STUDIES OF THE REGULATION OF GLUTAMATE DEHYDROGENASE IN *NEUROSPORA CRASSA*

by

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To My Parents

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ABSTRACT

An am^- mutant of Neurospora crassa, lacking the TPN-specific glutamate dehydrogenase enzyme, does not grow in a minimal medium containing glycine. In the presence of alanine this am^- mutant grows as well as the wild type Neurospora. The nucleotide pools of the am^- mutant grown in alanine were compared quantitatively and qualitatively with the same pools isolated from the am^- mutant grown in glycine. A possible explanation for the "glycine effect" was postulated.

The DPN-specific glutamate dehydrogenase enzyme was isolated and partially purified according to the method of Sanwal (1961b). An inhibitor, GMP, was found to bind the enzyme at the DPN site.

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INTRODUCTION

Neurospora crassa has two glutamate dehydrogenase enzymes; one enzyme specifically requires DPN and the other TPN (Sanwal and Lata, 1961b).

Most bacteria, higher plants and animals either possess only one glutamate dehydrogenase enzyme which is specific for one particular coenzyme, or an enzyme which may use both DPN and TPN.

A mutant of Neurospora crassa lacking the TPN-specific glutamate dehydrogenase enzyme grows very slowly on minimal medium but resembles the wild type Neurospora crassa when grown in the presence of alanine.

Certain nitrogen-containing compounds (glycine, serine, sarcosine, methylamine) will prevent the growth of the am^- mutant for several days. These compounds do not effect the growth of the wild type Neurospora crassa.

Purine nucleotides exert an effect upon the activity of the beef-liver glutamate dehydrogenase enzyme (Frieden, 1962; Wolff, 1962). The DPN-specific enzyme of Neurospora crassa is also affected by various purine nucleotides (Stachow and Sanwal, 1964). The TPN-specific enzyme is not affected. The

function of the DPN-specific glutamate dehydrogenase enzyme may be involved in supplying the glutamate precursor of glutamine which in turn could give rise to the purine nucleotide pools.

The object of this research was to investigate further the effect of purine nucleotides upon the DPN-linked glutamate dehydrogenase enzyme of Neurospora crassa.

HISTORICAL

The glutamate dehydrogenase enzyme was first observed in washed frog muscle by Thunberg in 1920. Since then much work has been published on the sources, extraction, purification, and kinetics of the enzyme. Today, the actual function(s) of the enzyme still remain a controversial subject.

There are two common methods by which ammonia can be incorporated into amino acid. One of these reactions involves the amination of fumarate and the production of aspartic acid (Gale, 1940). The second method involves the glutamate dehydrogenase system which catalyzes the production of glutamate from ammonia and α-ketoglutarate. The latter system appears to be the more common.

As would be expected glutamate dehydrogenase has been isolated from a large number of sources. It has been found in bacteria (Adler, Hellstrom, Gunther, and von Euler, 1937), pea seedlings (Damodoran and Nair, 1938), brain and liver (Dewan, 1938), brain, spleen and muscle (Copenhaver, McShan and Meyer, 1950), plancenta (Gaul, 1960) and in the fungi Neurospora crassa (Fincham, 1950) and Fusarium (Sanwal, 1961).

The year 1937 marked the modern beginnings of the

intensive study on glutamate dehydrogenase. In that year H. von Euler et al isolated the enzyme from yeast and reported that it required TPN as a coenzyme. Twenty years later it was demonstrated that baker's and brewer's yeast had two distinct dehydrogenases--one specific for DPN and one for TPN (Holzer and Schneider, 1957).

Glutamate dehydrogenase was obtained from acetone-dried pig or beef-liver extract (von Euler, Adler, Gunther, and Das, 1938; Dewan, 1939). The enzyme preparations reduced methylene blue or took up oxygen when DPN was present and produced ammonia and α-ketoglutarate as the final products. The reaction was reversible and highly specific for L-glutamic acid.

Both TPN and DPN served equally well as coenzyme for glutamate dehydrogenase isolated from liver (Mehler, Kornberg, Grisolia, and Ochoa, 1948). These results were confirmed with enzyme obtained from various rat tissue (Copenhaver et al, 1950).

Crystallized beef-liver glutamate dehydrogenase was obtained with ethanol extraction and sodium sulfate fractionation (Strecker, 1951; Olson and Anfinsen, 1952; 1953).

Mutants of Neurospora were reported to synthesize the

amino groups for a wide range of amino acids from ammonia (Fincham, 1950). The author suggested that "the reaction was α-ketoglutarate + ammonia $\longrightarrow$ glutamate and that the mutants had a defective glutamate dehydrogenase system". The activities of the different amino acids in transamination in vitro demonstrated a close correlation to their effectiveness in supporting growth of "amination deficient" mutants of Neurospora crassa lacking glutamate dehydrogenase (Fincham, 1951).

Sedimentation techniques were used to estimate the molecular weight of glutamate dehydrogenase at about 1×10^6 (Olson and Anfinsen, 1952). A more refined determination placed the molecular weight between 1 and 1.3×10^6 (Kubo, Iwatsubo, Watari, and Soyamo, 1959). Sedimentation experiments demonstrated that glutamate dehydrogenase was reversibly dissociated into four subunits of molecular weight approximately equal to 250,000 (Frieden, 1957). Crystalline bovine-liver enzyme was dissociated into fragments of low molecular weight - 30,000 to 60,000 (Jergensen, 1961). This latter treatment led to an irreversible loss of enzymatic activity.

The glutamate dehydrogenase enzyme from beef-liver

appeared to be specific for glutamate or α-ketoglutarate until it was demonstrated that α-ketovaleric, α-ketobutyric, and α-ketoisovaleric acids also functioned as substrates for this enzyme (Bassler and Hammer, 1958). The TPN-specific glutamate dehydrogenase isolated from Neurospora utilized many α-amino acids as substrate for oxidative deamination (Barratt, 1963). All these acids were less than 1% effective when compared to glutamate.

The association of crystalline glutamate dehydrogenase from beef-liver was found to be necessary for enzymatic function (Frieden, 1959). The enzyme contained 3.4 atoms of zinc per mole of enzyme. The dissociation of the enzyme by DPNH or 1,10 phenanthroline yielded four subunits. For each DPN or DPNH molecule bound to an active site, a second molecule could be bound to a non-catalytic but active site.

Adenosine nucleotides were reported to affect the velocity of the beef-liver glutamate dehydrogenase reaction and the effect depended upon which coenzyme was used (Frieden, 1959). At high DPNH concentration ADP increased the reaction rate three fold while ATP decreased the rate four fold. ADP was reported to prevent the DPNH-induced dissociation of the enzyme while ATP enhanced this dissociation. ATP did not

decrease the reaction rate when TPNH was used as coenzyme, although the oxidation of TPNH increased in the presence of ADP.

Several hormones have been reported to inhibit beef-liver glutamate dehydrogenase (Yielding, Tompkins, Munday, and Curan, 1961). ADP, however, completely reversed this steroid effect on glutamate oxidation or α-ketoglutarate reduction. High concentrations of ADP were required to reverse estradiol inhibition.

The dissociated form of glutamate dehydrogenase was thought to catalyze an alanine dehydrogenase reaction whereas the tetramer form was to participate in an oxidative amination of glutamate (Tompkins, Lemone, Yielding and Curan, 1961).

Two glutamate dehydrogenases were found in Fusarium, one specific for DPN and one specific for the TPN coenzyme (Sanwal, 1961a).

A DPN-specific and a TPN-specific glutamate dehydrogenase enzyme were isolated from Neurospora, in a 50% purified form (Sanwal and Lata, 1961b). Reasons for the requirement of two enzymes responsible for identical end products were advanced.

A) Transhydrogenase System

1. L-glutamate + $DPN^+ \rightleftharpoons$ α-ketoglutarate + DPNH + H^+

2. α-ketoglutarate + TPNH + H^+ + $NH_4^+ \rightleftharpoons$ L-glutamate + TPN^+

1 + 2 = TPNH + $DPN^+ \rightleftharpoons$ DPNH + TPN^+

B) The TPN-specific enzyme was thought to act in a biosynthetic role, while the DPN-specific enzyme performed a catabolic function.

An am^- mutant of Neurospora crassa which produced only the DPN-specific glutamate dehydrogenase was reported (Sanwal and Lata, 1961c). One gene mutation led to the loss of only one enzyme. Since the am^- mutant grew very slowly on minimal medium it was suggested that the DPN-specific enzyme was degradative whereas the TPN-specific enzyme was biosynthetic.

A marked decrease in the activity of the TPN-specific glutamate dehydrogenase was observed when the fungus was grown in the presence of glutamate and NH_4 (Sanwal and Lata, 1961d; 1962a). It was suggested that the DPN-specific and the TPN-specific glutamate dehydrogenase enzymes were under

the control of the same regulatory gene. Through a repressor substance this gene inhibited the synthesis of the DPN-specific enzyme. The TPN-specific glutamate dehydrogenase was repressed only when this substance combined with a small molecular weight compound. If there was a fixed level of repressor substance available in the cell a condition favouring derepression of DPN-specific enzyme would naturally lead to a corepression of the TPN-specific glutamate dehydrogenase.

Urea was found to regulate concurrently the formation of both cross-reacting material (CRM) and DPN-specific glutamate dehydrogenase in am^- mutants of Neurospora lacking the TPN-specific enzyme (Sanwal and Lata, 1962b). The CRM protein was antigenically related to the TPN-specific enzyme. It was suggested that the two coenzyme specific dehydrogenases could be controlled quantitatively "by another locus of the regulatory type".

An investigation of the thyroxine inhibition of liver glutamate dehydrogenase indicated that adenosine nucleotides reversed this inhibition (Wolff, 1962). Other nucleotides that were tested did not produce this affect. ADP also reversed the inhibition caused by GDP and GTP.

A number of possibilities for the role of glutamate dehydrogenase were suggested (Frieden, 1963).

1. GDP and GTP involved in succinate production controlled the interconversion of α-ketoglutarate from glutamate by exerting a control on the glutamate dehydrogenase enzyme.

2. Glutamate dehydrogenase could be important in the regulation of the first step of the urea cycle since oxidative deamination of glutamate produces a large source of ammonia.

3. The concentration of purine nucleotides could be controlled by the glutamine level in the cell.

4. Protein synthesis or gluconeogenesis could be partially controlled by glutamate dehydrogenase as the enzyme is an important link between carbohydrate and amino acid metabolism.

Three binding sites were proposed (Frieden, 1963).

1. A site for oxidized and reduced coenzyme.

2. A specific site for purine nucleotides.

3. A non-active site binding DPN.

IMP, GMP, GDP, and GTP were found to inhibit competitively the DPN-specific glutamate dehydrogenase enzyme of Neurospora (Stachow and Sanwal, 1964). These nucleotides had no effect upon the TPN-specific enzyme. The greatest inhibition was caused by GTP ($K_1 = 6 \times 10^{-5}$ M) and the least

by IMP (K_i = 1.5 x 10^{-4} M). ATP, alone, had no affect upon the DPN-specific glutamate dehydrogenase enzyme. ATP did, however, lower the amount of inhibition caused by GTP. It was suggested that the synthesis of purine nucleotides could be controlled by the availability of glutamate produced by the DPN-specific enzyme "to the partial exclusion of glutamate generated by the TPN-specific dehydrogenase".

Immunodiffusion experiments were used to demonstrate the presence of three antigenically different glutamate dehydrogenase enzymes in beef-liver (Talal and Tompkins, 1964a; 1964b). One form of enzyme (a), produced only glutamate dehydrogenase activity and was most apparent when experiments were performed in the presence of ADP. Form (c) had mainly alanine dehydrogenase activity and was readily observed when GTP was incorporated into the agar. Form (b) produced both enzymatic activities and was present in all conditions.

Glutamate dehydrogenase isolated from beef-liver was split into its α (dissociated) form and retained its ability to react with L-glutamate as a substrate with the same rate as the β (associated) form (Fisher, Gross, McGregor, 1965).

Both the α and β forms of the enzyme produced identical reaction rates with L-alanine as substrate.

Feedback Regulation

One of the main mechanisms of regulation of cellular metabolism is feedback or endproduct inhibition. This control mechanism is characterized by the inhibition of the first reaction in a metabolic sequence by its own endproduct.

This type of control was first observed when glucose phosphorylation in erythrocytes was inhibited by phosphoglyceric acid (Dische, 1941). This control was also found in the case of tryptophane which inhibited its own production (Novick and Szilard, 1954).

Isoleucine was reported to inhibit the enzyme threonine deaminase (Umbarger, 1956). The interaction between L-isoleucine and L-threonine deaminase was postulated to be a "negative feedback loop" that allowed the biosynthesis of isoleucine to occur when the level of L-isoleucine in the medium or the cellular pool had been reduced to a low level.

Uracil was found to inhibit the formation of pyrimidine intermediates produced earlier than the synthesis of orotic acid in mutants of E. coli requiring pyrimidine (Yates and

Pardee, 1956). A cellular mechanism linking pyrimidine production to the rate of pyrimidine uptake for nucleic acid synthesis was postulated.

In the pathway in which aspartate is converted to lysine, methionine, and threonine (Fig. 1) two aspartokinases have been demonstrated in E. coli (Stadtmon, Cohen, Le Bras, de Robichon-Szulmajester, 1961).

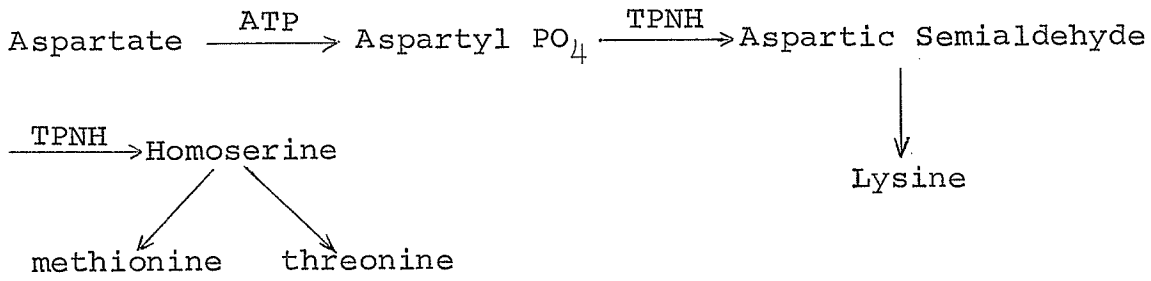


FIGURE 1.

Each enzyme was found to be controlled by the concentration of either L-lysine or L-threonine respectively.

β-aspartokinase isolated from the photosynthetic bacterium Rhodospeudomonas copsulatus was demonstrated to be insensitive to either L-lysine or L-threonine, although a combination of the two amino acids caused considerable inhibition (Datta and Gest, 1964). This phenomenon was termed "concerted feedback inhibition".

Systems controlled by endproduct inhibition have been described (Monod, 1963).

1. The regulatory enzyme is strongly and specifically inhibited by the terminal metabolite of the pathway. Intermediate metabolites do not inhibit the regulatory enzyme.

2. The enzymes which intervene in each pathway are not significantly sensitive to inhibition by the terminal metabolite.

"In the internally regulated machine, as in the living organism, processes are controlled by one or more feedback loops that prevent any one phase of the process from being carried to a catastrophic extreme" (Umbarger, 1956).

Induction and Repression

In microorganisms a particular protein may be present in very high concentration under certain conditions of cultivation but may be entirely undetectable under the influence of a new environment.

Proteins which increase in concentration with a corresponding increase in quantity of a metabolite are known

as inducible proteins whereas a protein decreasing in quantity with the addition of a metabolite is termed a repressible protein.

Many reports of induction and repression appear in the literature. D-serine was found to act as an inducer of D-serine deaminase, while threonine, L-leucine, and glycine induced the formation of L-serine deaminase (Pardee and Prestidge, 1955). The inhibition of ornithine transcarbamylase by arginine has been described (Gorini and Maas, 1957). The specific activity of glutamyl transferase in cultures of HeLa cells was reported to vary markedly with the growth medium (De Mars, 1958). Histidine-requiring mutants of Salmonella were used in demonstrating histidine repression in the synthesis of each histidine biosynthetic enzyme (Ames and Garry, 1959). The repression occurred to the same extent for each enzyme in the pathway and was termed coordinate repression.

A number of closely linked mutations affecting the synthesis of β-galactosidase in E. coli were described (Pardee, Jacob, Monod; 1959). A Z^+ mutation was expressed as an inability to synthesize β-galactosidase; a Y^+ mutation resulted in a loss of ability to produce galactoside-permease.

Other mutations in an i^+ region allowed the enzyme to be synthesized constitutively instead of inducibly as in the wild type. The Z and i mutations belong to different cistrons and the constitutive allele of the i cistron was recessive to the inducible allele. The kinetic nature of i^+ gene suggested that this i^+ allele controlled the synthesis of a substance which repressed the synthesis of β-galactosidase.

Tyrosinase in Neurospora was found to be controlled by at least three genes (Horwitz, Fling, MacLeod and Sueoka, 1960). The T locus, only, had a structure-determining role in tyrosine synthesis. The influence of the other two genes was of an indirect nature.

Two theories have enjoyed the most popularity in explaining induction and repression (Vogel, 1957; Jacob and Monod, 1961a; 1961b).

The Template Hypothesis (Vogel, 1957) described induction or repression as an interaction of inducer or repressor with "a macromolecule of a template nature". The template was involved in enzyme protein production. The inducer acted by assisting a template catalyst; the repressor retarded the activity of this catalyst. Therefore, the

inducers and repressors acted by affecting the rate of dissociation of a template product from its template (enzyme protein from the ribosomes).

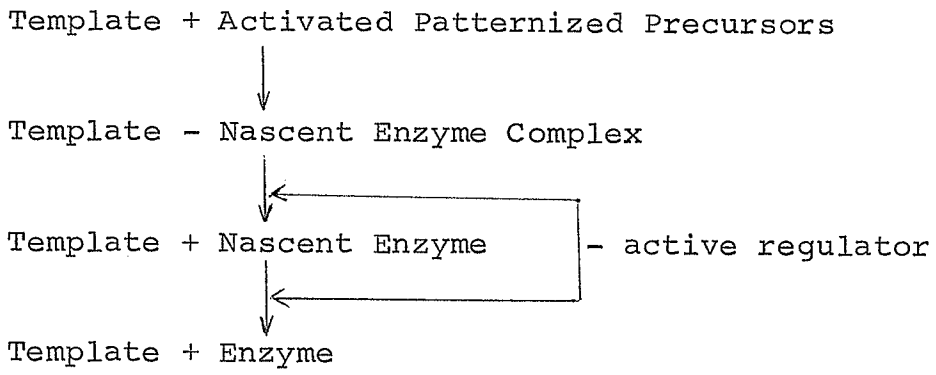


FIGURE 2.

The Operon Model (Jacob and Monod, 1961a, 1961b) explained repression and induction at the genetic level.

1. mRNA, a short lived intermediate, obtains information from a structural gene (DNA). In the process of ribosomal transcription mRNA is destroyed.

2. Synthesis of mRNA is a sequential and ordered process initiated at distinct regions known as operators located on the DNA strand. The operator controls the transcription ability of several adjacemt structural genes. The operator and its structural genes are referred to as an operon.

3. A regulatory gene produces a cytoplasmic substance

known as a repressor (RNA transcript of the regulatory gene). This repressor tends to associate reversibly with a specific operator blocking the whole operon and preventing the synthesis of the proteins governed by the structural genes.

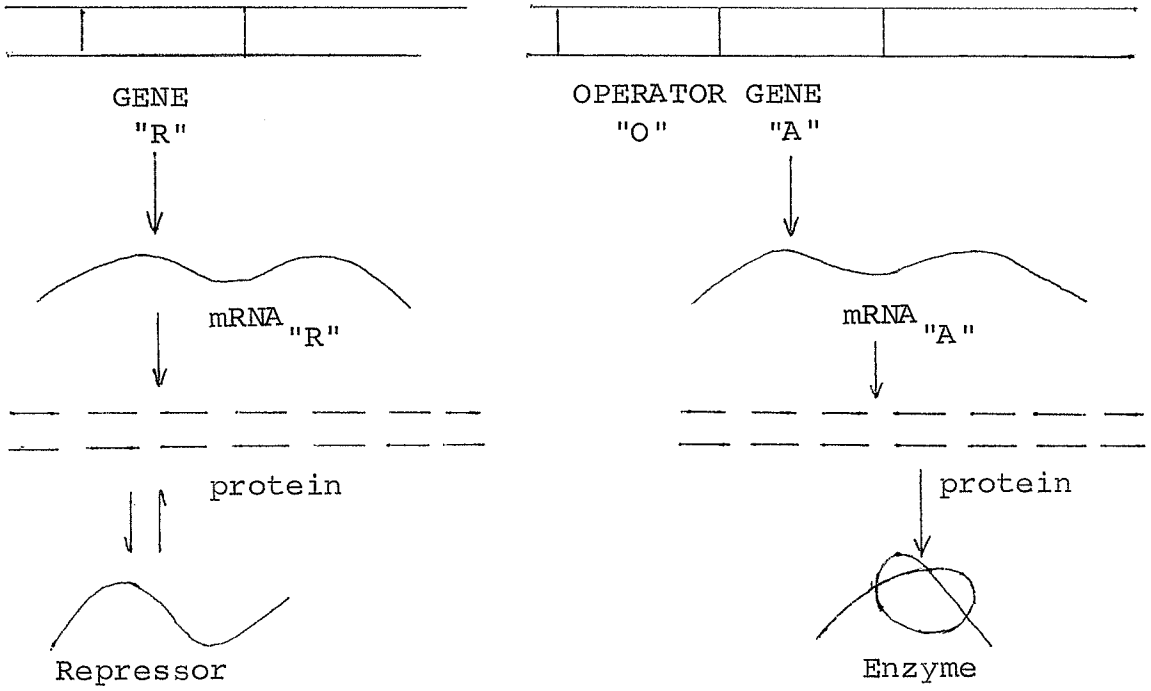
4. The repressors can combine with certain small molecules known as effectors.

$$R + F \rightleftharpoons R^1 + F^1$$

R = repressor F = effector

In inducible systems the R form of the repressor associates with the operator to block transcription of the operon. The presence of an effector (inducer) inactivates the repressor and allows transcription. In repressible systems, the R^1 repressor form is active. The transcription of the operon allowed in the absence of the effector is prevented in its presence.

FIGURE 3. Modern Scheme of Repression and Induction



1. Gene R is responsible for the synthesis of a protein that can exist in an active or an inactive conformation.

2. The R protein has an affinity for the O (operator) region of the DNA strand. With the repressor protein at O, RNA polymerase cannot initiate the synthesis of mRNA at the structural gene A. This structural gene possesses the information necessary for the production of a protein A.

3. The process of induction depends upon a small molecular

weight metabolite which maintains the repressor protein in an inactive state. Repression is dependent upon a low molecular weight metabolite which causes a conversion of repressor from an inactive to an active conformation.

MATERIALS AND METHODS

Chemicals and Equipment

All nucleotides were purchased from the Pabst Company, Sigma Chemical Company or Calbiochem. α-Ketoglutaric acid, reduced glutathionine, and alumina Cγ were obtained from the Sigma Chemical Company. L-alanine was purchased from Nutritional Biochemicals Corporation and glycine from the British Drug Houses Ltd. The formic acid was purchased from J. T. Baker Chemical Company; ammonium formate from Matheson Coleman and Bell; sodium formate and ammonium formate from the British Drug Houses Ltd. Dowex-1-Cl (200-400 mesh) was obtained from Sigma Chemical Company. The Bio-Gel P-2 (50-100 mesh) was purchased from Calbiochem.

All glass columns were purchased from Bellco Biological Glassware. The automatic fraction collectors were purchased from the Research Specialities Company and the Gilson Company. The Unicam Instruments SP-700 spectrophotometer and the Gilford Model 2000 recording spectrophotometer were used for all spectrophotometric work. The freeze drier was obtained from the Virtis Company; the RC-2 centrifuge from the Sorvall

Company; the shakers from Robin and Meyers Company and the New Brunswick Scientific Company.

Media

The basic medium used for growing Neurospora crassa was Vogel's medium-N (Stachow, 1965). The medium contained: sodium citrate, 120 gm; $MgSO_4.7H_2O$, 10 gm; NH_4NO_3, 100 gm; KH_2PO_4 (anhydrous), 250 gm; $CaCl_2.2H_2O$, 5 gm; trace element solution, 5 ml; 0.01% biotin, dissolved in de-ionized water to a final volume of one litre. The trace element solution had the following chemicals: citric acid, 5 gm; $ZnSO_4.7H_2O$, 5 gm; $FeSO_4.7H_2O$, 1 gm; $CuSO_4.5H_2O$, 0.05 gm; $MnSO_4.H_2O$, 0.05 gm; anhydrous H_3BO_3, 0.05 gm; $Na_2MoO_4.2H_2O$, 0.05 gm, dissolved in 100 ml of de-ionized H_2O. The stock solution listed above is 50 fold concentrated.

The growth medium of the wild type N. crassa contained the stock solution diluted 50 fold plus 2% sucrose. The am$^-$ mutant required alanine, at a concentration of 1 gm per litre, the stock solution diluted 50 fold, plus 2% sucrose. A solid medium contained 1.5% agar added to the above.

Growth Conditions

Stock preparations of Neurospora crassa were kept at 28° C on a solid medium. The fungus was grown for two days in the dark and then placed in the light for conidia formation. The conidia were used to inoculate liquid or solid medium. The conidia were washed from the solid medium with sterile de-ionized water and asceptically passed through two layers of cheese cloth. These conidia were used as inoculum.

Extraction of Nucleotides

Conidia from the am^- strain of N. crassa were isolated from the solid medium with sterile de-ionized water, passed through a double layer of cheese cloth and centrifuged at 12,000 x G for 10 minutes in sterile centrifuge tubes. The conidia were washed in sterile de-ionized water, re-centrifuged and used to inoculate 500 ml of liquid medium containing 2% Vogel's medium-N, 2% sucrose plus either .01 M alanine or .02 M glycine. The conidia were allowed to germinate for four hours with vigorous shaking.

The liquid medium containing the germinating conidia

was centrifuged at 12,000 x g for 15 minutes. The precipitate was washed and centrifuged twice with sterile, distilled water. The resulting precipitate was immersed in distilled water and filtered through a Buchner funnel to form a solid, dry material upon the filter paper. A small portion of this "cake" was weighed and placed in an oven at 80° C for 24 hours and then weighed again. The remainder of this "cake" was weighed, immersed in a small volume of water and placed in a Ten Broeck tissue grinder (kept cold by placing ice into the centre of the handle). Perchloric acid was added to a final concentration of 6% by volume (conidia contain about 70% water). The conidia and perchloric acid solution were ground until a homogenous mixture was obtained.

The mixture was centrifuged at 12,000 x g for 15 minutes. The precipitate was washed in a small volume of distilled water and again centrifuged at 12,000 x g for 15 minutes; the washings were added to the supernatant obtained from the first centrifugation.

Potassium hydroxide was used to neutralize the solution and to precipitate the perchlorate ions as potassium perchlorate. The solution was allowed to stand for thirty minutes and then centrifuged for 15 minutes at 12,000 x g.

The resulting precipitate was washed with a small volume of distilled water. The washings were added to the original supernatant and lyophilized until a dry powder formed.

The glass container with the lyophilized supernatant was washed with a small amount of water and centrifuged at 15,000 x g for 20 minutes. The precipitate was washed; added to the supernatant, and applied to a Dowex-1-formate column.

Conversion of Dowex-1-Chloride to Dowex-1-Formate

For convenience the resin was washed in beakers before it was added to the glass columns.

Dowex-1-chloride (200 to 400 mesh) was washed once with 4 N sodium hydroxide to convert the resin to the hydroxide form. The resin was then washed with 3 M sodium formate until a test with silver nitrate indicated that the eluant was free of chloride. The resin was washed once with 6 N formic acid and with water until the eluant had a pH between 6.0 and 7.0.

Pouring Columns

A slurry of the resin was added to a container with a

single outlet leading to the column. A magnetic stirrer was used to mix the slurry. The columns were poured to a height of 22 cm and allowed to pack for about 12 hours with water continuously passing through the resin.

Elution System

Two elution systems were employed - the formic acid system and the ammonium formate system. The technique involved gradient elution which required a mixer flask containing 500 ml of distilled water and a reservoir flask containing 500 ml of the required eluant (Hurlbert, 1957). The two flasks were connected by a rubber tube.

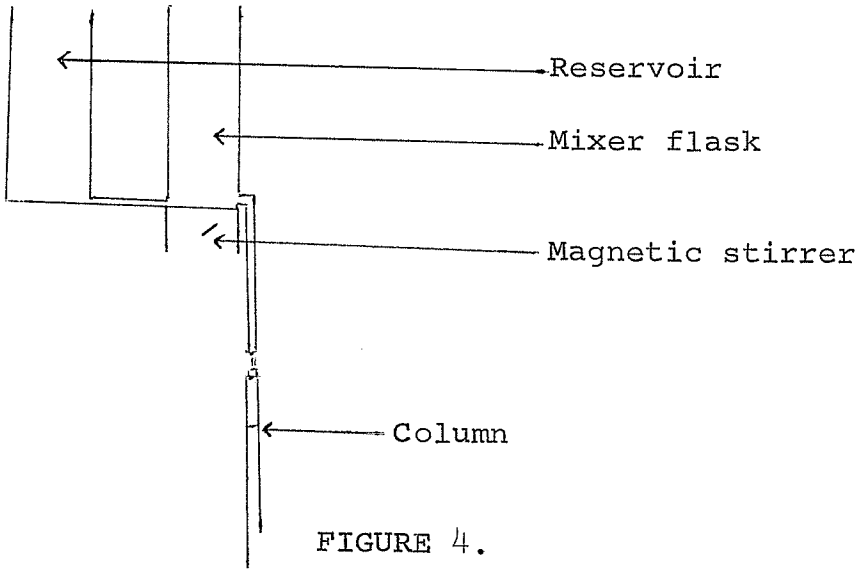


FIGURE 4.

The formic acid system required four different reservoir concentrations: 4 M formic acid, 4 M formic acid and 0.2 M ammonium formate, 4 M formic acid and 0.4 M ammonium formate, 4 M formic acid and 0.8 M ammonium formate.

The ammonium formate system required three different reservoir concentrations: 0.8 M ammonium formate, 1.6 M ammonium formate, and 2 M ammonium formate and 0.8 M formic acid.

The flow rate for both systems was about 1.0 ml/min/sq cm of cross section of resin bed. An automatic fraction collector was employed in collecting successive 6 ml fractions. The eluant was changed after 1.75 mixer volumes (875 ml) of the previous elution had passed through the column.

Each fraction was read on a spectrophotometer at 260 mμ.

Identification of Nucleotides

1. The wave length of maximum absorbance of the "unknown" nucleotides were read on a spectrophotometer and compared to standards.

2. The R_f values of the various nucleotide fractions were

compared to the R_f values of "known" nucleotides.

3. Comparisons were made between the various peak locations on a chromatogram with the location of standards.

Desalting of Nucleotides

A column, 55 cm by 0.9 cm, filled with large beads of Bio-Gel P-2 (50 to 100 mesh), was used in desalting techniques (Uziel and Cohn, 1965).

The nucleotide fractions were pooled according to peaks, neutralized with potassium hydroxide, centrifuged at 12,000 x g for 15 minutes, and lyophilized to a dry powder.

A small amount of distilled water was used to wash the tubes containing the dried nucleotide peaks. The solution was applied to a column of polyacrylamide gel and eluted with water. Successive fractions were collected and tested for nucleotide content spectrophotometrically.

Paper Chromatography

Whatman #1 sheets, 21 x 16.75 inches were used. A base line of 1.25 inches from the bottom of the sheet was drawn. Samples of nucleotides were added as small dots at the base line. The correct concentration of nucleotide could

be estimated with the aid of an ultraviolet lamp. Standard runs required approximately 150 µgms of nucleotide per spot.

Ascending chromatography was found to be adequate. The procedure required a covered chromatography jar and was accomplished at a temperature of 28° C. After sixteen hours the chromatograms were air dried and examined with an ultraviolet light.

Two solvent systems were used. System A consisted of isobutyric acid, concentrated ammonium hydroxide, and water at a ratio of 66:1:33 at a pH of 3.7. System B consisted of ethanol and ammonium acetate. In this latter system 77 grams of ammonium acetate were dissolved in 750 ml of water, adjusted to a pH of 7.5 with ammonium hydroxide and diluted to one litre. 300 ml of this solution was mixed with 700 ml of 95% ethanol.

Calculation of Nucleotide Concentration

The following relation was used to determine the concentration of the various nucleotide fractions (Bendich, 1957):-

$$\text{Molarity} = \frac{OD}{E}$$

E = the molecular extinction coefficient at maximum absorption.

OD = the maximum absorption of a sample multiplied by the total volume in millilitres.

In order to compare concentrations of various unidentified fractions the maximum absorption of these fractions multiplied by the total volume in milliliters was determined.

Conidia Germination

Conidia from the am^- strain and the wild type were harvested according to the method described (page 23). The wild type Neurospora was inoculated into a 100 ml medium of Vogel's medium-N plus 2% sucrose. The am^- strain was inoculated into 100 ml of a medium consisting of either Vogel's medium-N, sucrose (2%) and alanine (.01 M) or Vogel's medium-N, sucrose (2%) and glycine (.02 M). Approximately equal amounts of conidia were added. Counts of germinating conidia were taken in a population of 100 conidia randomly selected.

Enzyme Extraction

The DPN-specific glutamate dehydrogenase enzyme was extracted from the am^- mutant of Neurospora. The fungus was grown in Fernbach flasks, each containing one litre of medium. The liquid medium consisted of Vogel's medium-N with the usual nitrogen source omitted, 2% sucrose, and 0.5% urea.

The fungus was grown for 48 hours. The mycelium was collected in cheesecloth, washed with distilled water, and squeezed dry.

If a small amount of mycelium was obtained the enzyme was extracted by grinding the mycelium in 1.5 times its weight of alumina powder with a mortar and pestle. Tris-HCl buffer (0.05 M, pH 7.0) containing 1×10^{-3} M reduced glutathionine was added to the crushed cells (Sanwal and Lata, 1961b). The mixture was centrifuged at 12,000 x g for 15 minutes. The supernatant was the crude enzyme preparation.

If the crude enzyme extract was to be obtained from a large quantity of dried mycelium the cells were lyophilized and then ground to a fine powder in a Waring Blendor. The powder was suspended in 10 to 20 times its weight of Tris-HCl

buffer (0.05 M, pH 7.5) containing 1 x 10^{-3} M reduced glutathione. The suspension was stirred by a magnetic stirrer at 4^{o} C for 30 minutes. The extract was filtered through cheesecloth and centrifuged for 15 minutes at 12,000 x g. The resulting supernatant constituted the crude enzyme preparation.

Enzyme Purification

Solid ammonium sulfate was added slowly to the crude enzyme preparation to 0.30 saturation. The suspension was stirred for 30 minutes and centrifuged at 12,000 x g for 15 minutes. Solid ammonium sulfate was added to the supernatant to a concentration of 0.40 saturation, stirred for 30 minutes, and centrifuged at 20,000 x g for 20 minutes. The precipitate was dissolved in 0.10 M Tris-HCl buffer (pH 7.5) to 1/10 of the original volume. Alumina Cγ (40 mg/ml) was added to the ammonium sulfate extract at a volume of one millilitre per 5 ml of extract. The mixture was stirred intermittently for 30 minutes and centrifuged at 8,000 x g for 10 minutes. One millilitre of .01 M Tris-HCl buffer (pH 7.5) containing 1 x 10^{-3} M glutathione was added. The mixture was triturated

with a pipette and centrifuged at 8,000 x g for 10 minutes. One millilitre of 1.0 M phosphate buffer (pH 8.0) containing 1×10^{-3} M glutathione was added to the precipitate for each 5 ml portion of the original ammonium sulfate extract. The mixture was triturated with a pipette and centrifuged at 8,000 x g for 10 minutes. The resulting supernatant was examined on a spectrophotometer for activity. The treatment with 1.0 M phosphate buffer was continued until the enzyme activity in the washings was low.

Assay Procedures

DPN-specific glutamate dehydrogenase was assayed on a spectrophotometer at 340 mμ. The specific activity of the enzyme was determined by the decrease in optical density due to the conversion of DPNH to DPN. The assay mixture contained 20 μmoles of α-ketoglutarate, 120 μmoles of ammonium sulfate, 0.50 μmoles of DPNH, enzyme preparation and enough Tris-HCl buffer (pH 8.0) to give a final volume of 3.0 ml in a cuvette of 1 cm light path. A decrease in optical density of 0.001 OD was defined as 1 unit of enzyme activity.

All enzyme experiments were performed with 7 mM of

glutamate, 3.0 mM of DPN, enzyme preparation, Tris-HCl buffer (pH 9.0) to a final volume of 3.0 ml.

RESULTS

Germination Experiment

The germination rate of the wild type Neurospora is greater than the rate of the am^- mutant grown in alanine for the first five to six hours. After about six hours the wild type and the am^- mutant grown in alanine appear to have the same germination rate. A significant amount of germination of conidia isolated from the am^- mutant grown in glycine appeared only after the first eight to nine hours and remained very low even after twenty-two hours (Fig. 5).

Nucleotide Separation and Identification

The formic acid system was found to be far superior to the ammonium formate system for the separation of nucleotides. During standard runs all twelve nucleotides were separated on the formic acid system whereas only six nucleotides were satisfactorily separated with the ammonium formate system. Inadequate separation of the Neurospora nucleotide pools was also characteristic of the ammonium

formate system whereas good separation was accomplished with the formic acid system (Fig. 6, 7, 8).

Identification of nucleotide fractions obtained from either system was difficult due to the contaminating ions introduced by the elution systems.

The polyacrylamide gel was found to be very useful in desalting the nucleotide fractions obtained from the formic acid system (see Methods, page 28). Adequate absorption spectra were obtained for the "known" and "unknown" nucleotide fractions with this method of desalting (Fig. 9 and 10). The method was also used in desalting fractions employed in paper chromatography since any salt remaining in a particular sample produced a false R_f value. The correct R_f values are illustrated in Tables 1 and 2.

Twelve peaks were determined in the nucleotide extractions obtained from the am^- mutant grown on alanine or glycine. DPN, CMP, ADP, ATP and UTP peaks were positively identified while peak 1 appeared to contain the free bases. Four of the twelve peaks did not contain nucleotide since neither an absorption in the ultraviolet range nor the isolation of a spot on a paper chromatogram was determined. Two of the twelve peaks were definitely nucleotide but an

identification was not accomplished since the maximum absorptions, R_f values, and chromatogram positions were not similar to the standard (See Figure 8 and Tables 1 and 2).

Nucleotide Concentrations

The nucleotide pool concentrations of the am^- mutant grown in alanine was almost identical to the concentration obtained from the am^- mutant grown in glycine. The only significant difference was found in the free base level. The am^- mutant of Neurospora grown in glycine contained a 47.85% higher level of free bases than the same mutant grown in alanine (see Table 1).

Kinetic Studies

All enzymatic reactions were performed at a pH = 9.0, in 0.1 M Tris-HCl buffer. The reaction catalyzed by glutamate dehydrogenase was examined in the forward direction.

$$\text{DPN} + \text{glutamate} \rightleftharpoons \text{NH}_4 + \alpha\text{-ketoglutarate} + \text{DPNH} + \text{H}^+$$

The K_m value was defined as the concentration of

FIGURE 5

Number of germinating conidia per 100 counted in a given time.

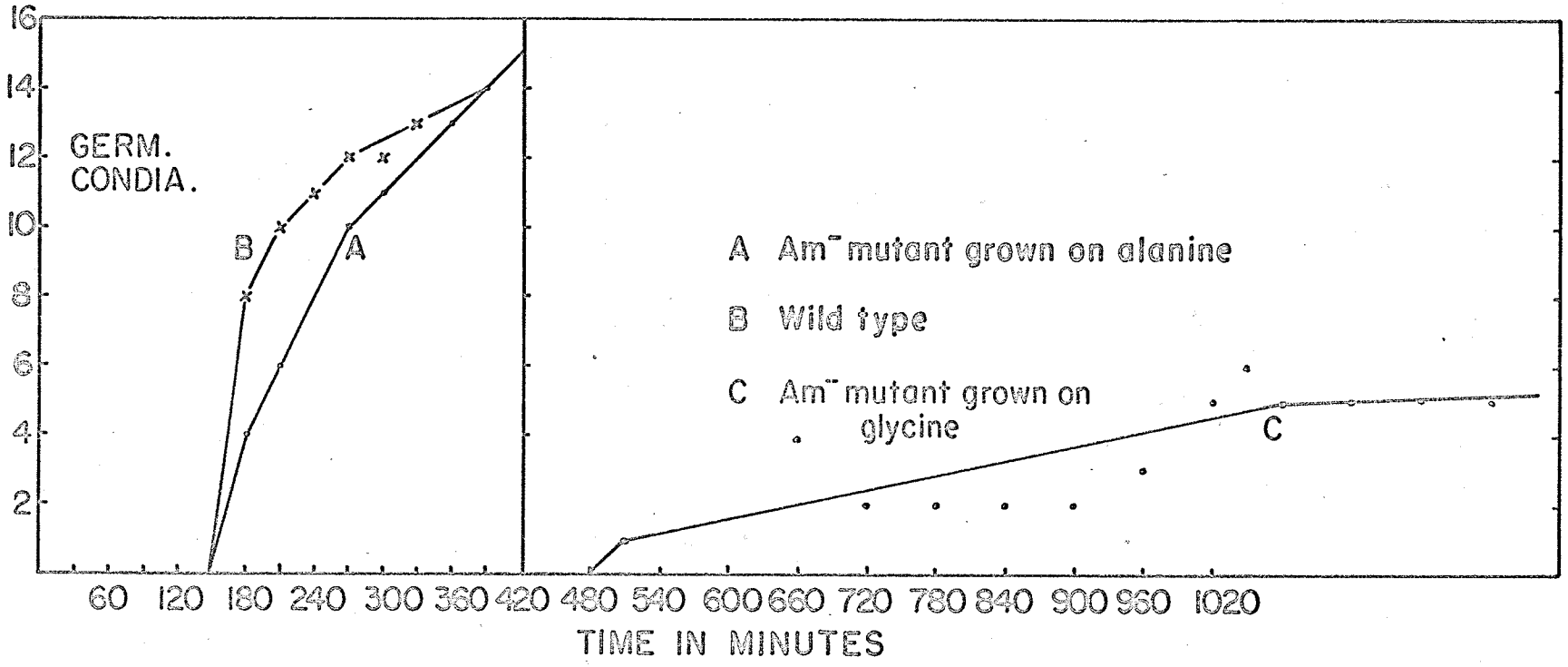

16
14
12
10
8
6
4
2
GERM.
CONDIA.
B
A
A Am⁻ mutant grown on alanine
B Wild type
C Am⁻ mutant grown on glycine
C
60 120 180 240 300 360 420 480 540 600 660 720 780 840 900 960 1020
TIME IN MINUTES

FIGURE 6.

Gradient elution chromatography on Dowex-1-Formate with the Formic Acid System and commercially prepared nucleotides.

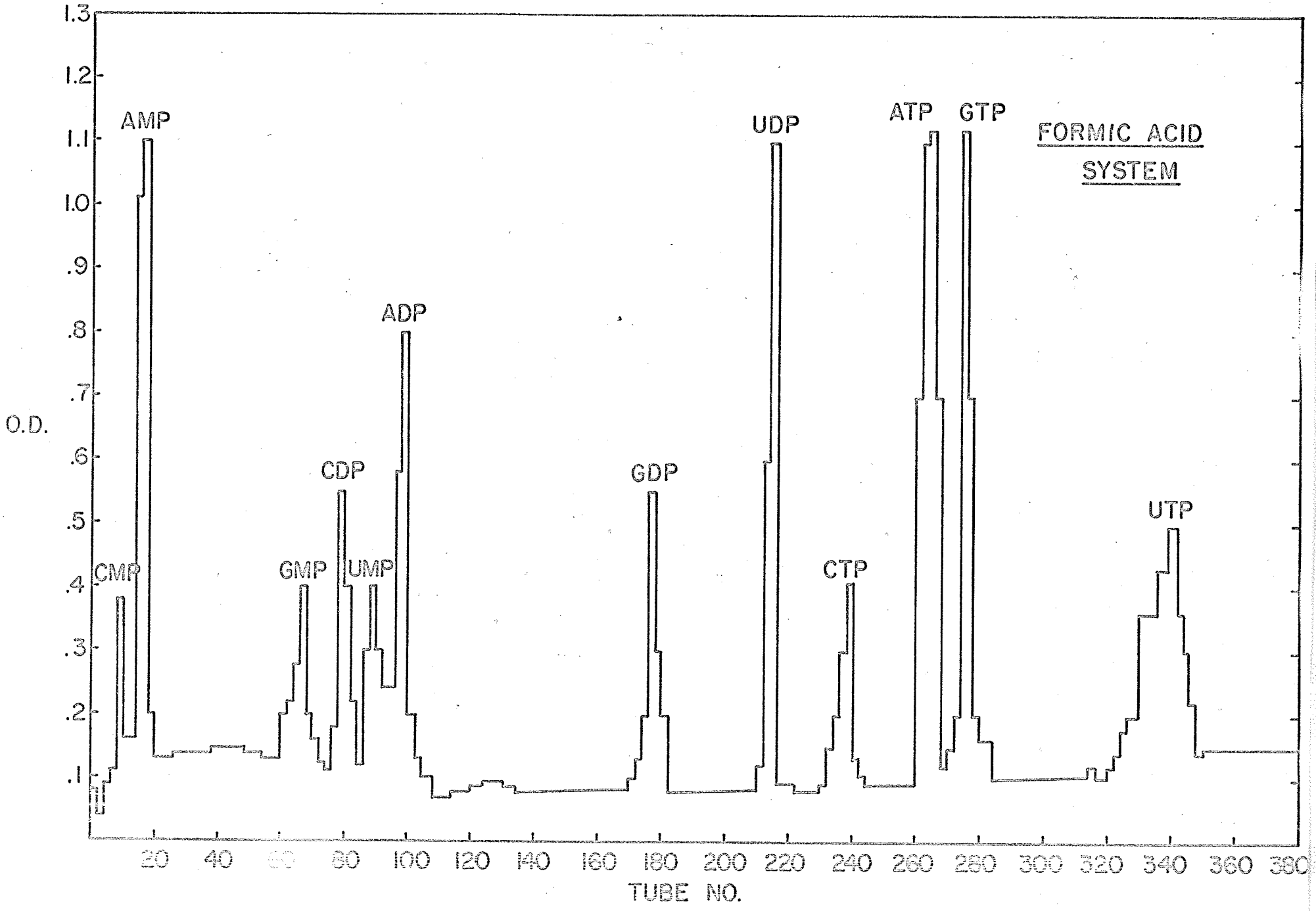

FORMIC ACID SYSTEM
AMP
CMP
GMP
CDP
UMP
ADP
GDP
UDP
CTP
ATP
GTP
UTP
O.D.
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
TUBE NO.

FIGURE 7

Gradient elution chromatography on Dowex-1-Formate with the Ammonium Formate System and commercially prepared nucleotides.

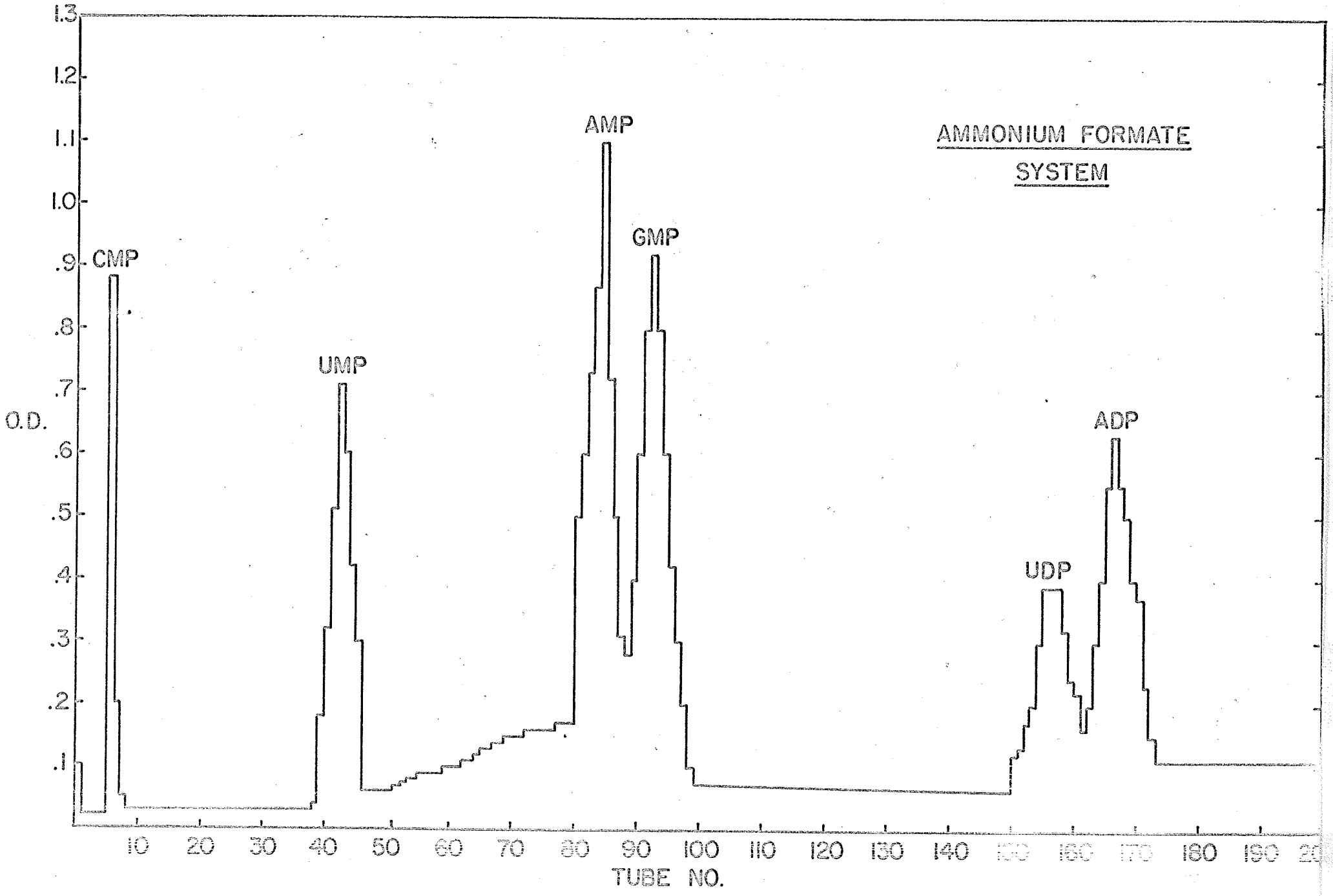

AMMONIUM FORMATE
SYSTEM
CMP
UMP
AMP
GMP
UDP
ADP
O.D.
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
TUBE NO.

FIGURE 8

Chromatography separation on Dowex-1-Formate of the nucleotide pools isolated from Neurospora crassa. The Formic Acid System was employed.

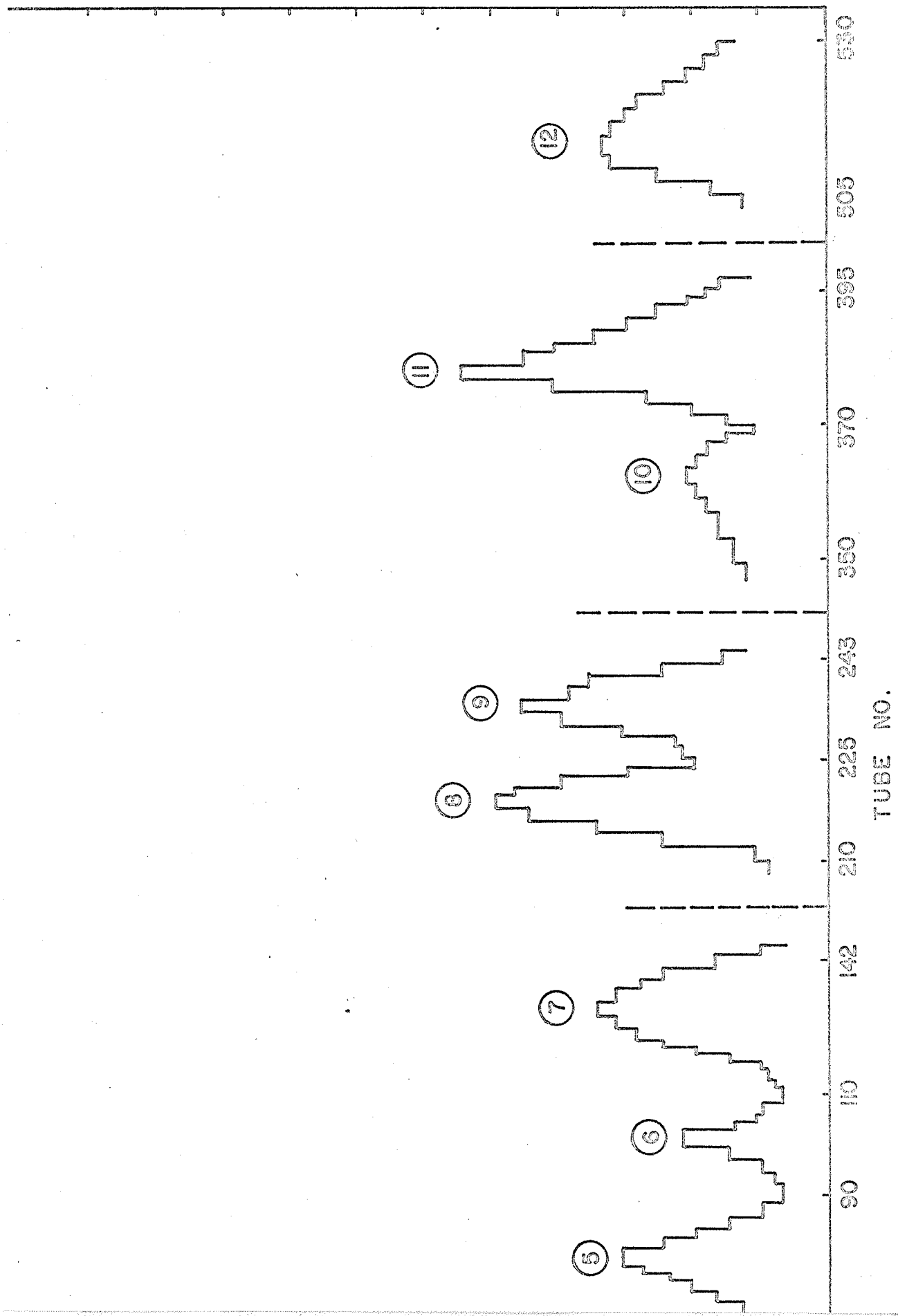

TUBE NO.
50
110
142
210
225
243
350
370
395
505
530
5
6
7
8
9
10
11
12

FIGURE 9

Absorption spectrum of two nucleotides and the free bases obtained from the chromatographic separation of the nucleotide pools of an am^- mutant of Neurospora.

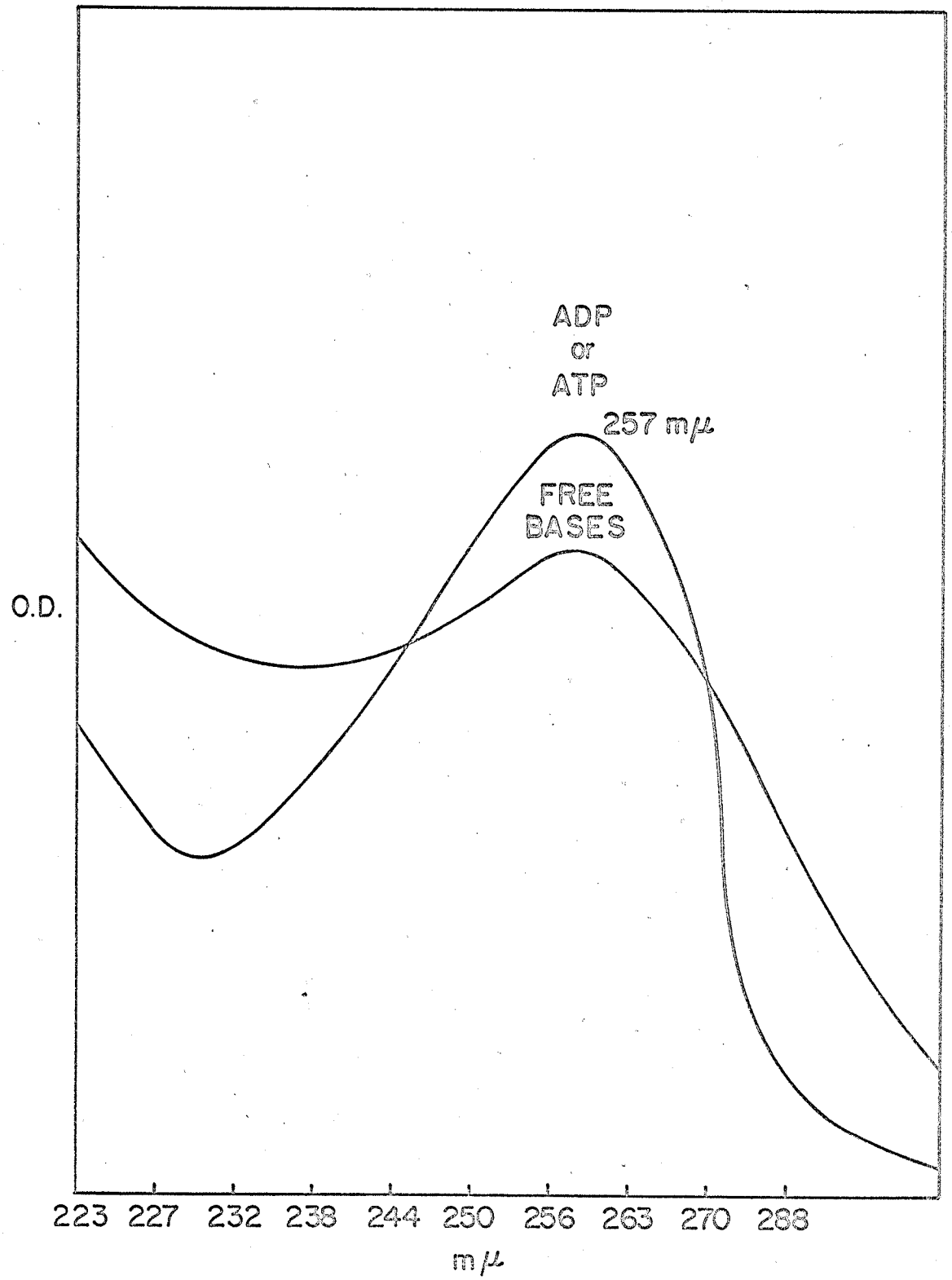

ADP
or
ATP
257 mμ
FREE
BASES
O.D.
223 227 232 238 244 250 256 263 270 288
mμ

FIGURE 10

Absorption spectrum of three nucleotides obtained from the chromatographic separation of the nucleotide pools of an am^- mutant of Neurospora.

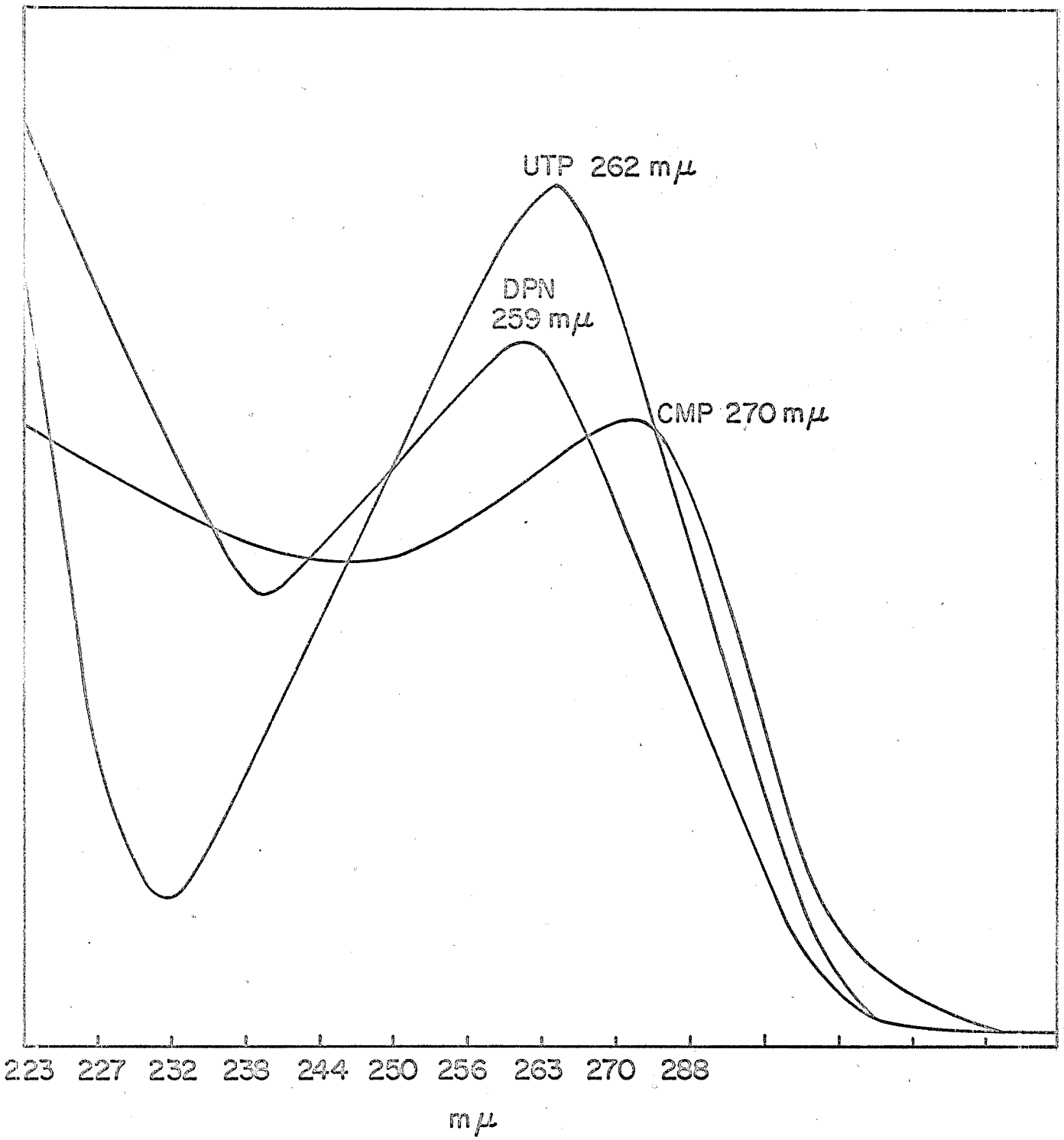

UTP 262 mμ
DPN
259 mμ
CMP 270 mμ
223 227 232 238 244 250 256 263 270 288
mμ

TABLE 1

Identification and Concentration of Nucleotides

Peak Number	Absorption Maximum	System A (R_f)	System B (R_f)	Nucleotide Identification	Alanine Medium	Glycine Medium	Wet Weight		Dry Weight	
							Alan.	Gly.	Alan.	Gly.
1	265 mμ	.357	.600	Free bases	7.410 units	14.105 units	4.0 g	4.0 g	.78 g	.768
2	259 mμ	.350	-	DPN	.936 μmoles	.903 μmoles				
3	270 mμ	.295	.150	CMP	1.696 μmoles	1.482 μmoles				
4	-	-	-	-	14.2 units	14.2 units				
5	253 mμ	.161	-	?	25.3 units	26.10 units				
6	-	-	-	-	16.92 units	18.48 units				
7	257 mμ	.290	.080	ADP	1.236 μmoles	1.506 μmoles				
8	-	-	-	-	32.08 units	31.50 units				
9	261 mμ	.031	-	?	33.92 units	32.76 units				
10	-	-	-	-	22.40 units	21.60 units				
11	258 mμ	.195	.050	ATP	3.392 μmoles	2.72 μmoles				
12	262 mμ	.050	.020	UTP	1.80 μmoles	1.67 μmoles				

Alan. = alanine
Gly. = glycine

TABLE 2

R_f Values of 5' Ribonucleotides

Nucleotide	System A	System B
AMP	.410	.150
ADP	.290	.080
ATP	.190	.050
Adenosine	.780	.060
Adenine	-	.640
CMP	.295	.150
CDP	.170	.080
CTP	.100	.030
Cytidine	.620	-
Cytosine	-	.660
GMP	.150	.110
GDP	.100	.060
GTP	.050	.020
Guanine	-	.850
Guanosine	-	.580
UMP	.200	.200
UDP	.100	.100
UTP	.050	.020

TABLE 2 (continued)

Nucleotide	System A	System B
Uridine	.500	-
Uracil	.055	.700
UDPG	.060	.260
IMP	.180	-
Inosine	-	.600
DPN	.330	-
DPNH	.400	-

substrate producing 1/2 the value of the maximum velocity when the enzyme was saturated with the second reactant.

The apparent K_m was determined for a given substrate from the point of intersection on the X-axis of a double reciprocal plot of velocity versus DPN concentration.

The K_i for an inhibitor (GMP) was defined as the concentration of inhibitor which inhibits the reaction by 50%. The K_i (DPN varied) was determined by replotting the slopes of the inhibition curves given in Figure 11 against the concentration of inhibitor (GMP) (See Figure 12).

The apparent slope K_i and the apparent intercept K_i were determined by replotting the slope or intercept of the inhibition curves shown in Figure 13 against the concentration of inhibitor (GMP) (See Figure 14).

The inhibition of glutamate dehydrogenase activity at changing pH levels was observed in the presence of a fixed inhibitor concentration (See Figure 14).

All points were plotted using the Least Squares Method (Dawes, 1963).

Kinetic Values

K_m (DPN) = 1.471 mM

K_i (real) = 1.65×10^{-4} M

K_i (inter.) = 1.50×10^{-4} M

K_i (slope) = 2.00×10^{-4} M

FIGURE 11

Double reciprocal plots of velocity versus DPN concentration at several fixed concentrations of GMP.

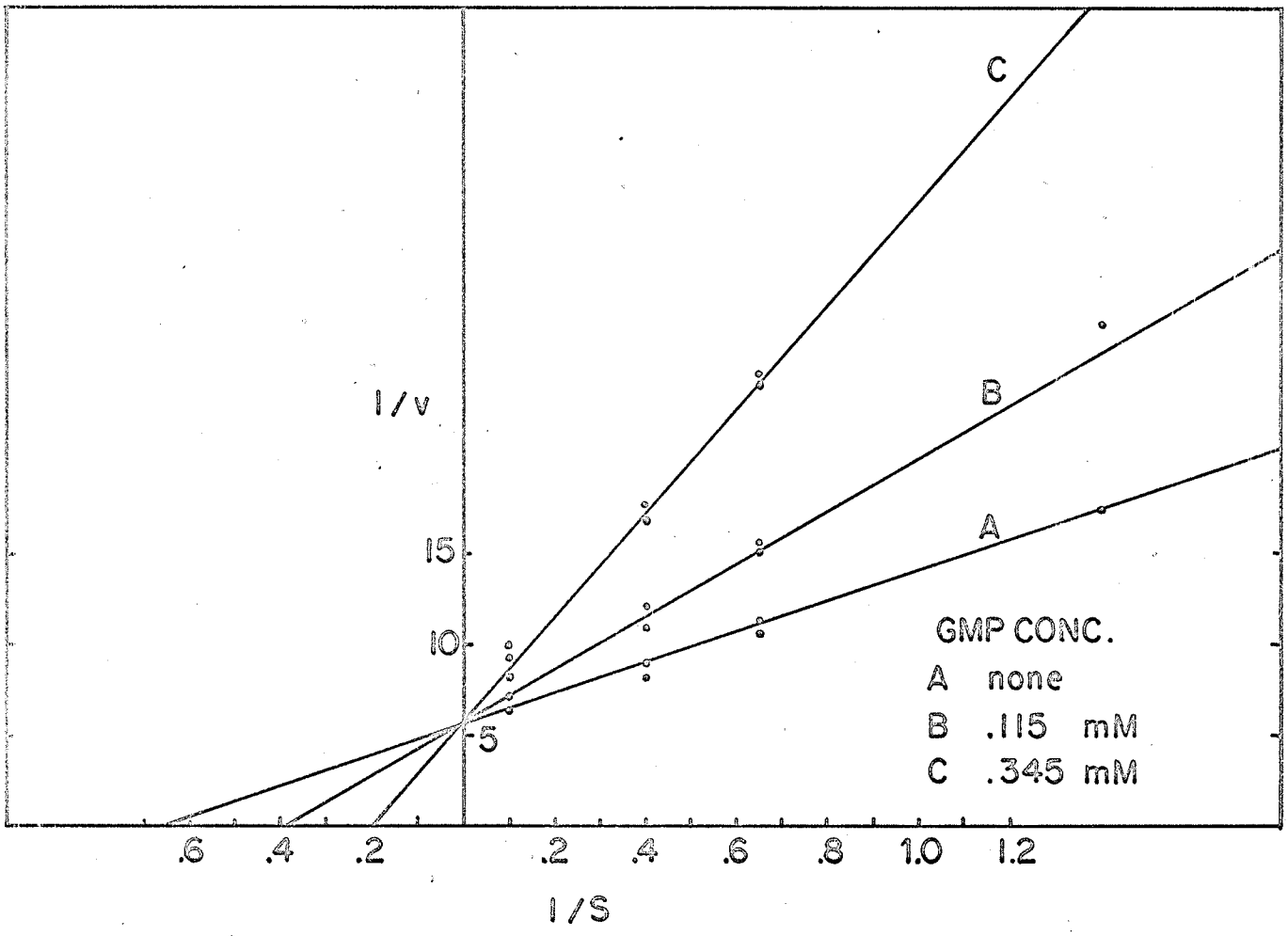

C
B
A
1/v
15
10
5
GMP CONC.
A none
B .115 mM
C .345 mM
.6
.4
.2
.2
.4
.6
.8
1.0
1.2
1/S

FIGURE 12

Replot of the slopes of the inhibition curves found in Figure 11 against the concentration of GMP.

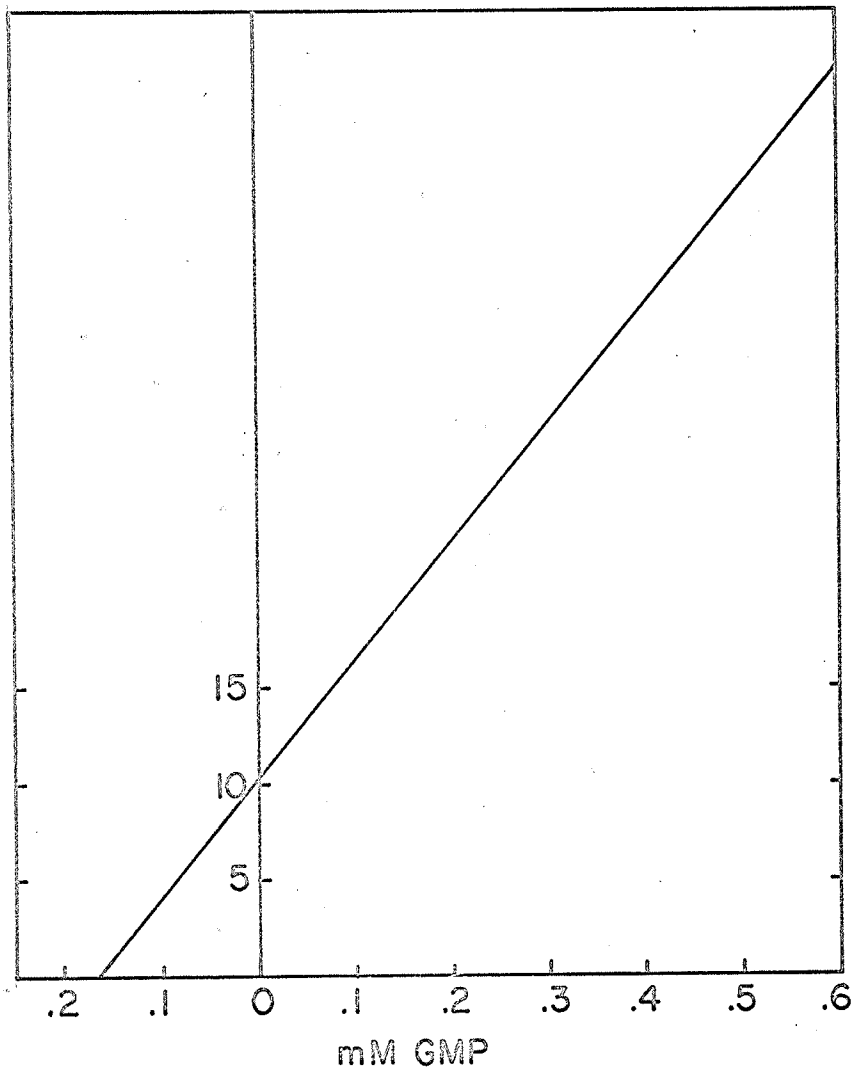

15
10
5
.2
.1
0
.1
.2
.3
.4
.5
.6
mM GMP

FIGURE 13

Double reciprocal plots of velocity versus glutamate concentration at several fixed concentrations of GMP.

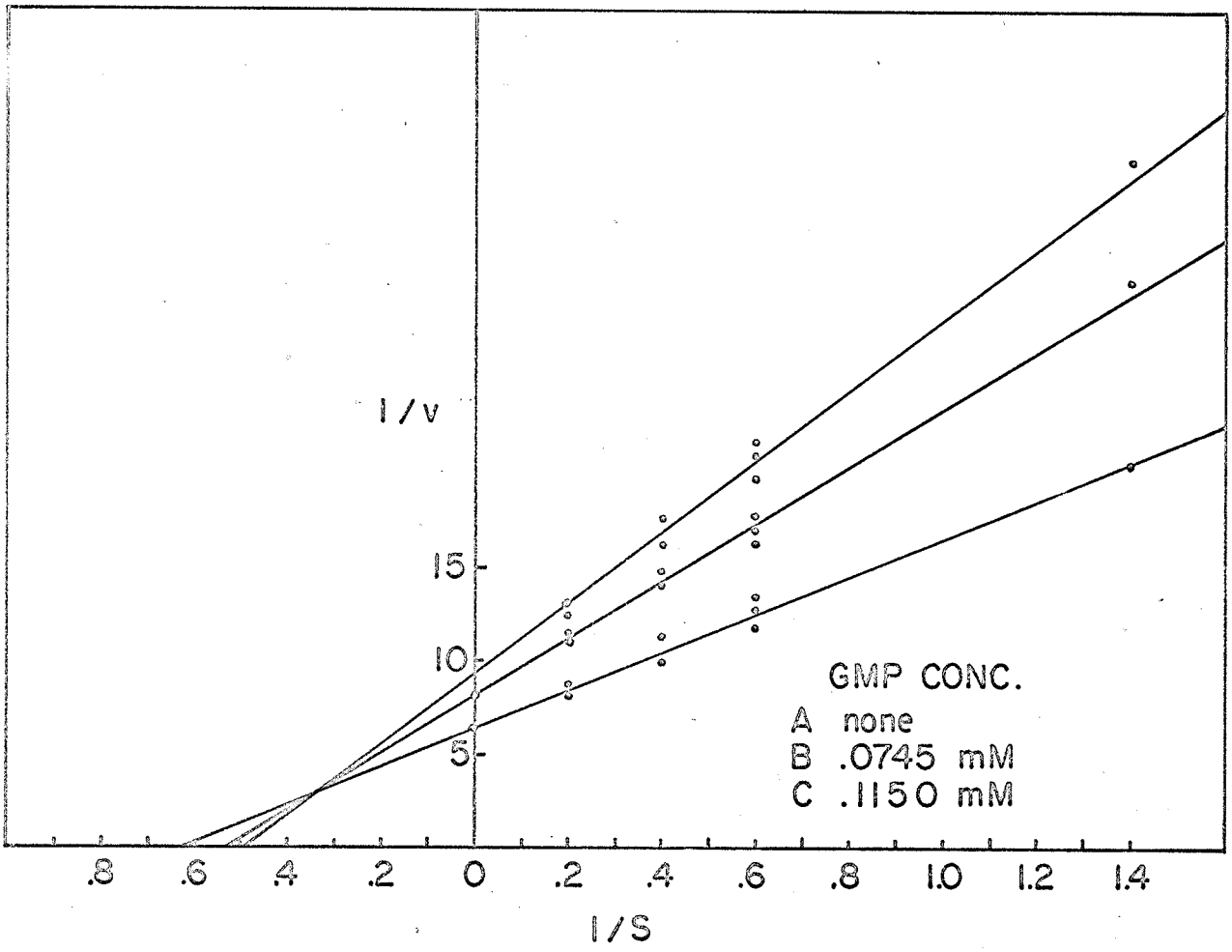

1/v
15
10
5
GMP CONC.
A none
B .0745 mM
C .1150 mM
.8
.6
.4
.2
0
.2
.4
.6
.8
1.0
1.2
1.4
1/S

FIGURE 14

Replot of the slopes or intercepts of the inhibition curves shown in Figure 13 against the concentration of GMP.

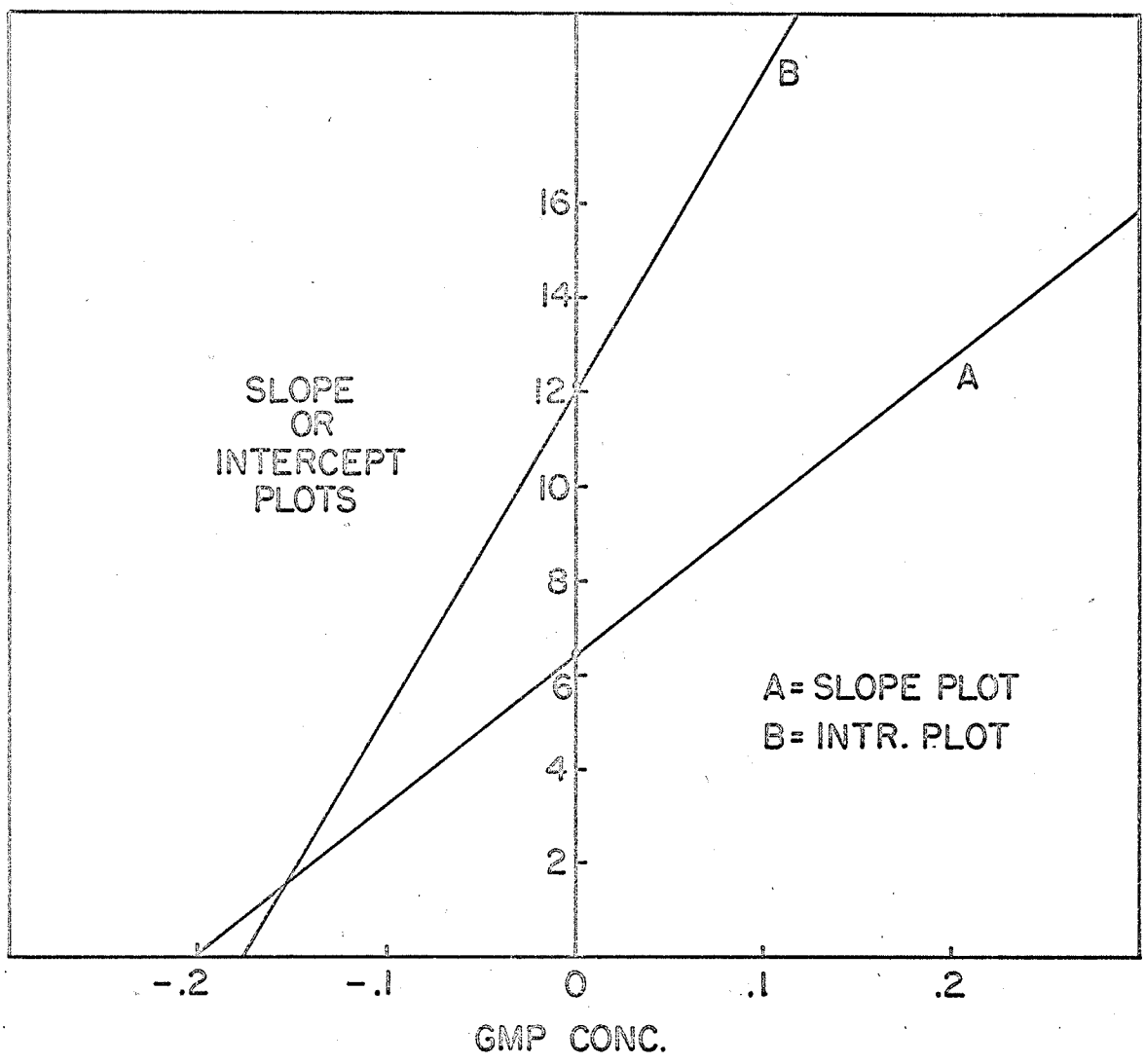
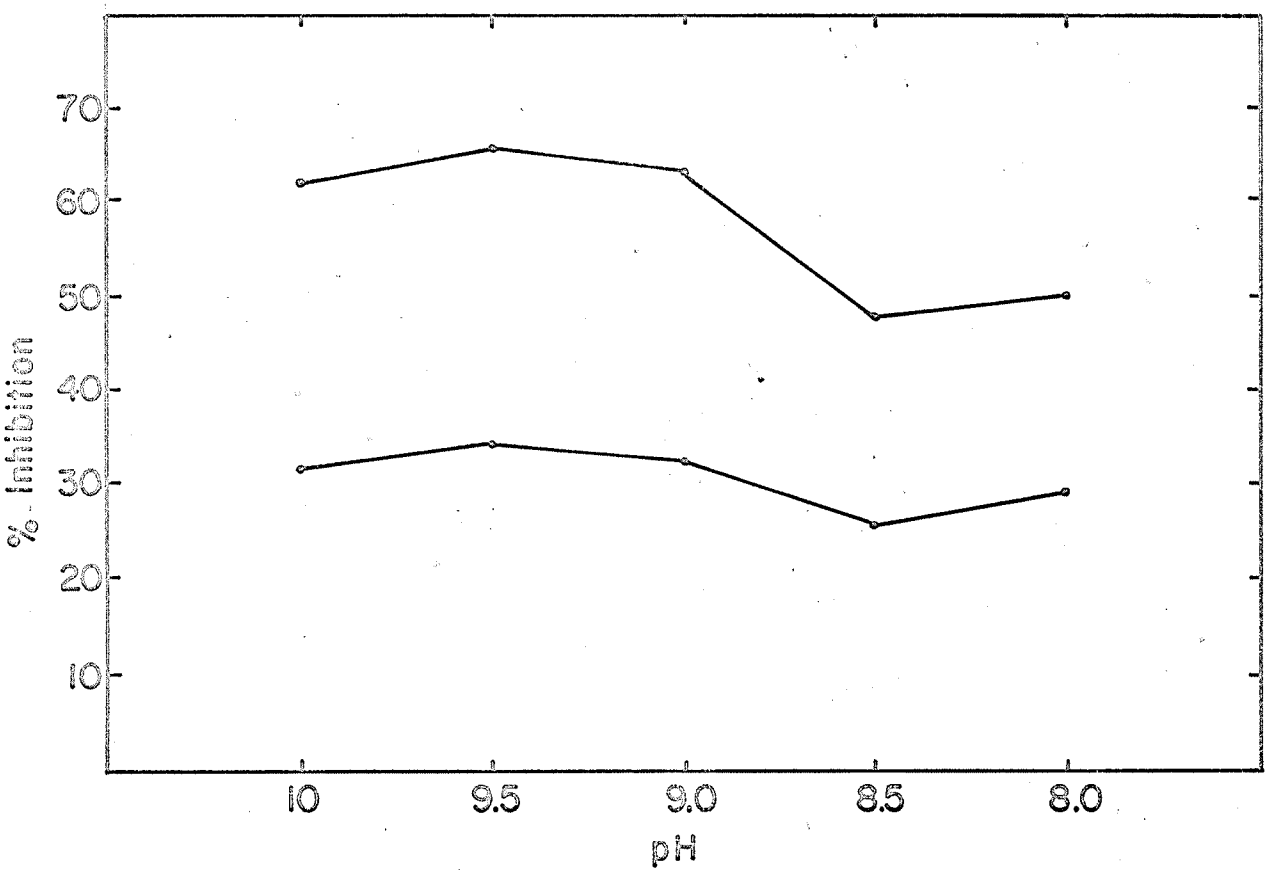

B
A
16
14
12
10
8
6
4
2
SLOPE
OR
INTERCEPT
PLOTS
A = SLOPE PLOT
B = INTR. PLOT
-.2
-.1
0
.1
.2
GMP CONC.

FIGURE 15

The percentage of inhibition caused by GMP at various pH values.


70
60
50
40
30
20
10
% Inhibition
10
9.5
9.0
8.5
8.0
pH

DISCUSSION

The am$^-$ strains of Neurospora, lacking the TPN-specific glutamate dehydrogenase enzyme, grow very slowly on minimal medium. It is thought that the TPN-specific enzyme participates in biosynthetic reactions, while the DPN-specific enzyme performs a catabolic function (Sanwal and Lata, 1961b). The requirement for glutamate in the am$^-$ strains of Neurospora is probably fulfilled by the DPN-specific enzyme located in a particular sub-cellular environment. Since the DPN-linked enzyme is normally catabolic, the glutamate production catalyzed by such an enzyme would be low and would account for the slow growth of the am$^-$ mutant on minimal medium.

In the presence of alanine the growth of the am$^-$ mutant resembles the wild type Neurospora. The "alanine effect" is easily explained. Alanine is converted to glutamate in vivo by transamination reactions (Fincham, 1951).

An explanation for the "glycine effect" is more difficult to find. Glycine and certain nitrogen-containing compounds (serine, sarcosine, methylamine) inhibit the growth of the am$^-$ mutant of Neurospora lacking the TPN-specific

glutamate dehydrogenase enzyme but have no effect upon the growth of the wild type. Glycine does not produce a direct inhibition of the DPN-specific enzyme (Stachow, 1965). Therefore the effect of glycine must be indirect. Two explanations are possible. Glycine may upset the concentration of purines within the cell causing a feed-back inhibition of the DPN-specific enzyme; or glycine may cause an inhibition of the enzyme directly at the genetic level.

It was suggested that beef-liver glutamate dehydrogenase was important in the synthesis of purine nucleotides (Frieden, 1963). Glutamine, which may be produced from glutamate is necessary for purine nucleotide synthesis (Buchanan and Hartman, 1959). Therefore, the concentration of the purine nucleotide pools may be controlled by the level of glutamate.

The purine nucleotides affect the enzymatic activity of the DPN-specific glutamate dehydrogenase of Neurospora, but have no effect upon the TPN-specific enzyme (Stachow and Sanwal, 1964). Guanosine and inosine nucleotides inhibited the enzymatic activity whereas ATP tended to reverse the inhibition caused by the other two purine compounds. ATP,

alone, did not affect the enzymatic activity. It was suggested that the DPN-specific enzyme was used in purine nucleotide synthesis to the partial exclusion of the glutamate produced by the TPN-specific glutamate dehydrogenase enzyme.

The concentration of the nucleotide pools of the am^- strain grown in alanine was compared with the level of the same pools in the am^- mutant grown in glycine. There was no significant difference in the level of the nucleotide pools, although a higher level of free bases was found in the am^- mutant grown in glycine. Neither the isolated free bases nor the commercial preparations inhibited the DPN-specific enzyme *in vitro*. It was concluded that the "glycine effect" is not the result of an increase in the level of purine nucleotide pools.

The regulation of glutamate dehydrogenase has been explained at the genetic level (Sanwal and Lata, 1962; Stachow, 1965). The model offers a possible explanation for the "glycine effect". According to the hypothesis the TPN-specific and the DPN-specific enzyme genes are located in different operons. The operon containing the structural gene for the DPN-specific enzyme also contains a repressor

gene responsible for producing an aporepressor. This aporepressor (R_2) is specific for an operator (O_2) which is found in the operon containing the TPN-specific glutamate dehydrogenase gene. Another aporepressor (R_1) is found outside either operon and is specific for operator (O_1) which belongs to the operon containing the DPN-specific glutamate dehydrogenase gene.

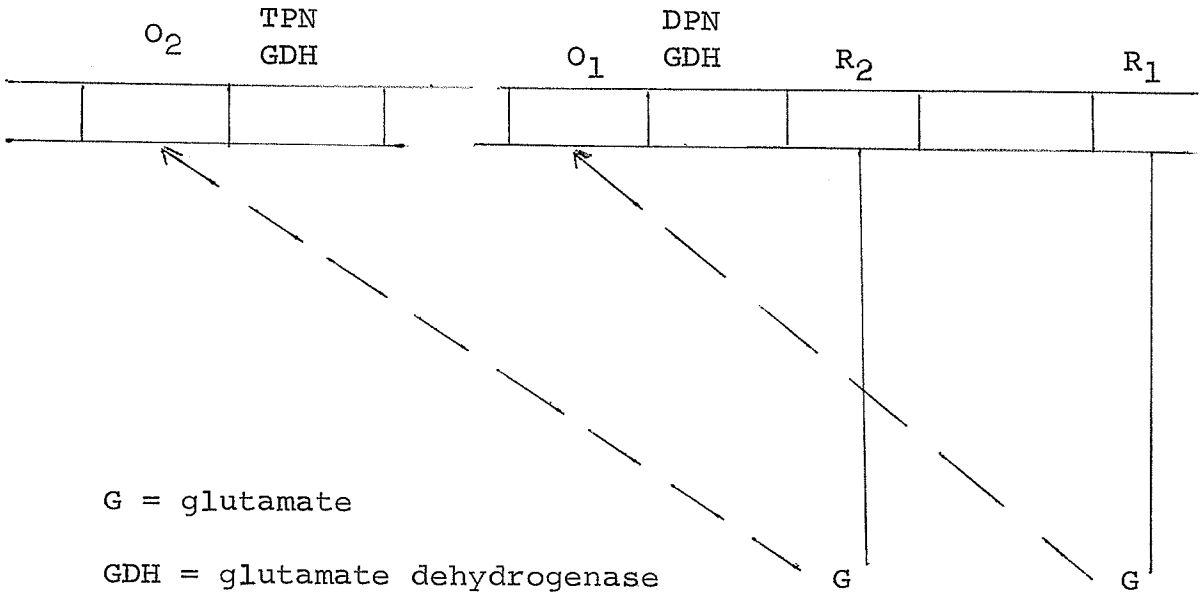


FIGURE 16

There is a decrease in the activity of the TPN-specific glutamate dehydrogenase and a corresponding increase in the

activity of the DPN-specific enzyme when Neurospora is grown in the presence of glutamate and ammonia (Sanwal and Lata, 1962). This effect was also produced by amino acids that are either formed or give rise to glutamate in vivo. The genetic model (Figure 16) was used to explain this concurrent regulation.

Glutamate, in the presence of ammonia, binds to the aporepressor (R_1) rendering it inactive. Therefore, the equilibrium between active and inactive aporepressor (R_1) is shifted in favour of the inactive form. The transcription ability of the DPN-specific glutamate dehydrogenase gene and the R_2 gene is increased resulting in an increase in total synthesis. A higher concentration of the aporepressor (R_2) results in a decrease in the synthesis of the TPN-specific glutamate dehydrogenase enzyme.

Glycine, like glutamate and ammonia, has the ability to bind to the aporepressor (R_1). Glutamate has a higher affinity and ammonia a lower affinity than glycine for the aporepressor (R_1). Glycine simply reduces the amount of aporepressor (R_1) available for binding with ammonia. In the presence of glycine this "repressor substance" still has an affinity for the operator region (O_1). When glycine is

added to the medium the synthesis of the DPN-specific glutamate dehydrogenase enzyme is either entirely prevented or drastically retarded.

Kinetic Analysis

The mechanism of the glutamate dehydrogenase reaction was found to be an Ordered Bi-Ter reaction with DPN binding first followed by L-glutamate. The products were released in the order: ammonia, α-ketoglutarate and DPNH (Stachow, 1965).

GMP competes with DPN for an active site on the DPN-specific glutamate dehydrogenase enzyme (Figure 11) but does not compete for the glutamate binding site (Figure 13). Therefore, GMP probably produces inhibition by binding at the DPN site on the enzyme.

It is possible that GMP binds at an entirely different site on the enzyme causing a conformational change. This change would alter the DPN binding site. Perhaps, GMP causes inhibition at a distance. To test this hypothesis the percentage change in the inhibition of the enzyme by GMP at various pH values was determined.

It was assumed that changes in pH would affect the

ionization of GMP and DPN in a similar manner (both contain amino purine and phosphate). If DPN and GMP bind at the same site a change in pH should not produce a change in the percentage of inhibition caused by GMP. Therefore, a plot of the percentage of inhibition at various pH values would, ideally, produce a straight line parallel to the X-axis. If GMP and DPN bind at different sites a change in pH would probably alter one site more than the other. A straight line sloping downward and approximately parallel to the Y-axis would be obtained.

The line was found to be approximately straight and parallel to the X-axis. Therefore, GMP and DPN compete for the same site (See Figure 15).

Summary

The concentration of nucleotide pools obtained from the am^- mutant of Neurospora crassa grown in alanine was almost identical to the concentrations determined for the am^- mutant grown in glycine. Therefore, the "glycine effect" was not the result of an increase in the level of purine nucleotide pools. The regulation of the glutamate dehydrogenase enzyme was explained at the genetic level.

The K_m value for DPN, and the real, intercept, and slope K_i values for the inhibitor, GMP, were determined. The inhibitor (GMP) was found to bind the enzyme at the DPN site.

REFERENCES

1. Alder, E., Hillstrom, V., Gunther, G., and von Euler, H.; Uber den enzymatischen abbaw und aufbaw der glutanunsaure. III. In Bacterium coli. Hoppe-Seyler's Z. physiol. chem. 255: 14 (1937).

2. Ames, B. N. and Garry, B.; Coordinate repression of the synthesis of four histidine biosynthetic enzymes by histidine. Proc. Nat. Acad. Sci. U. S. 45: 1453 (1959).

3. Barratt, R. W.; Effect of environmental conditions on the TPN-specific glutamate dehydrogenase in Neurospora crassa. J. Gen. Microbiol. 33: 33 (1963).

4. Bassler, K. H. and Hammer, C. H.; Reduktive amenierung von α keto valeriansaure an glutamersause dehydrogenase. Biochem. Z. 330: 446 (1958).

5. Bendich, A.; In Methods of Enzymology. S. P. Colowick and N. O. Kaplan (Editors) Vol. 3, 715, Academic Press, Inc., New York (1957).

6. Buchanan, J. M. and Hartman, S. C.; Enzymic reactions in the synthesis of purines. Advances in Enzymology 21: 197 (1959).

7. Copenhaver, J. H. Jr., McShan, W. H., and Meyer, R. K.; The determination of glutamic acid dehydrogenase in tissue homogenates. J. Biol. Chem. 183: 73 (1950).

8. Damodaran, M. and Nair, K. R.; Journal of Biochem. 32: 1064 (1938).

9. Datta, R. and Gest, H.; Control of enzyme activity by concerted feedback inhibition. Proc. Nat. Acad. Sci. U. S. 52: 1004 (1964).

10. Dawes, E. A.; In Quantitative Problems in Biochemistry. P. 270. E & S Livingstone Ltd., Edinburgh and London.

11. De Mars, R.; Inhibition by glutamine of glutamyl transferase formation in cultures of human cells. Biochim. et Biophys. Acta 27: 435 (1958).

12. Dewan, J. G.; Biochem. Journal 32: 1378 (1938).

13. Fincham, J. R. S.; Mutant strains of Neurospora deficient in amination ability. J. Biol. Chem. 182: 61 (1950).

14. Fincham, J. R. S.; Transaminases in Neurospora crassa. Nature 168: 957 (1951).

15. Fischer, H. F., Gross, D. G. and McGregor, L. L.; The independence of the substrate specificity of glutamate dehydrogenase on its state of aggregation. Biochim. et Biophys. Acta 99: 165 (1965).

16. Frieden, C.; Kinetic studies on the diphosphopyridine nucleotide cytochrome c reductase from heart. Biochim. et Biophys. Acta 24: 241 (1957).

17. Frieden, C.; Glutamate dehydrogenase. I. The effect of coenzyme on the sedimentation velocity. J. Biol. Chem. 234: 809 (1959).

18. Frieden, C.; The unusual inhibition of glutamate dehydrogenase by guanosine di- and triphosphate. Biochim. et Biophys. Acta 59: 484 (1962).

19. Frieden, C.; Glutamate dehydrogenase. V. The relation of enzyme structure to the catalytic function. J. Biol. Chem. 238: 3286 (1963).

20. Gale, E. F.; Enzymes concerned in the primary utilization of amino acids by bacteria. Bact. Rev. 4: 135 (1940).

21. Gaul, G. Preparation and properties of glutamic dehydrogenase from human placenta. Biochim. et Biophys. Acta 40: 552 (1960).

22. Gorini, L. and Maas, W. K.; The potential for the formation of a biosynthetic enzyme in Escherichia coli. Biochim. et Biophys. Acta 25: 208 (1957).

23. Holzer, H. and Schneider, S.; Anreicherung and trennung liver DPN-specifischen und liver TPN-spezifishen

glutaminsaure-dehydrogenase aux Hepe. Biochem. Z 3320: 240 (1957).

24. Horowitz, N. H., Fling, M., MacLeod, H., and Sueoka, N.; Genetic determination and enzymatic induction of tyrosinase in Neurospora. J. Mol. Biol. 2: 96 (1960).

25. Hurlbert, R. B. In Methods of Enzymology. S. P. Colowick and N. O. Kaplan (Editors) Vol. 3, p. 785 Academic Press, Inc., New York (1957).

26. Jacob, F. and Monod, J.; Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318 (1961).

27. Jacob, F. and Monod, J.; On the regulation of gene activity. Cold Spring Harbour Symp. 26: 193 (1961).

28. Jergensen, B.; Glutamic acid dehydrogenase - A protein of unusual conformation. J. Am. Chem. Soc. 83: 3161 (1961).

29. Kubo, H., Iwatsubo, M., Watari, H., and Soyama, T.; Polymerization and molecular form of glutamic dehydrogenase. J. Biochem. 46: 1171 (1959).

30. Mehler, A. H., Kornberg, A., Santiago, G., Ochoa, S.; The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate. J. Biol.

Chem. 174: 961 (1948).

31. Monod, J.; Allosteric proteins and cellular control systems. J. Mol. Biol. 6: 306 (1963).

32. Novick, A. and Szilard, L.; Experiments with the chemostat on the rate of amino acid synthesis in bacteria. Dynamics of Growth Processes. Princeton University Press.

33. Olson, J A. and Anfinsen, C. B.; The crystallization and characterization of L-glutamic acid dehydrogenase. J. Biol. Chem. 197: 67 (1952).

34. Olson, J. A. and Anfinsen, C. B.; Kinetic and equilibrium studies on crystalline L-glutamic acid dehydrogenase. J. Biol. Chem. 202: 841 (1953).

35. Pardee, A. B. and Prestidge, L. S.; Induced formation of serine and threonine deaminases by Escherichia coli. J. Bact. 70: 667 (1955).

36. Pardee, A. B., Jacob, F. and Monod, J.; The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by Escherichia coli. J. Mol. Biol. 1: 165 (1959).

37. Sanwal, B. D.; Diphosphopyridine nucleotide and triphosphopyridine nucleotide linked glutamic

dehydrogenases of Fusarium. Arch. Biochem. and Biophys. 93: 377 (1961a).

38. Sanwal, B. D. and Lata, M.; The occurrence of two different glutamic dehydrogenases in Neurospora. Can. J Microbiol. 7: 319 (1961b).

39. Sanwal, B. D. and Lata, M.; Glutamic dehydrogenase in single-gene mutants of Neurospora deficient in amination. Nature 190: 286 (1961c).

40. Sanwal, B. D. and Lata, M.; Concurrent regulation of glutamic acid dehydrogenase of Neurospora. Arch. Biochim. Biophys. 97: 582 (1962a).

41. Sanwal, B. D. and Lata, M. The regulation of glutamic dehydrogenase and an antigenically related protein in amination deficient mutants of Neurospora. Arch. Biochim. Biophys. 98: 420 (1962b).

42. Stachow, C. S. and Sanwal, B. D.; Differential effects of purine nucleotides on the activity of two glutamic dehydrogenases of Neurospora. Biochem. Biophys. Res. Comm. 17: 368 (1964).

43. Stachow, C.; Ph.D. Thesis, University of Manitoba (1965).

44. Stadtman, E. R., Cohen, G. N., Le Bras, G. and de Robichon-Szulmajester, H.; Feedback inhibition and repression of

aspartokinase activity in Escherichia coli and Saccharomyces cerevisiae. J. Biol. Chem. 236: 2033 (1961).

45. Strecker, H. J.; Crystallization of L-glutamic acid dehydrogenase from liver. Arch. Biochem. Biophys. 32: 448 (1951).

46. Talal, N. and Tompkins, G. M.; Allosteric properties of glutamate dehydrogenases from different sources. Science 146: 1309 (1964a).

47. Talal, N. and Tompkins, G. M.; Antigenic differences associated with conformational changes in glutamate dehydrogenase. Biochim. et Biophys. Acta 89: 226 (1964b).

48. Tompkins, G. M., Lemone, K., Yielding, K. L. and Curan, J.; Steroid hormone activation of L-alanine oxidation catalyzed by a subunit of crystalline glutamic dehydrogenase. Proc. Nat. Acad. Sci. U. S. 47: 270 (1961).

49. Umbarger, H. E.; Evidence for a negative feedback mechanism in the biosynthesis of isoleucine. Science 123: 848 (1956).

50. Uziel, M. and Cohn, W. E.; Desalting of nucleotides by

gel filtration. Biochim. Biophys. Acta 103: 539 (1965).

51. Vogel, H. J.; Repressed and induced enzyme formation: A unified hypothesis. Proc. Nat. Acad. Sci. U. S. 43: 491 (1957).

52. von Euler, H., Adler, E., and Eriksen, T. S.; Z. physiol. Chem. 248: 227 (1937).

53. Wolff, J.; The effect of thyrosine on isolated dehydrogenases. J. Biol. Chem. 237: 236 (1962).

54. Yates, R. A. and Pardee, A. B.; Control of pyrimidine biosynthesis in Escherichia coli by feedback mechanism. J. Biol. Chem. 221: 757 (1956).

55. Yielding, K. L., Tompkins, G. M., Munday, J. S. and Curan, J. F.; The effects of steroid hormones on the glutamic dehydrogenase reaction. Biochem. Biophys. Res. Comm. 2: 303 (1960).