine was used as substrate.


100
80
60
40
20
% Remaining Activity
5
10
15
20
25
30
Time (Minutes)
45°C
55°C
65°C

Temperature Effect

The stability of the enzyme was studied by incubating it at different temperatures and assaying the aliquot of the enzyme at five mintues intervals. The enzyme seemed to be quite stable as it showed only 3% inactivation at five minutes at 45°C and a maximum of 17% inhibition at 30 minutes at this temperature. However, it became labile at 55°C where it lost 21% of its activity at the first five minutes and 49% at the end of 30 minutes. It showed a substantial inactivation at 65°C where at the end of five minutes it lost about 54% of its activity and 91% at the end of 30 minutes. Similar results were reported for the rabbit enzyme by Lewis (33). The results are shown in Figure 25.

Conclusion

Human liver guanine deaminase is an acidic protein with a molecular weight of 120,000 and is composed of two subunits. The purification scheme used in this study resulted in a 3248 fold purification with 33% yield. The affinity label, agarose hexane guanine triphosphate is important in the purification of the enzyme since it is readily available. The synthesis of the label has been successfully attempted in this laboratory although the ligand is commercially available. The affinity label greatly minimizes the number of the steps of several more column chromatography as used in the earlier procedures, and helps in achieving the pure enzyme preparation in a shorter period. Amino acid analysis and isoelectric focusing supported the data that is is an acidic protein. SDS gel electrophoresis and gel filtration also were in accordance to the dimer nature of the enzyme as was the isoelectric focusing.

There are conflicting reports on the molecular weight and kinetic parameters of the enzyme from different sources, so the results of this investigation are not in a position to support a particular study, although they closely parallel to most of the workers' observations. This enzyme is very interesting enzyme and recently clinicians have started to observe guanine deaminase in the onset of the various types of diseases as in viral hepatitis, kidney diseases, etc. (88, 89, 90) and a study with the clinical approach will be interesting to do particularly at the molecular level.

Strong inhibition by PHMB suggests the possibility of the presence of -SH groups at the active site of the enzyme. pH effects of the enzyme were quite interesting in that they indicate the involvement of hydroxide ions

quite clearly as the velocity increased constantly without any abrupt changes. A sharp peak or decline in velocity after pH 8.0 strongly suggests a pH optimum of 8.0 for this enzyme. A summary of the results obtained in this study is presented in Table 6. and the comparative data on guanine deaminase from various organs and tissues of various species in Table 6.

Table 5

Summary of the Results Obtained in this Investigation

	Step	Result
1.	Purification factor	3248
2.	Specific activity	21.5
3.	Native molecular weight	120,000
4.	Subunit molecular weight	59,000
5.	Isoelectric point	4.76
6.	Amino acid analysis	Acidic protein. High in Glu, Asp, Gly, Ala, Val Leu residue
7.	C-terminal amino acid	Alanine
8.	N-terminal amino acid	Valine
9.	Km with guanine	1.538×10^{-5}M
10.	Km with 8 - azaguanine	2×10^{-4}M
11.	Optimum pH	8.0
12.	Effect of varying pH at kinetic parameters	As shown in Figures 18 - 20.
13.	Ionization groups (pKa) values	5.8 and 8.0
14.	Inhibition by iodoacetate	Slight inhibition
15.	Inhibition by p - hydroxymercuribenzoate	Strong noncompetitive inhibitor
16.	Time Inactivation by PHMB	As shown in Figure 25
17.	Inhibition by 5 - aminoimidazole 4 - carboxamide	Competitive inhibitor
18.	Temperature stability	Loss of activity above 45°C

Table 6

SUMMARY OF COMPARATIVE DATA ON GUANINE DEAMINASE

FROM VARIOUS ORGANS AND TISSUES FROM VARIOUS SPECIES

ource	Reference	Degree of purification	Substrate specificity	Michaelis constants (moles/liter)	Competitive inhibitors	Inhibitor constants	pH optimum	Molecular weight
Rabbit liver	(i) Rousch & Norris (22)		guanine	$5x10^{-6}$			near 8.0	
			8 - aza-guanine	$7x10^{-3}$			6.3	
	(ii) Kream & Chargaff		guanine	N.D.	N.D.	N.D.	N.D.	
			8 - aza-guanine					
	(iii) Lewis (56)		guanine				6.5	
	(iv) Currie et al (29)	200-fold	guanine	$1.05x10^{-5}$			7.7	170,000 and 525,000 (gel filtration on sephadex G-200).
			8 - aza-guanine	$1.02x10^{-4}$			5.9	
			1-methyl-guanine	$2.70x10^{-3}$			7.1	
	(v) Lewis (33)	9000-fold	guanine	$1.2x10^{-5}$M	AIC[1]	$3.05x10^{-5}$	6.8	56,000 (gel filtration on sephadex G-200) 54,000 (SDS polyacrylamide gel electrophoresis).
					THFA[2]	$2.58x10^{-4}$		
					AICA[3]	$5.68x10^{-4}$		
			8 - aza-guanine	$3.33x10^{-4}$M			6.0	
			6-thio-guanine	$8.0x10^{-4}$M				

Continued

ource	Reference	Degree of purification	Substrate specificity	Michaelis constants (moles/liter)	Competitive inhibitors	Inhibitor constants	pH optimum	Molecular weight
. Rat liver	(i) Schmidt (17)		guanine				9.2	
	(ii) Mansoor et al (53)		guanine				9.0	
	(iii) Kumar & Krishnan (27)	360-fold	guanine 8-aza-guanine					
	(iv) Baker & Siebeneick (59)	200-fold						
. Rat brain	(i) Mansoor et al (53)	80 to 100-fold	guanine	1.92×10^{-5}			6.6	
				1.98×10^{-5}			9.0	
			8-aza-guanine isoguanine 6-thio-guanine					
) Soluble fraction	(ii) Kumar et al (27)	590-fold	guanine	2.90×10^{-4}			8.0	
			8-aza-guanine					
) Light-mitochondria	"	49-fold		1.05×10^{-3}			7.5	
) Heavy mitochondria	"	38-fold						
. Sheep brain	(i) Mansoor et al (53)	80 to 100-fold					6.5 and 8.5	

Continued

ɔurce	Reference	Degree of purification	Substrate specificity	Michaelis constants (moles/liter)	Competitive inhibitors	Inhibitor constants	pH optimum	Molecular weight
. Rabbit brain	(i) Lewis (33)	1200-fold	guanine	1.22×10^{-5} (pH7.0)	AIC THFA AICR	2.78×10^{-5} 3.12×10^{-4}	6.50[4]	58,000 (gel filtration Sephadex G-200).
			8-aza-guanine	1.43×10^{-4} (pH 7.0)				
			6-thio-guanine[5]					
. Ling cod muscle	(i) Roy (47)	200-fold	guanine	3.30×10^{-5}			6.0	
			8-aza-guanine					
. Mouse liver (soluble fraction0	(i) Kumar et al (27)	crude extracts	guanine 8-aza-guanine	5.0×10^{-6} (B)			5.6 and 8.5	
		195-fold		6.3×10^{-5} (A)			7.0 8.0	

Continued

Source	Reference	Degree of purification	Substrate specificity	Michaelis constants (moles/liter)	Competitive Inhibitor inhibitors	constants	pH optimum	Molecular weight
8. Rabbit liver	(vi) Fogle & Bieber (34, 35)	1250	guanine	5.6×10^{-6}M			8.0	110,000
9. Rabbit liver	(vii) Bergstrom & Bieber (62)	7100	guanine	3.63×10^{-6}M			8.0	110,000 (2 subunits of 52,000 each).
			8-aza-guanine	2.3×10^{-4}M			6.0	
					3-deaza-guanine	3.7×10^{-6}M	7.0	
10. Human liver	This thesis	3248	guanine	1.538×10^{-5}M			8.0	120,000 (59,000 each 2 subunits)
			8 - azaguanine	2×10^{-4}M	5-AIC	5×10^{-5}M		

*SDS Sodium dodecyl sulfate

1. AIC 5 -aminoimidazole - 4 - carboxamide
2. THFA Tetrahydrofolate
3. AICR 5 - aminoimidazole - 4 - carboxamide riboside
4. At 1200-fold purification of rabbit brain deaminase, a single sharp pH optimum is observed at pH 6.5.

   At 100-fold purification, however, the pH-rate profile shows a broad plateau between pH 5.5 and pH 8.5 with observable maxima at pH 6.5 and pH 8.5.
5. 6 - thioguanine - the utilization of 6 - thioguanine as substrate by brain deaminase is only observed at extremely high concentration of enzyme. The liver enzyme is quite capable of utilizing this analog at normal concentrations of enzyme.

Bibliography

1. Parks, Jr., R.E. (1963) in Biochemical Frontiers in Medicine. (Busch, H. ed) p. 245, Little Publishing Co., N.Y.

2. Kanzawa, F., Hoshe, A. and Kurentani, K. (1971) Chem. Pharmac. Bull. 19, 1737 - 1738.

3. De Lamirande, G., Allard, C., and Cantero, A. (1958) Cancer Research 18, 952 - 958.

4. Brown, G.B., Bendich, H., Roll, P.M. and Sugirua, K., (1949) Proc. Soc. Exptl. Biol. and Med. 72, 50.

5. Balis, M.E. (April 1959) Cancer Res., 19 (3) Part 1, 297.

6. Kidder, G.W., Dewey, V.C., Parlis, R.E., Jr. and Woodside, G.L. (1949) Science 109, 511 - 514.

7. Mandel, H.G., and Carlo, P.E. (1953) J. Biol. Chem. 201, 335

8. Balis, M.E. (1958) International Cancer Congress, 35.

9. Moore, E.C. and Le Page, G.A. (1958) Cancer Research, 18, 1075.

10. Bennett, L.L., Skipper H.E., Stock, C.C, and Rhoads, C.P. (1955) Cancer Research 15, 485.

11. Jones, W., (1904) Z. Physiol. Chem., 42 35.

12. Jones, W., and Partridge C.L. (1904) Z. Physiol. Chem., 42, 343.

13. Lesch, M. and Nyhan, W.L. (1968) Amer. J. Med., 36, 561 - 570.

14. Schittenhelm, A., (1904) Z. Physiol. Chem., 43, 228.

15. Jorpes, E. and Myrback, K. (1941) Die Metnoden der Ferment forschung, Leipzig, 1, 1090.

16. Graff, S. and Maculla, A. (1935) J. Biol. Chem, 110, 71.

17. Schmidt, G. (1933) Z. Physiol. Chem., 219, 191.

18. Hitchings, G.H. and Falco, F.A. (1944) Proc. Natl. Acad. Sci. U.S., 30, 294 - 297.

19. Kalckar, Herman M. (1947) J. Biol. Chem., 167, 429 - 443.

20. Kalckar, Herman M. (1947) J. Biol. Chem., 167, 461 - 475.

21. Vischer, E. and Chargaff, E. (1948) J. Biol. Chem., 176, 703.

22. Rousch, A. and Norris, E.R. (1950) Arch. Biochem. and Biophys., 29, 124 - 129.

23. Kream J. and Chargaff, E. (1952) J. Am. Chem. Soc., 74, 4274.

24. Rakosky, J., Zimmerman, L.N. and Beck, J.V. (1955) J. Bacteriol., 69, 566.

25. Galanti, B., Russo, M., Nardiello, S. and Giusti, G. (1975) Enzyme, 20, 90 - 97.

26. Galanti, B., Russo, M., Nardiello, S. and Giusti, G. (1976) Enzyme 20, 342 - 348.

27. Jumar, K.S., Sitaramaya, A. and Krishnan, P.S. (1972) Biochem J., 128, 1079 - 1088.

28. Levin, R., Ha.., J.C. and Harris, C.A. (1963) Cancer, 16, 267 - 272

29. Currie, R., Bergel, F. and Bray, R.C. (1967) Biochem. J., 104, 634 - 638.

30. Baker, B.R., (1966) J. Med. Chem 10, 59 - 61.

31. Lewis, A.S. and Glantz, M.D. (1974) J. Biol. Chem., 249, 3862 - 3866.

32. Lewis, A.S. and Glantz, M.D. (1975) J. Biol. Chem., 250, 8220 - 8221.

33. Lewis, A.S. (1973) Ph.D Thesis, City Univ. of New York.

34. Fogle, P.J. (1974) Ph.D Dissertation, Arizona State University.

35. Fogle, P.J. and Bieber A.L. (1975) Prep. Biochem, 5, 59 - 77.

36. Kluge, H., Hartman, W., Wieczorek, R. and Zahletn, W., (1974) Acta. Biol. Med. Ger, 33, 49 - 56 in Chem. Abstracts Am 82, 14497.

37. Jordan, W.K., March, R., Honchim, O.B. and Pope, E. (1959) J. Neurochem., 4, 170 - 174.

38. Hans, K.S. (1975) Soul uital chapchi, 15, 175 - 183 in Chem. Abst. Am, 84, 146674d.

39. Carpene, E. Falasca, A., Hakin, G. and Seolain, G. (1976) Boll. Soc. Ital. Biol. Sper., 52, 7 - 8 in Chem Abst. Am. 86, 2746X.

40. Knights, E. Whitehouse, J., Hul, A. and Santos, G. (1965) J. Lab. Clin. Med., 65, 355 - 360.

41. Zindovic, L., Kanjuh, V. and Trpinae, P. (1971) Estratto da Biochem. Exp. Biol, 10, 61 - 65.

42. Schittenhelm, A. (1909) Z. Physiol. Chem., 63, 289.

43. Rabinowitz, J.C. and Barker, H.A. (1956) J. Biol. Chem., 218, 161 - 173.

44. Pierre, L.L. (1965) Nature 20, 66 - 67.

45. Lisa, S.P. and Ludwig, P. (1959) Ann. Entomol. Soc. Amer. 52, 548 - 550.

46. Hodge, L.D. and Glassman, E. (1967) Genetics, 57, 571 - 577.

47. Roy, J.E. (1966) Can. J. Biochem., 44, 1093 - 1098.

48. Roy, J.E. and Roy, K.L. (1967) Can. J. Biochem., 45, 1263 - 1269.

49. Lindahl, P.E. and Svard, E.P.O. (1957) Acta. Chem. Scand., 11, 846 - 853.

50. Farina, B. and Leone, E. (1963) Biochem. J., 89, 7P.

51. Farkus, W.R. and Singh, R.D. (1975) Biochem. Biophys Acta., 377, 166 - 173.

52. Kizaki, H., Matsuo, L. and Sakurada, T., (1977) Clinica Chimica Acta., 75, 1 - 4.

53. Mansoor, M., Kalyankar, G.D. and Talwar G.P. (1963) Biochim et. Biophys., Acta. 77, 307.

54. Kumar, K.S., Tewari K.K. and Krishnan, P.S. (1965) J. Neurochem., 12, 1003 - 1004.

55. Norstrand, I.F. (1972) M.S. thesis, Brooklyn College, City Univ. of New York.

56. Lewis, A.S. (1964) M.S. thesis, Brooklyn College, City Univ. of New York.

57. Panzica, M. (1968) M.S. thesis, Arizona State University.

58. Kumar, S.K. and Krishnan, P.S. (1972) Biochem. Biophys. Res. Commun. 39, 1087 - 1093.

59. Baker, B.R. and Siebeneick, H. (1971) J. Medicinal Chem., 14, 799 - 801.

60. Prodarnov, K. (1970) Bull. Exp. Biol. Res., 70, 1368.

61. Day, D.J., (1974) Ph.D. Dissertation, Arizona State University.

62. Bergstrom, J.D. and Bieber, A.L. (1979) Arch. Biochem. Biophys., 194, 107 - 116.

63. Hertig, R.L. (1970) Ph.D thesis, Illinois Inst. of Tech.

64. Zamenhof S. in Methods in Enzymology, 3, 702, Academic Press, N.Y.

65. Canalco, 4935 Cordell Ave., Bethesda, Md.

66. Weber, K. and Osborn, M. (1969) J. Biol. Chem., 244, 4406 - 4412.

67. Edman, P. (1950) Acta. Chem. Scand., 4, 283.

68. Ambler, R.B. (1967) Methods in Enzymology, XI, 436, Acad. Press, N.Y.

69. Moore, S. and Stein, W. in Methods in Enzymology, 6, 819 - 831, Acad. Press, N.Y.

70. Andrews, P. (1965) Biochem, J., 96, 595 - 606.

71. Vesterberg, O. (1971) in Methods in Enzymology Jacoby ed., 389 - 412, Acad. Press, N.Y.

72. Vesterberg, O. (1971) Biochim, Biophys. Acta., 243, 335 - 348.

73. Lineweaver, H. and Burk, D. (1934) J. Amer. Chem. Soc., 56, 658 - 666.

74. Dixon, M. (1953) Biochem, J. 55, 161 - 171.

75. Rossi Carlo, Solaini Giancarlo, (1978) Biochim. Biophys. Acta., 526, 235 - 246.

76. Mitchell, H.K. and McElroy, W.D. (1946) Arch. of Biochem. and Biophys., 10, 343.

77. Dixon, Malcolm and Webb, E.C. (1958) Enzymes Acad. Press Inc., N.Y.

78. Hirschberg, E., Kream, J., Gelhorn, A. (1952) Cancer Res., 12, 524.

79. Greenstein, J.P., Carter, C.E., Chalkley, H.W. and Lenthardt, F.M. (1946) J. Nat. Cancer Inst., 7, 9.

80. Korotkoruchko, V.P. and Honecharenko, O.H. (1962) Ukr. Biokhim, Zh., 34, 720.

81. Block, W.D. and Johnson, Doris V. (1955) J. Biol. Chem., 217, 43.

82. Gelhorn, A. (1953) Cancer 6, 1030.

83. Passananti, Thomas (1963) Medical World News, 84, 20.

84. Hirschberg, E., Murrary, M.R., Peterson, E.R., Kream, J., Schafranek, R. Pool, J.L. (1953) Cancer Res. 13, 153.

85. Kety, S.S. and Elkes, J. (1961) Regional Neurochemistry, Pergamon Press, N.Y.

86. Irving H. Fox, Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency. A Molecular Defect. Edited by Polara et al., Academic Press, N.Y. (1975) 47 - 50.

87. Hirs, C.H.W. (1967) Methods in Enzymology XI, 197 - 199, Acad, Press, N.Y.

88. Galanti, B., Russo, M. (1976) Enzyme 21 342-348.

89. Prodarnov, K. and Astrug, A. (1971) Clinica. Chimica, Acta, 35, 445 - 447.

90. Giusti, G., Galanti, B., and Mancini, A. (1969) Enzymology, 38, 373 - 381.