COMPARATIVE BIOCHEMICAL STUDIES OF PYRIMIDINE MUTANTS OF *NEUROSPORA CRASSA*

by

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INTRODUCTION

Although the basic pattern of pyrimidine metabolism in mammalian and bacterial systems has been elucidated, little is known of pyrimidine metabolism in the fungi, and the inter-relationships of genetic potentials and pyrimidine metabolism have not been clarified in any organism.

Neurospora crassa offers a rather unique tool among organisms for determining genetic-metabolic interrelations in that detailed, classical genetic analyses can be made concomitantly with the biochemical analyses. Biochemical studies are simplified by the fact that Neurospora can be grown in chemically-defined media. The principles of genetic and physiological studies of Neurospora have been reviewed(5,62,78).

Among the several available and interesting areas of metabolism approachable from the chemical-genetic point of view, the metabolism of pyrimidines was chosen for three reasons: 1) little is known about pyrimidine metabolism, per se, in fungi, 2) the genetic relations to pyrimidine metabolism are unknown, and 3) deoxyribonucleic acid(DNA), the postulated carrier of genetic information(18), is unique, in contrast to ribonucleic acid, in that it contains the pyrimidines, thymine, 5-methylcytosine, and in the case of the T-even bacteriophages, 5-hydroxymethylcytosine(6). Uridine is known to be a precursor of thymidine in E. coli(28), Neurospora(55), T_1 bacteriophage(2), and is possibly a precursor to 5-hydroxymethylcytosine in E. coli via the dihydropyrimidines(13). Thus, a study of the biosynthesis of these pyrimidines and their precursors would, in effect, be a beginning in the study of the autocatalytic properties of genes and DNA. Furthermore, fundamental

principles elucidated by these studies might also have unforeseen implications in studies of eccentric DNA metabolism as found in virus and neoplastic growth.

During the course of these studies, Dr. V. W. Woodward has isolated and characterized the physiological properties of pyrimidine mutants of Neurospora, and Mr. Y. Suyama has done the genetic analyses. Details of their studies will be presented in future publications and therefore will not be elaborated in this paper, except as necessary to present perspective to the over-all problem.

LITERATURE REVIEW

Pyrimidine Biosynthesis in Neurospora crassa. The available knowledge of the genetics of pyrimidine biosynthesis in Neurospora, at the time these studies were instigated, can be largely attributed to the work of Mitchell and his coworkers.(57-63)

The main features of Mitchell's findings are summarized as follows(Plate I)[1]: All pyrimidine mutants responded to the nucleotides, the nucleoside, or the free form of uracil, and the nucleoside or nucleotides of cytosine, but not to cytosine alone(62). Mutants pyr-2 (38502) and pyr-4 (36601) accumulated orotic acid and orotidine (57,62), and lacked the enzymic activity of orotidylic acid decarboxylase, an enzyme active in the prototroph (77). Neither pyr-2 nor pyr-4 grew in medium supplemented with orotic acid or orotidine, however, pyr-1 (263) and pyr-3 (37301) both responded to these compounds(78). Furthermore, the double mutants of either pyr-1 or pyr-3 with pyr-2 or pyr-4 failed to accumulate these compounds(60). It was therefore concluded that pyr-1 and pyr-3 affected intermediary reactions leading to orotic acid. The order in

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1. All plates in the appendix.

which pyr-1 and pyr-3 affected the biosynthetic pathway and the orotic acid precursors was not clear. Possible precursors to orotic acid were suggested by the observations that two pyrimidine mutants with partial genetic blocks were stimulated by oxalacetic acid and aminofumaric acid(59).

None of the above mutants utilized thymine, thymidine, or 5-methylcytosine for growth(78). Since thymine is known to reside in DNA, and since uniformly labeled C^{14}-cytidine was converted to thymine in Neurospora(55), the non-utilization of thymine or thymidine suggested that the formation of thymidine probably occurs irreversibly via uridine as shown by Friedkin (28) in E. coli extracts.

H. K. Mitchell[1] has reported the enzymatic conversion of ureidosuccinic acid and dihydroorotic acid to orotic acid, and the accumulation of ureidosuccinic acid and dihydroorotic acid by pyr-1. Details of these experiments were not available at this writing.

A genetic map of the pyrimidine mutants in Neurospora is illustrated in Plate II. Three of the loci, pyr-1, pyr-3, and pyr-2, are located on the right arm of linkage group IV. Pyr-4 apparently lies in another linkage group(3). The locus of pyr-3 was found to contain at least 6 mutations, some of which were distinguishable by their temperature sensitivity, response to a pyrimidine suppressor gene, and by genetic analysis some of the mutants were thus considered to be pseudoallelic(63).

According to McNutt(55), ribonucleosides are utilized as precursors of the carbon skeleton of DNA in a pyrimidine-requiring mutant of Neurospora. The mutant source was not reported. Uniformly labeled C^{14}-cytidine was fed and the specific activities of the nucleosides of RNA and DNA were determined.

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[1]Personal communication.

He concluded that the distribution of labeled carbon between the pyrimidine and the sugar of pyrimidine ribosides and deoxyribosides indicated that the entire carbon skeleton of the pyrimidine riboside was inserted into the pyrimidine ribosides of the RNA and converted to the pyrimidine deoxyriboside of the DNA. An interesting observation was that this mutant, although unable to grow in the absence of a pyrimidine source, had some capacity to synthesize the pyrimidine nuceloside skeleton, as evidenced from the activity dilution of administered cytidine.

J. S. Fairley and co-workers(22,37) reported the ability of pyr-3e (1298) to utilize α-amino-n-butyric acid-3-C^{14}(or threonine) for growth in the absence of exogenous pyrimidine, and suggested the labeled compound was utilized as a source of the 3-carbon chain of the pyrimidine ring. Significant labeling was found only for the pyrimidines, cytosine and uracil, and for isoleucine among a number of compounds examined. Even for these compounds, however, considerable dilution of the radioactive carbon was observed. The metabolic relations of these administered compounds remains unclear.

Pyrimidine Metabolism in Bacterial and Mammalian Systems. Within the past five years, a wealth of information has been accumulated on the nature of pyrimidine metabolism in mammalian and bacterial systems. It is beyond the scope of this section of the paper to critically review all of the research that has appeared in this area. The interested reader is referred to recent review articles for the current status of pyrimidine metabolism(12,44). The general pattern that has emerged is illustrated in Plate III. At present, there is some disagreement on the relative importance of the orotic acid and uracil pathways for the biosynthesis of RNA precursors. Kornberg(44) and Cooper, et al.(16) suggested that the orotic acid pathway was primarily for biosynthesis and that catabolism (or "scavengering" among fastidious organisms) was the

primary function of the uracil pathway. However, Canellakis(10,11) and Mokrasch and Grisolia(64) have shown that, under certain conditions, the uracil pathway may be equally important in the biosynthesis of RNA precursors.

Mention should be made of the comparative studies of bacterial pyrimidine mutants. Yates and Pardee(85,86) reported on a series of pyrimidine mutants of E. coli. Mutants singly deficient in the enzymes, ureidosuccinic synthetase, dihydroorotase, dihydroorotic dehydrogenase, and blocked in the conversion of orotic acid to uridine-5'-phosphate were studied. The genetics of these mutants has not been studied, to our knowledge.

Slotnick and Weinfeld(70) found mutants of E. coli strain W that required either β-alanine or pantothenic acid also responded to dihydrouracil and β-ureidopropionic acid, but uracil, orotic acid, dihydroorotic acid, dihyrouridine, and dihydrodeoxyuridine were ineffective in supporting growth.

Crawford, et al(17) reported a comparative study of various uridine-requiring species of Lactobacilli in the enzymatic conversion of uracil and orotate to uridine-5'-phosphate. They found a clear correlation between the pyrimidine growth requirements of the Lactobacilli studied and the presence of specific enzymes capable of forming nucleotides from them.

MATERIALS AND METHODS

Strains

The studies were begun by isolating over 100 pyrimidine-requiring mutants from the prototroph, ST 73, after ultraviolet irradiation by the filtration and selective plating technique of Woodward, et al(78). Mutants 263 (pyr-1), 38502 (pyr-2), 37301 (pyr-3a), 37815 (pyr-3b), and 36601 (pyr-4) were kindly supplied by M. B. Mitchell. Mutants isolated in this laboratory were designated by the prefix KS (Kansas State College).

Growth Analyses

The minimal medium used throughout these experiments were described elsewhere(82). For the reader's convenience, the composition of the minimal medium is included in the appendix. The pyrimidines and their derivatives were supplemented on a micro-molar equivalent basis. (The molecular weight of uracil was taken as 100, rather than 112, as a standard.) In other instances, the nutrilites were merely added on a weight-volume basis(mg./100 ml. medium, mg. per cent). The growth measurements reported were made from dried(24 hr. at 60°C) mycelial pads harvested from 40 ml. of liquid medium in 250 ml. Erlenmeyer flasks. During the growth period, the cultures were incubated at 27°C, and replicas were harvested at 24 h. intervals following inoculation. For the routine inocula, three day-old cultures grown on 5 ml. agar slants supplemented with 10 mg. per cent uridine and incubated at 27°C were harvested in 5 ml. sterile distilled water, and one drop from a 10 ml. pipette was added to one flask for incubation. Woodward(83) has estimated the standard deviation of treatments within an experiment, by the above techniques, to be on the order of 5 mg. per 100 mg. dry weight.

Chemicals

Standard media and reagent chemicals (certified reagent grade) were obtained from Fisher Scientific Company. Pyrimidines and their derivatives were secured from the California Foundation for Biochemical Research and the Nutritional Biochemicals Corp., with the exception of orotidine, β-ureidopropionic acid, and 5-carboxymethylhydantoin, which were prepared in this laboratory.

Orotidine was isolated from the mycelia of pyr-4 by the anion exchange method of Leiberman et al(50). Concentration was determined by the use of the reported extinction coefficient of orotidine(57,50). Paper chromatograms in n-butanol-acetic acid-water solvent[1] and n-butanol-water-ammonia solvent[2] showed a single ultraviolet-absorbing component free of orotic acid and exhibiting the migration characteristic of nucleosides. That the compound was orotidine was further evidenced by its appropriate elution peak from the anion exchange column(50) and by its characteristic acid hydrolysis curve(57).

β-ureidopropionic acid was synthesized from β-alanine and potassium cynate and purified as the free acid from Dowex 50 H-ion exchange resin by the method of Fink, et al(26). After two recrystallizations from hot methanol, the product melted at 173°C;[3] previously reported, 169-170°C,(4). A single PDAB reactive component, free of ninhydrin-reactive substance, and showing a negative flame test for potassium was obtained on paper chromatograms in several solvents with R_f values in agreement with those reported by Fink, et al(26.)

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[1] 2:1:1 v/v

[2] 86:14 v/v +1% NH_4OH

[3] All melting points were uncorrected.

DL-5-carboxymethylhydantoin was synthesized from DL-ureidosuccinic acid by the method of Nyc and Mitchell(66). After three recrystallizations from hot water, the compound melted in the range 214-217°C; previously reported, 214-216°C(66), 214-217°C(47). The product, as well as authentic hydantoin, failed to react with PDAB reagent by the methods of Fink, et al(25) for the dihydropyrimidines.

Paper Chromatography

Cabinets sufficiently large to contain 46 by 57 cm. filter paper sheets were constructed of plywood and glass from the plans of Comar(14). Solvent tray assemblies were obtained from the Scientific Glass Apparatus Company.

Samples were applied in 5 ul. aliquots with a 5 ul. micropipette (Microchemicals Specialities Co.) at 3 cm. intervals along a line 10 cm. from the long edge of 46 by 57 cm. Whatman No. 1 or No. 3 MM filter paper sheets. The R_f values of the controls were essentially the same for both grades of paper, but the heavier paper was more suitable for culture concentrates and mycelial extracts in that less spot distortion and trailing was obtained.

After the cabinets were equilibrated with solvent vapor for 12-24 hr., the samples were partitioned by a descending solvent at room temperature (20-25°C). When the solvent front had traveled to within 1-3 cm. from the edge of the sheet, the sheets were removed and the solvent front marked with a pencil. The sheets were then thoroughly dried in a ventilated room.

Ultraviolet-absorbing spots were located by viewing the chromatograms under an ultraviolet lamp with maximum emmission at the wave-length, 2537 Å(Mineralight, Model SL; Ultraviolet Products, Inc.). The minimal concentrations for the detection of uracil and orotic acid were about 10 and 100 mu moles per sq. cm., respectively.

Amino acids were detected by spraying the filter sheets with water-saturated n-butanol (86:14 v/v) solution containing 0.1 to 0.2 per cent ninhydrin(15). After drying the chromatograms in a hood, they were placed in an oven (35°C) for 60-90 min. and kept in the dark for 24 hours or longer (19).

Dihydropyrimidines and ureido acids were detected by the spray method of Fink, et al(25). The principle of this method is based on the fact that compounds with the general formula, $RNHCONH_2$, give a yellow color with acidified, p-dimethylaminobenzaldehyde(PDAB). The following procedure was employed (after ninhydrin treatment, if desired). When solvents had evaporated from the paper, it was uniformly sprayed with 0.5N sodium hydroxide and allowed to dry for at least 30 minutes. The alkali hydrolyzes the dihydropyrimidines to the corresponding ureido acid. The paper was uniformly sprayed with a solution containing 1 gm. of PDAB(recrystallized from ethanol), 10 ml. of concentrated hydrochloric acid, and 100 ml. of ethanol, dried for a few minutes in a hood, hung in a ventilated room for 2 to 6 hr. (depending on the rate of color development), and viewed in transmitted white light to facilitate the outlining of colored spots. The sodium hydroxide spray was generally omitted if only R_f values of compounds not requiring preliminary hydrolysis were to be determined. The minimum limit for visual detection of the dihydropyrimidines and ureido acids was on the order of 1 ug. per sq.cm.

R_f values reported in this paper are defined by the convential relation:

$$R_f = \frac{\text{distance from origin to center of spot}}{\text{distance from origin to solvent front}} .$$

Marker compounds for chromatography were dissolved in distilled water at a concentration of 4 mg. per ml. Five ul. were routinely taken for control samples, with the exception of the more chromogenic compounds, β-ureidopropionic

acid, ureidosuccinic acid, citrulline, and urea, of which 2.5 ul. were taken. Due to the uncontrollable variability of R_f values between sheets within an experiment, and between experiments, marker compounds were routinely partitioned on the same sheet with the unknowns.

Spectrophotometry

All spectrophotometric studies were made with a Beckman ultraviolet spectrophotometer, Model DU.

Binary analyses were made according to the method described by Loring(51). The theory of binary spectrophotometric analysis states: "The analysis of a binary mixture of components A and B, having different absorption spectra, is made on the assumption that the total optical density, D, at each of the two wavelengths, λ_1 and λ_2, is the sum of the densities due to each component at each wavelength."(51).

Ion Exchange Analyses

Ion exchange analyses of cultural filtrates and mycelial extracts were made according to the procedure of Leiberman and Kornberg(47). The procedure was as follows. Solutions were adjusted to pH 7.0 with 1 M KOH (brom thymol blue as an internal indicator) and absorbed on a column of Dowex 1, formate form (200 to 400 mesh, 10% cross-linkage, height 7 cm., diameter 1 cm.). The eluent was formate buffer (0.055M sodium formate adjusted to pH 3.2 with formic acid), and the rate of flow was adjusted to 0.5 to 1.0 ml. per minute; fractions of 10 to 15 ml. were collected.

Appearance of ureido compounds and dihydropyrimidines in the effluent fractions was determined by the colorimetric procedure of Kortiz and Cohen(43).

Enzyme Purification and Assay

Culture. Strains were grown under forced aeration at 27° C in 5 l. round-bottom flasks containing 3200 ml. of minimal media supplemented uridine or uracil. After 76 or 96 hr. incubation, the mycelia were harvested, washed once in distilled water(10°C), pressed dry, and immediately frozen at -20° C for not more than 6 hr. before preparation of acetone powders.

Preparation and Extraction of Acetone Powders. The method described below for the preparation and extraction of acetone powders is essentially as described by Wallach and Grisolia for calf liver dihydrouracil hydrase(81).

Frozen (-20°C) mycelia were homogenized for 2-3 minutes in a chilled Waring blendor with 5 vol. acetone(-20°C), filtered through a Buchner funnel, taken up again in 5 vol.(-20°C) acetone, and homogenized for 30 sec.. The resulting homogenate was filtered through a Buchner funnel with suction from a water pump. Just before the sediment became dry, a dental dam was stretched across the top of the funnel (to prevent denaturation by moisture) and suction was applied for 8 hr. with a vacuum pump. The dried cake was crushed gently, placed in a vacuum dessicator over calcium chloride, and evacuated 0.5-1 hr. with a vacuum pump. The powder was either extracted immediately, or stored overnight in the evacuated dessicator at -20°C. The powder was suspended in ice-cold distilled water or tris buffer(0.25M, pH9.0) in the proportion, 1 gm. of powder to 12 ml. liquid, gently stirred for 10 min. at 0-5°C, and centrifuged for 10 min. at 0-5°C at ca. 4500 x g. The supernatant was set aside at 0-5°C, the residue reextracted and centrifuged as above, and the supernatants combined.

Assay for Enzyme Activity. One ml. of trishydroxymethylaminomethane (Tris) buffer (0.25 M, pH 9.0) containing 50 uM of dihydrouracil was pipetted into duplicate or triplicate centrifuge tubes for 5 timed intervals for each

preparation, and placed in a 30° C water bath for at least 15 min. to equilibrate the temperature. The homogenates (freshly prepared) were brought to 30°C, and 1 ml. was rapidly pipetted into each tube. Five ml. of ice-cold 10 per cent perchloric acid was immediately added to zero time tubes. Subsequent time intervals were also precipitated by 5 ml. ice-cold 10 per cent perchloric acid. The reactions mixtures were incubated at 30°C in a water bath. Protein was removed by centrifugation at ca. 4500 x g for 10 min. at 5°C. Protein was assayed by the method of Lowry(53) with human serum standards previously assayed by the biuret method(72).

Reaction mixtures were assayed by the colorimetric method of Archibald(1) for the formation of β-ureidopropionic acid as follows. To 4 ml. of reaction mixture, 2 ml. H_2SO_4-H_3PO_4 (3:1 v/v) mixture and 0.25 ml. aqueous (3 per cent) diacetylmonoxime were added. The tubes were shaken, capped with glass marbles, and heated in a boiling water bath for 15 min. (light was excluded from the tubes during heating, and thereafter). The 15 min. period of heating, rather than 10 min. by the original procedure, gave lower blank values with dihydrouracil as substrate(81). The tubes were removed after heating and placed in a cold water bath for at least 10 min.. Absorption was determined in the spectrophotometer at 490 mu against either zero reaction time or a solution containing 1 ml. substrate, 1 ml. distilled water, and 5 ml. 10 per cent $HClO_4$ for 100 per cent transmission adjustments. Authentic β-ureidopropionic acid was used for the concentration standards.

EXPERIMENTAL

Spectrophotometric Analyses of Culture Filtrates of Pyrimidine Mutants

Experiment 1. Strains were grown 72 hr. at 27°C on 2 mM eq. of uridine and the culture filtrates examined with the ultraviolet spectrophotometer for absorption. The wavelengths of maximum absorption of the pH (4.5) of the culture filtrate for orotic acid and uridine were found to be 280 and 260 mu, respectively. Minimal media was used as a blank for 100 per cent transmission adjustments. The optical densities of the cultural filtrates are shown in Table 3.[1]

Experiment 2. The method of binary spectrophotometric analysis was employed in this experiment(51). Samples of known concentrations of uridine, orotic acid, and equimolar mixtures of the two were made up in HCl (pH 4.0). The extinction coefficients of each of the pure components was determined at 260 mu and 277 mu. Analysis of the known mixtures at the two wavelengths revealed less than 5 per cent error in the determination of orotic acid concentration in the presence of uridine. Table 4 shows the results of a typical binary analysis of culture filtrates.

Paper Chromatography of Culture Filtrates and Mycelial Extracts

Experiment 3. The strains were grown under forced aereation for 72 hr. at 27°C in 5 l., round-bottom flasks containing 3 l. of minimal medium, supplemented with 22.4 mg. per cent of uridine where necessary.

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[1]All tables are in the Appendix.

Mycelia were harvested with cheesecloth and the media were filtered on a Buchner funnel. One l. of filtered medium of each strain was lyophilized to dryness and the residue partially dissolved in 25 ml. of hot distilled water. Insoluble residue was removed by filtration through a Buchner funnel. Excess media salts were precipitated by allowing the filtrate to stand in the cold overnight. Three concentrations (5, 10, 15 ul.) of concentrated filtrate were applied to Whatman No. 1 filter paper in 5 ul. aliquots, partitioned in water-saturated phenol solvent, and sprayed to detect ureido and amino acids. Table 6 presents the average R_f values obtained in two independent trials.

Experiment 4. One l. of growth media, after filtration through cheesecloth, was filtered through a 2 in. layer of infusorial earth on a Buchner funnel. The filtrate was neutralized to pH 7.0, allowed to stand overnight in the cold, excess salts were filtered off, the filtrate lyophilized to dryness, and partially dissolved in 20 ml. of distilled water. Excess salts were centrifuged off, and 10 ul. were applied to Whatman No. 3 MM filter paper in 5 ul. aliquots. After partitioning the samples in butanol-acetic acid-water solvent, the papers were dried, examined under ultraviolet light, and sprayed for the detection of pyrimidine reduction products. The results are shown in Table 7.

Experiment 5. For each strain, duplicate 250 ml. Erlenmeyer flasks, initially containing 40 ml. of minimal media (supplemented with 20 mg. per cent uracil in some cases) were harvested by filtration at 24 hr. intervals. One set of duplicates for a given strain was incubated in standing cultures (non-aereated) and the other set was incubated on a rocking shaker (aereated). The mycelial pads were lyophilized or dried in an oven (40°C for 24 hr.). Duplicate culture filtrates were pooled, a 20 ml. aliquot was lyophilized, and the resulting residue was dried in an evacuated dessicator over calcium

chloride. The hydroscopic residue was pulverized and 1 ml. of distilled water was added. After heating the concentrated suspension in a 60°C water bath for 10 min. with agitation, the residue was allowed to settle and come to room temperature.

Routinely 10 ul. of the concentrated filtrate were applied in 5 ul. aliquots to Whatman No. 3 MM filter paper for chromatography. Distortion of the spots from the media concentrates in the butanol-acetic acid solvent, apparently due to complex formation with the media salts, made it necessary to correct the controls for this error. This was done by adding an equivalent concentration of concentrated minimal filtrate from wild type to the control samples at the origin before partitioning the chromatogram.

Mycelial extracts for chromatography were prepared by extracting mycelial pads in distilled water (four times the mycelial dry weight) in a 100°C water bath for 10 min.. Ten ul. of extract were routinely applied in 5 ul. aliquots to Whatman No. 3 MM filter paper.

Tables 8, 9, 10, 11, 12, show the results of chromatography in several solvents. The concentrations reported in Table 13 were estimated from the optical densities of the spots by the method of Fink, et al(25) with dihydroorotic acid and ureidosuccinic acid as concentration standards.

Experiment 6. Strains were cultured at 27°C in 250 ml. Erlenmeyer flasks containing 40 ml. of minimal media with 50 mg. per cent of orotic acid. Cultures were harvested at 24 hr. intervals after inoculation, 30 ml. of filtrate were lyophilized to dryness, and taken up in 1 ml. of distilled water for application to chromatograms. Ten ul. of concentrated filtrate from the inoculation time intervals, 48,72,96,120, and 144 hr., were applied to Whatman No. 3 MM paper and partitioned in butanol-acetic acid-water solvent. The spray techniques for pyrimidine reduction products were then employed.

Ion Exchange Analyses of Culture Filtrates and Mycelial Extracts

Experiment 7. The mutant, pyr-1, was grown at 27°C for 56 hr. under forced aeration in 10 l. of minimal media supplemented with 4 mg.-per cent uridine. Mycelia were harvested by cheesecloth filtration, and the culture filtrate set aside at 0°C.

Extraction and analysis of mycelia. Mycelia were washed twice with minimal medium and pressed dry(moist weight, 70 g.). After shredding and suspending the mycelia in 140 ml. of boiling water, they were heated in a 100°C water bath for 10 min., filtered, adjusted to pH 7.0(initial pH 6.3) with 1 M KOH (brom thymol blue was used as an internal indicator), and stored in the frozen state. The extract contained 20.3 mg. of chromogenic material expressed as ureidosuccinic acid when assayed by the colorimetric procedure of Koritz and Cohen(43).

A column of Dowex 1-X10(200-400 mesh, formate form, height 7.5 cm., diameter 1.0 cm.) was prepared. The mycelial extract was brought to room temperature, and passed slowly through the column. After washing the column with 200 ml. of distilled water, it was eluted with 0.055 M sodium formate buffer (adjusted to pH 3.2 with formic acid) at a flow rate of 1.0-1.5 ml. per minute. The effluent fractions were collected by hand in 10 ml. portions.

Ten ul. samples of each fraction were applied to Whatman No. 3 MM filter paper, dried, and sprayed for the detection of dihydropyrimidines. Chromogenic yellow material was found in fractions 2 through 21 (3 to 28 resin bed volumes (RBV)), with maximum concentration appearing in fractions 14 through 17 (18-22 RBV), as estimated visually.

The fractions were pooled into two groups: pool 1 contained fractions 14, 15, 16, 17 and pool 2 contained the remaining chromogenic fractions (1-17 and 23-35 RBV). Pool 1 was concentrated *in vacuo* at 85°C to 4 ml., streaked on the large end of 6 Whatman No. 1 sheets, and partitioned in the n-butanol-acetic-water solvent. After partitioning and drying, vertical, 2 cm. strips from the center of each sheet were sprayed for the detection of dihydropyrimidines as a guide for the location of bands on the remainder of the sheet. Only one band was detected with Rf 0.34; dihydroorotic acid, Rf 0.35. Horizontal strips from each sheet containing the bands were eluted by descending technique for 36 hr. with distilled water. After concentrating the eluant *in vacuo* at 60°C to 4 ml., 4 ml. of methanol were added, and the mixture allowed to stand overnight at 0°C. The sparse crystals were dried and taken up in 1 ml. of distilled water.

Pool 2 was passed through a column of Dowex 50-X12(200-400 mesh, H-ion form, 10 cm. by 1.0 cm.) to remove sodium ions and the effluent concentrated by lyophilization to dryness. A sample of the meager residue was taken up in 1 ml. of distilled water.

Neither pool 1 nor pool 2 contained sufficient material for organic analysis after purification, therefore they were examined by paper chromatography. Table 14 shows the results.

Analysis of Culture Filtrate-Run No. 1. Two liters of culture filtrate were brought to room temperature, adjusted to pH 7 with 1 M KOH, and filtered through a Buchner funnel to remove mycelial fragments and precipitated salts.

The neutralized, filtered medium was passed through a Dowex 1-X10(200-400 mesh, formate form, 25 cm. by 1 cm.) at a rate of 5 ml. per minute. After washing the column with 500 ml. of distilled water, it was eluted with formate buffer at a flow rate of 5 ml. per minute. The effluent front was collected by hand in 50 ml. fractions.

The fractions were analyzed by the colorimetric procedure of Koritz and Cohen(43). Absorption at 235 mu (near the absorption maximum of dihydropyrimidines) were also determined.

Fractions 7, 8, 9, 10, 11 (14-22 RBV), which gave a maximum absorption at 235 mu and also by the colorimetric method, were pooled (pool 1) and all remaining fractions were pooled (pool 2). The pooled fractions were passed through Dowex 50-X12 columns (200-400 mesh, H-ion form, 10 x 1 cm.) to remove sodium ions, and then lyophilized to dryness. The residues were taken up in 1 ml. of distilled water and chromatogramed by standard procedure. Table 15 summarizes the results of this experiment.

Analysis of Culture Filtrate-Run No. 2. In run no. 1, evidence was obtained for dihydroorotic acid; however, ureidosuccinic acid was undetected. This was attributed to insufficient quantity of growth media; therefore, a larger quantity was analyzed.

Four liters of culture filtrate were brought to room temperature, neutralized to pH 7 with 1 M KOH, and the resulting precipitate together with mycelial fragments was removed by filtration.

The neutralized, filtered medium was passed through a Dowex 1 column as in run no. 1. The column was washed with 200 ml. of distilled water, and eluted with formate buffer at a flow rate of 3.5 ml. per minute. The effluent front was collected in 25 ml. fractions, assayed for absorption at 235 mu and 260 mu against formate buffer, and analyzed colorimetrically by the method of Koritz and Cohen (43). The effluent front pattern obtained by the colorimetric analysis is illustrated in Plate VI.

The two elution peaks, corresponding to dihydroorotic acid (fractions 5 through 31), and ureidosuccinic acid (fractions 33 through 41) were separately pooled, and sodium ions, formic acid and water removed as in run no. 1.

The residues were taken up in 1 ml. of distilled water and chromatogramed by routine procedure. The results of chromatography are presented in Table 16.

Control Columns. Control columns, by the conditions of these experiments, yielded the following effluent front pattern, expressed in resin bed volumes required for elution: 5-carboxymethyhydantoin, 6 to 12; dihydroorotic acid, 13 to 20; and ureidosuccinic acid, 25 to 50(47). Campbell(8) found that β-ureidopropionic acid appeared in 25 to 31 resin bed volumes.

Experiment 8. Pyr-1 was grown 96 hr. under sterile, forced aeration at 27°C in 20 l. of minimal medium containing 2.5 uM eq. of uridine. 145 gm. of moist mycelia were harvested, immediately suspended in 280 ml. of boiling distilled water, and boiled 10 min.. The hot extract was filtered through a Buchner funnel, and the residue again suspended in 100 ml. boiling distilled water, boiled 10 min., and allowed to stand 3 hr. before filtering. The filtrates were then pooled.

Five l. of culture filtrate were filtered through infusorial earth, neutralized to pH 7 with 1 M KOH, and stored at 0°C until further treatment. After thawing and filtering off precipitated salts, the filtrate was lyophilized to dryness. The dried residue was suspended in 20 ml. of distilled water, heated at 60°C for 10 min., cooled to room temperature, and excess salts were centrifuged down.

The mycelial extract and filtrate concentrate were passed through Dowex 1 columns (15 cm. x 1 cm.) at absorption flow rate of 5 ml. per min.. After the columns were washed with 1 l. of distilled water, they were eluted with formate buffer in 25 ml. fractions.

After the effluent fractions were assayed colorimetrically by the method of Koritz and Cohen(43), and for alkaline degradation at 235 mu for dihydropyrimidines by the method of Yates and Pardee(85), the appropriate fractions

were pooled, passed through Dowex 50 columns, lyophilized to dryness, taken up in 2 ml. of distilled water, and chromatogramed. The effluent front patterns of the culture filtrate and the chromatographic results are shown in Plate VII and Table 17, respectively.

Fraction IA (Table 17) was streaked on the large end of Whatman No. 3 MM filter paper, developed in the butanol-acetic acid-water solvent, and a vertical 2 cm. strip was cut out for spraying to locate the dihydroorotic acid bands. That the fraction was not pure is shown in Table 18. Horizontal bands, corresponding to dihydroorotic acid, was excised from the sheets, and eluted in KOH (pH 12.0). The ultraviolet spectra of the sample, and authentic dihydroorotic acid in KOH (pH 12.0) similarly eluted, were determined immediately after elution. A blank strip of similar area from the chromatogram was also eluted in KOH at the same pH for 100 per cent transmission adjustments. The spectra are shown in Plate VIII. No absorption peaks were observed at pH 7.0 and pH 2.0 for either dihydroorotic acid or the sample.

Fraction IB (Table 17) was purified on chromatograms as was done for fraction IA, and bands corresponding to ureidosuccinic acid were eluted, concentrated, and recrystallized as described in experiment 7. The crystals gave a melting point of 173-176°C; previously reported, 171-172°C(4), 178-180°C(66). The infrared spectrum of these crystals has yet to be determined at this writing.

Experiment 9. Mutant 28-III was cultured with 2.5 uM eq. of uridine, extracted, purified on Dowex 1 and Dowex 50, purified on chromatograms, and eluted as described in experiment 8 for pyr-1. The spectrum of the eluted, purified fraction that occurred in 12-25 REV and gave an appropriate R_f value for dihydroorotic acid in butanol-acetic solvent, is shown in Plate VIII.

Enzyme Studies

Experiment 10. Pyr-1 and 74A were grown for 76 hr. on medium supplemented with 5 mg. per cent uridine under the conditions described in the "Materials and Methods" section of this paper.

Ten gm. of frozen mycelia of pyr-1 and 17 gm. of 74A were converted to acetone powders, extracted, and assayed for dihydrouracil hydrase activity as described in the "Materials and Methods."

The results of the colorimetric assay for the formation of β-ureidopropionic acid are shown in Table 19.

Experiment 11. Strains were cultured 96 hr. with 11.2 mg. per cent uracil (U) supplement, or minimal medium alone (M), harvested, converted to acetone powders, extracted, and assayed as described in "Materials and Methods." Extracts were made on the basis of 24 ml. Tris buffer per gm. of acetone powder. The quantities of starting material were (in frozen weight): pyr-1, 7.5 gm.; 73aU, 25.5 gm.; and 73aM, 23.5 gm.

In this experiment, the controls, substrate plus buffer alone, and homogenate plus buffer alone were assayed for endogenous activity. Neither control was found to produce significant increase in chromogene (Table 20).

The reaction mixture series and controls were chromatogramed in the butanol-acetic acid-water solvent, examined under ultraviolet light for the formation of uracil, treated with nihydrin for the location of β-alanine, and treated with NaOH and PDAB for the detection of substrate and β-ureidopropionic acid.

The product, β-ureidopropionic acid, was further characterized by purification on an anion exchange column. Duplicate tubes from 73aM at the reaction

times, 120 and 180 min. (calculated to contain 3.7 mg. BUP) were pooled, adjusted to pH 7 with 1M KOH, the potassium chlorate salts precipitated at 0°C, and the mixture was filtered. The mixture was passed through a Dowex 1-X10 (200-400 mesh, formate form 7.5 x 1 cm.) column, eluted with formate buffer (pH 3.2) at a flow rate of 0.8-1.0 ml. per min., and collected in 10 ml. fractions for a total of 300 ml.. The fractions were assayed by the Archibald method (1). The chromogene was found to appear in the effluent at 26-28 RBV, the appropriate fractions for β-ureidopropionic acid(8). The results of this experiment are presented in Tables 20 and 21, and Plate IX.

Experiment 12. Strains were cultured 96 hr. on 11.2 mg. per cent uracil supplement(U) (or minimal medium(M)), converted to acetone powders, extracted with 24 ml. ice-cold distilled water per gm. of powder, and assayed for dihydrouracil hydrase activity as described in the "Materials and Methods" section. The quantities of frozen mycelia recovered were: 73aU-1, 25 gm.; 73aU-2, 25 gm.; and pyr-3, 29.5 gm. 73aU-1 and 73aU-2 were duplicated cultures.

In this experiment, protein was assayed by the method of Lowry(53), with human serum standards whose protein concentration was estimated by the biuret method(72).

An attempt was made to purify the extract of 73aU-1 by the method of Wallach and Grisolia(81) for dihydrouracil hydrase of calf liver. The extract 73aU-1 was stored 4 days at -20°C after the comparative assay before the purification procedure was initiated.

The results of the comparative assay and the purification procedure are presented in Tables 22, 23, 24, 25 and illustrated in Plate X.

Experiment 13. In this experiment, strains were grown in medium with 11.2 mg. per cent uracil supplement (U) or in minimal medium (M), in 3 l. of

liquid with continuous aeration at 27°C. The mycelial pads were harvested and frozen immediately at -20°C for 2 hr. The yields were(in gm. frozen weight): 73aM, 21.8; 73aU, 25; pyr-3, 19; and pyr-1, 3. The acetone powders were prepared as described in "Materials and Methods", and extracted in 24 ml. ice-cold distilled water per gm. of powder. After the low-speed centrifugation step, the supernatants were cooled to 0°C, and centrifuged at 10,000 x g for 30 min. in a Servall vacuum centrifuge in which the rotor had previously been cooled to 0°C. The supernatants were stored at -20°C for 6 hr., thawed to 30°C, and assayed for dihydrouracil hydrase activity as described previously. The results of this experiment are summarized in Tables 26 and 27, and Plate XI.

Growth Responses of Pyrimidine Mutants

Experiment 14. The experimental procedures for determining the growth response of mutants are outlined in the "Materials and Methods" section of this paper. In addition, it should be mentioned that whenever growth response was obtained with a new supplement, subsequent transfer of the mycelia to minimal medium revealed that the response was not due to the selection of wild-type nuclei or suppressor genes. The results of the experiment are presented in Tables 28 and 29.

RESULTS

Spectrophotometric Analyses of Culture Filtrates of Pyrimidine Mutants

In initial growth experiments, it was found that only one KS mutant, KS-1, responded to orotic acid (Table 1). This observation suggested a

metabolic block after orotic acid for the majority of the mutants, as in pyr-2 and pyr-4, and that orotic acid might accumulate in the growth media. A determination of the ultraviolet absorption of the mutant culture filtrates revealed that only pyr-2 and pyr-4 accumulated large amounts of ultraviolet-absorbing material (Table 3). The spectrum of pyr-2 culture filtrate after diluting 1:15 with KOH (pH 12.0) gave: λmax., 286 mu; λmin., 248 mu. By using the reported extinction coefficient of orotic acid at pH 12.0, 7.52×10^{-3}(66), it was calculated that the original media of pyr-2 contained 0.37 mg. of orotic acid per ml..

The small absorption values at 280 mu in the culture filtrates of mutants other than pyr-2 and pyr-4 might be attributed to either the uridine supplement only, or to the resultant of uridine supplement plus a small amount of orotic acid. The method of binary analysis was employed to distinguish between these two alternatives.

Table 4 shows the results of a typical binary analysis of culture filtrates. The calculated values for orotic acid concentration in the filtrates of the KS-mutants were only about 0.1% of the concentration in the pyr-2 filtrate. Possibly these trace values were due to small quantities of protein that escaped filtration since protein with aromatic amino acids absorbs in the region of 275-280 mu. Indeed, Kalckar(40) has reported a similar method for estimation of protein in the presence of nucleic acid.

The filtrate of pyr-2 contained 0.37 mg. of orotic acid per ml. by the method of experiment 1; the same sample in experiment 2 was estimated to contain 0.1 mg. per ml.. The lower value by binary analysis was expected since the calculations contained a correction for uridine absorption.

Subsequent growth studies revealed that the failure of certain KS-mutants

to respond to orotic acid, although they apparently did not accumulate orotic acid, was in some cases, a function of the concentration of the supplemented orotic acid and the time interval of incubation. In other cases, it appeared that non-specific radiation damage elicited these responses, since some of the progeny of backcross generations responded to orotic acid. Table 2 illustrates the responses of the "non-utilizers" of orotic acid to 10 mg. per cent of that compound for the UV-1 and backcross generations.

Further evidence that some of these mutants were not of the pyr-2 or pyr-4 types was obtained from the positive growth responses to orotidine (Table 5).

Finally, Suyama has shown (76) by genetic tests that the major locus of these mutants (both UV-1 and backcross generations) was allelic or very closely linked to the pyr-3 locus.

An interesting side-light which appeared in these experiments was the positive growth responses of most of the mutants to deoxyuridine and deoxycytidine (Table 1). Apparently, Neurospora possesses enzymic mechanisms for the conversion of these deoxynucleosides to the corresponding ribosides. This observation is in agreement with the findings in other organisms that these deoxy- and ribonucleotides are interconvertible(44).

Paper Chromatographic Studies of Accumulated Pyrimidine Intermediates

Recent work by others has revealed that in bacterial and mammalian systems, the pyrimidines may be either synthesized or reduced via the corresponding dihydropyrimidines, ureido acids, and β-amino acids. (For a review of dihydropyrimidine metabolism, see Carter(12); Plate III. The seeming

biochemical universality of those reactions prompted the examination of the culture filtrates of pyrimidine mutants and wild type for reduction products of orotic acid and uracil that might accumulate from catabolism or biosynthesis.

From the hypothesis that pyrimidine metabolism of Neurospora is analogous to that of bacteria and mammals, it follows that mutants pyr-2 and pyr-4, which accumulate orotic acid, should also accumulate dihydroorotic and ureidosuccinic acid. Not only were these expectations verified, but also a new metabolic pathway in Neurospora was indicated that involved the interconversions of uracil, dihydrouracil, β-ureidopropionic acid and β-alanine.

The results of experiment 3 (Table 6) suggested that wild type and the mutants, KS-5 and pyr-3, when grown on uridine, accumulated β-ureidopropionic acid, β-alanine, and dihydrouracil, in addition to an unknown compound that was reactive to both ninhydrin and PDAB. Since the compounds were not detected in the filtrate of wild type when grown on minimal medium, it was assumed that they arose from the catabolism of uridine; however, in the mutants, the intermediates could have accumulated as a result of a shift of the catabolic pathway toward synthesis with subsequent accumulation resulting from a metabolic block in that pathway. This problem will be discussed later in this paper in light of growth responses of pyrimidine mutants to the uracil pathway intermediates.

If the uracil reductive pathway is operative in Neurospora, it should be possible to demonstrate the reductive intermediates in the culture filtrate when supplemented with uracil as well as with uridine. Furthermore, these intermediates should give appropriate R_f values in a solvent other than phenol. These expectations were verified in experiment 4.

The results of experiment 4 (Table 7) suggested the accumulation of

dihydrouracil by the mutants and by wild type when grown in uracil supplemented medium, but not by wild type grown in minimal. The apparent absence of β-ureidopropionic acid in this experiment might have been a function of the incubation period. (In this experiment, the cultures were incubated 24 hr. longer than in experiment 3.) This view was supported by the observation that pyr-3 had detectable amounts of uracil and dihydrouracil in the filtrate at 96 hr. but not at 120 hr.

A new spot was observed in this experiment from the filtrate of pyr-1 with the R_f value of dihydrcorotic acid. Three unknowns were observed, but were apparently not unique to the mutants or wild-type grown in uracil medium since they were also observed in the filtrate of wild-type when grown in minimal. Possibly the ultraviolet-absorbing material at the origin was nucleic acid or nucleotide(s), since in other experiments, it was observed that this solvent does not move those compounds. These compounds could have arisen from cell lysis, since, under aeration, a voluminous, white froth appeared on the culture medium surface in 72-96 hr., suggesting the denaturation of proteins.

In a comparative study of accumulation products of mutants, it appeared that the presence or absence of a given compound could be a function of the limits of detection of the technique, the supplement added and its concentration, the phase of growth examined, and whether mycelia or growth media were examined. Experiment 5 was designed to circumvent some of these difficulties.

Two new mutants were introduced in experiment 5. Mutants 28-III and 6-III(lg) were derived from the double mutant, KS-12. The genetics and physiological characteristic of these mutants will be described later in this paper.

In general, when a compound was found in the culture filtrate, it was also found in the mycelia. No compound was found under aereated conditions

that was not present under non-aereated conditions, considering all time intervals of incubation.

Table 8 shows the results of chromatography of mycelial extracts and concentrated culture filtrates in butanol-acetic acid-water solvent. An unknown spot with R_f 0.16 was observed in all mycelia and growth media. This substance, or another substance with the same R_f value, was also ninhydrin positive. This was probably the same unknown observed in the phenol solvent of experiment 3. The spots with R_f 0.35 suggested dihydroorotic acid. This view was supported by the fact that these spots were absent if the sodium hydroxide spray was omitted prior to spraying with PDAB. Spots with R_f 0.41 that reacted with PDAB alone were identified as ureidosuccinic acid. That they were not citrulline was evidenced by the absence of ninhydrin-positive material with R_f 0.41 (Table 9). The R_f values of 0.57 and 0.62 were identified as dihydrouracil and β-ureidopropionic acid since they also gave appropriate behavior to the PDAB spray with and without prior alkaline treatment. Urea gave a similar R_f value to β-ureidopropionic acid in this solvent, but urea was not found in pyr-3 or 73aU filtrates in the phenol solvent of experiment 3.

Table 9 presents the results of treatment of the filter papers with ninhydrin reagent. These were the only ninhydrin-positive substances found in the media and the major ninhydrin-positive substances in the mycelia. The substance with R_f 0.51, in addition to giving the appropriate lilac color of β-alanine was identical to the R_f value of β-alanine. The additional observation of β-alanine in the phenol solvent of experiment 3 supports the view that Neurospora catabolizes uracil or uridine via dihydrouracil and β-ureidopropionic acid to β-alanine.

Table 10 presents the R_f values of ultraviolet-absorbing materials found

in the mycelia and concentrated media after chromatography in n-butanol-acetic acid-water solvent. As might be expected, the uracil supplement was found in the mycelia and filtrate at all time intervals. Wild-type and 6-III grown on minimal were also observed to contain traces of uracil in their mycelia in increasing amounts from 72 to 120 hr. That wild-type also accumulated traces of uracil in its growth medium when grown in minimal medium has been suggested by the cross-feeding and spectrophotometric experiments by Woodward(83). A compound with an R_f value of uridine was observed in the mycelia of all mutants, 73a, and 6-III when grown with uracil supplement, at the 72 hr. incubation period. Strongly-absorbing spots with R_f values of orotidine and orotic acid were observed in both the media and mycelia of pyr-4 and pyr-2 at 96 and 120 hr. incubation periods, but were not detected in the media and mycelia of the other mutants or wild-type. The accumulation of orotic acid and orotidine by pyr-2 and pyr-4 was in agreement with the spectrophotometric studies in experiment 1 and 2 and the report of Mitchell, et al(60).

Table 11 shows the results of chromatography of concentrated culture filtrates in the lower phase of a phenol-water mixture. Additional evidence was obtained for dihydrouracil, ureidosuccinic acid, and dihydroorotic acid; however, the pair, β-ureidopropionic acid and urea were still ambiguous. Strong background absorption in the phenol solvent prevented the detection of pyrimidines by the ultraviolet lamp.

Table 12 represents the R_f values obtained by chromatography of concentrated culture filtrates in the mixture, isoamyl alcohol-pyridine-water. In this solvent, clear evidence was seen for ureidosuccinic acid, dihydroorotic acid, β-ureidopropionic acid, and dihydrouracil. The intense background absorption of the pyridine in this solvent precluded the observance of ultraviolet-absorbing components.

An attempt was made to determine the relative concentrations of dihydroorotic acid and ureidosuccinic acid occurring in the growth media. The optical densities of chromatogram spots of a concentration series of dihydroorotic acid and ureidosuccinic acid were determined and the concentrations of the sample spots also estimated from their optical densities in the Beckman spectrophotometer according to the methods of Fink, et al.(25). Table 13 presents the results of these estimates. Although estimates were not made of the variance of the measurements (Fink, et al., report 20 per cent variance), it was clear that the presence or absence of an accumulate and its concentration were a function of the growth phase examined. Similar observations were made from the mycelial extracts although quantitative measurements were not made. In general, the pattern of accumulation increased to 96 hr. and declined thereafter. If one examines the plots of mycelial dry weight versus incubation period (Plates IV and V), as a first approximation, 96 hr. was the peak of logarithmic growth, and thereafter was the stationary or decline phase. A reasonable explanation of this phenomenon can be made on the basis of the work of Strauss(73), who showed that Neurospora, in a similar minimal medium, depleted the exogenous nitrogen source in a 96 hr. growth period. Thus, any accumulated nitrogenous compounds could be expected to be depleted as the peak of exponential growth was approached and thereafter.

Evidence has been presented to support the hypothesis that the metabolic blocks in the mutants pyr-1, pyr-2, pyr-4 28-III, and KS-12 were in the pathway of orotic acid synthesis; however, the metabolic aberrancy(ies) of pyr-3 and 6-III were unknown. Neither of these mutants accumulated detectable amounts of the intermediates in the orotic acid pathway, yet they utilized only orotic acid and not DL-ureidosuccinic acid, DL-5-carboxymethylhydantoin, or

L-dihydroorotic acid(83).[1] Possibly these compounds were impermeable, as was the case for ureidosuccinic acid in Ehrlich ascites tumors(45), or the enzymes for their utilization require some sort of long-term adaptation. Erratic, irregular growth has been observed with these compounds among replicate flasks by pyr-3-like strains(83). In experiment 14, the growth stimulation of a pyr- mutant by L-dihydroorotic acid is presented. At any rate, in this experiment, it seemed worthwhile to test for the presence of dihydroorotic dehydrogenase and dihydroorotase by the relatively simple techniques established in previous experiments.

If mutants pyr-3 and 6-III, as well as wild-type, were grown on a large concentration of orotic acid, it seemed that, if the necessary enzymes were present, orotic acid would be catabolized to its precursors which would tend to accumulate in front of the metabolic block until orotic acid approached depletion. Furthermore, it seemed that dihydrouracil and β-ureidopropionic acid could also arise by the decarboxylation of the corresponding accumulated analog in the orotic acid pathway; therefore, mutants 28-III and pyr-1 were grown with orotic acid.

In experiment 6, mutants pyr-1, 28-III, pyr-3, 6-III, and wild-type 73a were grown with 50 mg. per cent orotic acid, harvested at 24 hr. intervals from 72 to 144 hr., the culture filtrates lyophilized, and chromatogramed for the detection of pyrimidine reduction products. The spray techniques revealed only two compounds for each of the mutants, pyr-1 and 28-III, that corresponded to dihydroorotic acid and ureidosuccinic acid. Mutants pyr-3, 6-III, and wild-type 73a failed to accumulate detectable quantities of these compounds. In no case, were dihydrouracil or β-ureidopropionic acid observed. We cannot

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[1] Experiments conducted after this thesis was written indicated pry-3 will respond to L-dihydroorotic acid. See also the section of this thesis entitled "Growth Responses of Pyrimidine Mutants."

conclude from these results that pyr-3, 6-III, and 73a lacked enzymes for reducing orotic acid, particularly since Mitchell[1] reported the presence of these enzymes in Neurospora. The apparent absence of dihydrouracil and β-ureidopropionic acid strengthened the view that these compounds arose from the reduction of uracil, rather than by decarboxylation of the corresponding analog of the orotic acid pathway, or by reversal of the uracil reductive pathway.

Ion Exchange Analysis of Culture Filtrates and Mycelial Extracts

The ultimate goal in the study of accumulation products is their isolation in pure, crystalline form with subsequent identification by organic and physical techniques. Ion exchange analysis offers a convenient means for identifying and resolving complex chemical mixtures.

In the analysis of the mycelial extract of pyr-1(exp. 7), an effluent band was found to appear in the appropriate fractions for dihydroorotic acid(18-22 RBV) that gave R_f values in two solvents to indicate dihydroorotic acid (Table 14). In addition, paper chromatography revealed dihydrouracil in both fraction pools. Since dihydrouracil is not absorbed specifically on a formate column, it is to be expected that it should occur throughout the column, if not completely washed out.

In experiment 7, run no. 1, the effluent fractions from a Dowex-1 column purification of 2 l. of pyr-1 culture filtrate revealed a chromogenic band in the appropriate fractions (14-22 RBV) for dihydroorotic acid whose identity was confirmed by paper chromatography (Table 15.).

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[1]Personal communication.

Run no. 2 of experiment 7, in which 4 l. of culture filtrate of pyr-1 were purified on a Dowex-1 column, elution peaks were obtained that corresponded to dihydroorotic acid(5-31 REV) and ureidosuccinic acid(33-41 REV). (See elution pattern in Plate VI.) The identity of these compounds, in addition to dihydrouracil, was confirmed by paper chromatography (Table 16).

In experiment 8, in which twice as much mycelial growth was extracted than in experiment 7, not only was an effluent peak(12-23 REV) corresponding to dihydroorotic acid, but also effluent peaks corresponding to β-ureidopropionic acid and ureidosuccinic acid were observed. Paper chromatography affirmed the identity of these compounds in addition to dihydrouracil (Table 17). Further purification of fraction IA (Table 18) followed by elution of the band corresponding to dihydroorotic acid and determination of the ultraviolet spectra (Plate VIII) gave further proof that the sample was indeed dihydroorotic acid. The ultraviolet spectra were in agreement with those reported for dihydroorotic acid by Batt, et al.(4).

Analysis of a culture filtrate of pyr-1 by anion exchange columns also revealed the presence of dihydroorotic acid, ureidosuccinic acid, and β-ureidopropionic acid in the appropriate effluent bands (Plate VII). The presence of these compounds, in addition to dihydrouracil, was confirmed by paper chromatography (Table 17).

In experiment 9, a fraction was isolated from the mycelia of mutant 28-III in the appropriate effluent band for dihydroorotic acid that gave the appropriate R_f value and appropriate ultraviolet spectra for dihydroorotic acid (Plate VIII).

Attempts were unsuccessful in these experiments in obtaining sufficient quantities of all the desired compounds in pure, crystalline form for further

analysis. That goal could be accomplished, however, if at least four modifications in technique were made: 1) replace the ammonium tartrate in the growth medium with an ammonium salt of an acid that does not have a carboxyl group to be exchanged on the formate column, 2) "scale up" the column, e.g., prepare columns of greater height and diameter, or else pool the appropriate effluent bands from several columns, 3) begin with larger quantities of material, and 4) repurify the effluent front bands on fresh formate columns (after removing sodium ions and formic acid), and/or repurify with columns of filter paper disks.

Enzyme Studies

Although the molecular structures and primary products of genes remain to be clarified, there is little doubt that the physiological effects of genes are mediated via enzymes. Genes, individually and in concert, control the rates of step reactions in multi-enzyme systems, and thereby determine the metabolic pathways. In many instances gene mutation causes the block, complete or incomplete, of a step reaction; the consequence of such a block is the accumulation of precursors and the appearance of a growth requirement for the product of the blocked reaction.

When the routes of biosynthesis have been traced through a study of the growth requirements induced, and by the identification of precursors accumulated in a particular class of mutants, additional proof of the metabolic sequences and gene-enzyme relations can be obtained by the study of the isolated enzymes in vitro. Finally, if the mechanism(s) of gene action is to be defined, specific alterations in structure and function of a given enzyme should be related to a specific gene mutation. This final goal awaits refinements in

sequential analysis of proteins, the definition of the mechanism(s) of enzyme function, and the definition of protein biosynthetic mechanisms.

The enzyme, dihydrouracil hydrase, was chosen in these studies for two reasons: 1) to obtain additional verification of the uracil reductive pathway in Neurospora, and 2) because of the relatively simple assay technique. Unfortunately, mutant 28-III was not available when these studies were made, for it would have been of interest to compare the specific activities of 28-III and pyr-1 for dihydrouracil hydrase. These mutants exhibited differential growth responses to β-ureidopropionic acid. Mutant 28-III grew on dihydrouracil but not on β-ureidopropionic acid, whereas the growth of pyr-1 was stimulated by both compounds.[1]

In experiments 10 through 13, the evidence appeared ample that dihydrouracil was hydrolyzed to β-ureidopropionic acid in acetone powder extracts of wild-type 73a and in mutants pyr-3 and pyr-1.

If one examines the results of all four experiments, it may be seen that much variability in the extent and nature of the reactions was obtained. Possibly variables in the physiological states of the strains and uncontrolled variables in the isolation and assay procedure account for the differences between experiments. At any rate, before further comparative studies are made, the variations in specific activity should be defined for at least such variables as: 1) the period of incubation, 2) the nature and concentration of supplemented pyrimidine, and 3) the temperature of incubation. That the physiological state of a strain influences the specific activity of particular

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[1]See the section of this thesis entitled, "Growth Responses of Pyrimidine Mutants."

enzymes has been noted by Wagner(79) with Neurospora and by Yates and Pardee (85) with E. coli.

Nevertheless, in spite of the variability between experiments, certain generalities can be made. In the extracts of 73aU, most of the reaction occurred in the first 15 min. of incubation. In future experiments, extracts of 73aU should be diluted to study the proportionality of enzyme to reaction rate. In experiment 11 (Table 9), 73aU exhibited the greatest reaction velocity, 73aM was intermediate, and pyr-1 was low. Although protein concentration was not estimated in experiment 11, approximate relative comparisons in activity can be made since the extracts were made on the basis of 24 ml. Tris buffer per gm. of acetone powder. That the product formed in these reactions was β-ureidopropionic acid was evidenced by paper chromatography of the reaction mixtures (Table 21), and by the appearance of a chromogene from Dowex-1 columns in the appropriate effluent band for β-ureidopropionic acid. Neither substrate plus buffer nor extract plus buffer gave a detectable reaction (Table 20).

In experiment 12, the reaction curves were quantitatively and qualitatively different from those of experiment 11 (Plate X). After 30 to 60 min. incubation, the product declined to zero in 180 min. instead of continuing to increase. The only known new variable in this experiment, as compared to experiment 11, was that the acetone powders were extracted in distilled water instead of Tris buffer as in experiment 11. These results are interpreted, from the kinetics of the reactions, to either represent multiple reactions of dihydrouracil hydrase and the β-ureidopropionic acid splitting enzyme, or action of dihydrouracil dehydrogenase to form uracil. (It should be recalled that the substrate is also chromogenic by the colorimetric method for assay

of β-ureidopropionic acid.) The complexity of amino acids and peptides in the extracts precluded the identification of β-alanine formation by paper chromatography. We cannot be certain whether uracil was formed in these reactions since paper chromatography revealed uracil both in the reaction mixtures and at zero reaction time (Table 21).

Tables 24 and 25 present the results of an attempt to purify the acetone powder extract of 73aU-1. The extract had been stored at -20°C after the comparative assay. Apparently storage in the cold activates the β-ureidopropionic splitting enzyme or dihydrouracil dehydrogenase because negative values were obtained in the 90 min. assay for all of the purification steps except the 2nd ammonium sulfate fraction in which a high positive activity was obtained. This fraction should contain dihydrouracil hydrase in highest activity according to Wallach and Grisolia(21).

It should be noted in experiment 12 that, on an equal protein basis, 73a exhibits greater dihydrouracil hydrase activity than pyr-3. This observation could be the result of: 1) different degrees of adaptation, 2) greater lability of dihydrouracil hydrase in pyr-3, 3) relatively higher activity of the β-ureidopropionic acid splitting enzyme in pyr-3, 4) the presence of inhibitors in pyr-3 extract, or 5) some combination of the above factors. It would seem premature to interpret the apparent low activity of pyr-3 and pyr-1 (experiment 11) in terms of gene-enzyme relations.

In experiment 13, (Tables 26 and 27, Plate XI) pry-3 and pyr-1 exhibited a low, but positive activity, whereas 73aU and 73aM gave negative optical density values at 490 mμ in the colorimetric assay. This experiment was essentially the same as experiment 12 with the exception that the extracts were stored at -20°C for 6 hr. instead of being immediately assayed as in experiment 12. Thus it appeared that the supposition made in experiment 12; e.g.

that storage in the cold activates either the β-ureidopropionic acid splitting enzyme or dihydrouracil dehydrogenase, was supported by these results.

Further studies of the enzymatic reactions of the uracil reductive pathway, as well as comparative assays of strains, are being planned.

Growth Responses of Pyrimidine Mutants-- Results and Discussion

The detection of dihydrouracil, β-ureidopropionic acid, and β-alanine in the culture filtrates of wild-type and the pyrimidine mutants when grown with uracil or uridine supplement suggested that growth responses might be obtained with these compounds in the absence of pyrimidines. In addition, the accumulation of ureidosuccinic acid and dihydroorotic acid in several mutants suggested that other mutants might yield growth responses to these compounds.

Table 29 presents the growth weights of certain mutants when cultured on the proposed pyrimidine intermediates. Mutants 6-III, 28-III, and pyr-1 have been found to be consistently stimulated by dihydrouracil. No other mutant, with the exception of KS-12, the double mutant of 6-III and 28-III, responded to dihydrouracil. KS-12, which does not yield traces of growth on minimal, yields about 30 mg. dry weight after 2 weeks culture with 10 mg. per cent dihydrouracil. Isolation of the resultant growth to minimal medium showed that no reversion to wild type or contamination had occurred. That the dihydrouracil was not contaminated with traces of uracil was evidenced by the lack of response of KS-5 (which responds to 0.1 mg. per cent uracil with no growth on minimal) to 10 mg. per cent dihydrouracil.

The observation that pyr-3 accumulated dihydrouracil, β-ureidopropionic acid, and β-alanine when grown on uracil or uridine and did not accumulate

dihydroorotic acid or ureidosuccinic acid suggested that growth response could be obtained on these substrates. This hypothesis has been partially verified by Woodward(83), however, the responses were of a peculiar type. Among triplicate flasks of pyr-3 inoculated in 10 mg.per cent of the substrates, except β-alanine, after preculture on uridine agar slants, highly variable growth was obtained after 96 hr., ranging from 0 to 60 mg. of dry weight, whereas no growth was obtained in minimal flasks. These observations suggest that adaptive enzyme or permeability phenomena may be involved.

That some type of adaptation was involved in the utilization of uracil and orotic acid has been observed by Woodward(83). Pyr-3 and certain KS-mutants were transferred serially on orotic acid and uracil agar slants for 5 conidial generations and were tested quantitatively after each transfer for growth responses to both compounds in liquid flasks. The results clearly indicated that preculture on uracil markedly enhanced the growth responses to uracil and decreased the responses to orotic acid, and conversely for preculture on orotic acid. Similar experiments are now underway to test for adaptation phenomena in the utilization of the precursors of uracil and orotic acid.

No pyrimidine mutant has exhibited growth stimulation with as much as 50 mg.per cent β-alanine. This observation could be predicted solely on the basis of the low growth efficiency of dihydrouracil and β-ureidopropionic acid.

Since Maas(54) has observed the coupling of β-alanine and pantoic acid to form pantothenic acid by an enzyme of E. coli, and Slotnick and Weinfeld(70) observed growth stimulation of pantothenate-requiring mutants of E. coli by β-alanine, β-ureidopropionic acid, and dihydrouracil, but not uracil, it would be interesting to obtain a series of pantothenate mutants in Neurospora to test the efficiency of the uracil reductive pathway as a source of β-alanine.

In addition, the E. coli mutants should prove useful for the bioassay of the uracil intermediates in Neurospora.

Growth response of 6-III and pyr-1 to β-ureidopropionic acid were only slightly greater than the growth on minimal. Woodward(83) has concluded from many tests that the response of pyr-1 to this compound was significantly greater than minimal medium, whereas that of 6-III was not. However, these tests were made after preculture on uridine. Experiments are now being planned to test the growth response of these mutants after preculture on uracil, dihydrouracil, and β-ureidopropionic acid with the hopes of elucidating possible adaptive mechanisms. That the enzymes of the uracil pathway were adaptive has been shown by Canellakis in rats(11) and by Campbell(8) in Clostridium uracilicum.

No mutant has yet been found that yielded significant growth response to L-dihydroorotic acid, DL-5-carboxymethylhydantoin, or DL-ureidosuccinic acid, with the exception of the partial mutant, substrain 3, derived from the cross KS 138-3-1a x KS 12-2-4A, which responded to L-dihydroorotic acid and was inhibited by DL-ureidosuccinic acid (Table 29).[1] The inhibition could be due to the presence of the D-form of ureidosuccinic acid. Mutant 6-III was inhibited by L-dihydroorotic acid, DL-ureidosuccinic acid (Table 28), as well as by DL-5-carboxymethylhydantoin and DL-aspartic acid(Woodward, 83). These observations are not interpretable except on the basis of inhibition by the D-form; however, the dihydroorotic acid was obtained as the L-form.

One could speculate that 6-III represented a partial metabolic block

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[1]Two other substrains, 11 and 18, derived from the above cross, have been isolated recently that yielded good response to L-dihydroorotic acid and DL-ureidosuccinic acid.

between dihydroorotic acid and uridine-5'-phosphate (accounting for the growth on minimal) which was not accelerated by the mass action effect when precursors were added, but rather the precursor inhibited other reactions; however, with such a metabolic lesion, one could expect to find some of the precursors accumulating, an expectation which was not verified in the accumulation experiments.

Another speculation to explain the inhibition of 6-III and the rare growth response of pyr-mutants to ureidosuccinic acid and dihydroorotic acid is that these compounds are not true biosynthetic intermediates in Neurospora, but that orotic acid formation is by closely related analogs to these compounds. Hypothetical reactions leading from argininosuccinic acid, an intermediate in the biosynthesis of arginine in Neurospora(23), via one of the two anhydrides of argininosuccinic acid, 2-ornithinyl-dihydroorotic acid, to form orotic acid may prove to be an alternate pathway for orotic acid synthesis. This point of view will be elaborated further in the discussion section of this paper.

A final, and more experimentally feasible, hypothesis to account for the almost complete absence of mutants responding to dihydroorotic acid and ureidosuccinic acid may be made on the basis of the findings of Yates and Pardee(86) with pyrimidine mutants of E. coli. They found that the formation of the three "constitutive" enzymes required for the formation of orotic acid was controlled by a mechanism of enzyme repression. End products of the metabolic pathway, derived from uracil, inhibited the formation of these enzymes. Pyrimidine starvation caused the specific activities of these enzymes to increase by one to two orders of magnitude. Furthermore, they found that the precursors of orotic acid were ineffective as inducers of these enzymes. Thus, if a similar situation exists in Neurospora, the routine preculture of mutants on uracil and uridine, as has been the practice in this laboratory,

would suppress the formation of enzymes for the conversion of ureidosuccinic acid and dihydroorotic acid to orotic acid. That the mycelia of the mutants contained uracil and uridine when cultured with uracil was shown in the accumulation experiments, and presumably the conidial inocula should also be rich in these compounds. Thus, transfer of these conidia to dihydroorotic acid or ureidosuccinic acid could not result in growth until the endogenous pyrimidine supply was depleted and the appropriate enzymes were formed. In addition, if permeability to these compounds was low, as was the case in E. coli, the growth rate might be slow until substrains with higher permeability could be selected.

Genetics of Pyrimidine Mutants-- Results and Discussion

Mr. Y. Suyama(76) has kindly permitted the author to include a summary of his current genetic data obtained from the pyrimidine mutants of Neurospora. The briefness of the following discussion is not meant to imply that the available information on the genetics of pyrimidine mutants is brief, but rather the purpose here is to present certain generalizations to orientate the reader to the over-all problem.

The order of the mutants, pyr-1, pyr-3a, and pyr-2, on the linkage map of group IV has been found to be the same as reported by Barratt, et al(3) (Fig. 2), although more precise values for the linkage distances have been found.

Two new loci have been obtained, 28-III and 6-III(lg). Mutant 28-III in crosses to pyr-1 and pyr-3 has been located about 0.05 map units from pyr-1. The close linkage of 28-III to pyr-1, together with the similar accumulation products and growth responses of each, lead to the tentative conclusion that

these mutants were pseudoallelic. Final proof of this hypothesis must, of course, await the demonstration that each mutant affects the same enzyme.

Mutant 6-III(lg), as well as 28-III, were derived from the double mutant KS-12. The lg-mutant (termed laggard) was characterized by a delayed lag in the growth 48 hr. longer than the lag of wild-type that is relieved by pyrimidine or dihydrouracil supplementation. This mutant has not been observed to be a temperature-sensitive type. Crosses of lg to pyr-3a or tryp-4 indicated lg was located 5 map units distal to pyr-3a. No pyr-locus has hitherto been reported in that region.

Among all of the KS-mutants tested to date, only KS-12 was not located at the pyr-3 locus. The pyr-3 locus appears to be a complex short region that can be sub-divided into at least 8 to 10 sites. Recombination values within this locus are on the order of 10^{-2} to 10^{-3} map units and are apparently additive between sites according to classical recombinational analyses of more widely separated hereditary units. Mitchell and Mitchell(63) studied a number of mutations at the pyr-3 locus, but found that although recombinational analysis was complicated by sterility and pseudo-wild formation, there was indication of pseudo-alleles or sites at the pyr-3 locus.

No mutants at the pyr-2 or pyr-4 loci have been obtained in these studies. That pyr-4 was not on the right arm of linkage group IV has been confirmed.

DISCUSSION AND CONCLUSIONS

An ironic truism in biochemical genetics is that to devise effective selection procedures to obtain an array of biochemical mutants of a particular metabolic system, one should first know the metabolic intermediates involved, their possible cross-inhibition relations and feed-back mechanism, and their permeability relations; yet that knowledge cannot be readily obtained until an array of mutants in that system are available!

In the mutation and selection experiments, over 100 independently isolated mutants have been obtained, but all of these tested to date, with the exception of KS-12, have been found to be mutations at the pyr-3 locus. KS-12 has been one of the more useful mutants in elucidating the metabolic relationships for it was a double mutant of 28-III and 6-III, both of which were new loci hitherto unreported in Neurospora. Apparently the pyr-3 locus is quite stable since preliminary experiments to obtain back mutations at that locus have been unsuccessful (Woodward, 83). Therefore, it is concluded that some factor or factors in the selection techniques favor the isolation of mutations at the pyr-3 locus. If these factors could be defined, the long sought goal of plant breeders and geneticists--to produce specific mutations at will--might be approached.

In the initial studies of accumulation products, pyrimidine mutants were found that would not utilize orotic acid but yet did not accumulate that compound. Crossing experiments revealed these mutants were at the pyr-3 locus (with the exception of KS-12); however, KS-1 and pyr-3 at the same locus did respond to orotic acid. Subsequent experiments revealed that most of the backcross progeny of the "non-utilizers" of orotic acid would respond to

orotic acid and that preculture on orotic acid agar slants enhanced that response. Nevertheless, the backcross progeny and strains precultured on orotic acid were still mutations at the pyr-3 locus. From these evidences it is concluded that the metabolic lesion affected by the pyr-3 locus does not involve the conversion of orotic acid to orotidine-5'-phosphate, but rather the anomalous effects were due to mutations at other loci, cytoplasmic damages, or permeability barriers. These experiments emphasize that caution should be taken in interpreting negative growth responses in terms of gene functions and metabolic reacions. Haskins and Mitchell(35) arrived at similar conclusions in the study of the tryptophan cycle in Neurospora. Their conclusion(62) was that "the assumption that the gene affected by the mutation controls, in a direct fashion, the reaction which, on the basis of the results of growth experiments appears to be blocked, does not seem to be a safe one." Yet Mitchell(60) concluded from the negative growth response of pyr-3 to orotic acid, and from the fact that the double mutant, pyr-3-pyr-4, did not accumulate orotic acid, that orotic acid must therefore not be a direct intermediate in pyrimidine biosynthesis in Neurospora. Subsequent work by Mitchell(62,78) and the experiments reported in this paper showed that pyr-3 did utilize orotic acid. Furthermore, KS-mutants at the pyr-3 locus which did not initially respond to orotic acid could be made to do so by backcrossing to the wild-type parent, and by preculturing on orotic acid.

On the basis of the above considerations, the observations that certain pyr-mutants accumulated dihydroorotic acid and ureidosuccinic acid, that other mutants gave growth response to those compounds, that Mitchell[1] has

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[1]Personal communication.

obtained enzymatic conversions of these compounds to orotic acid, and that other precursors to orotic acid have been found in Neurospora, mammals, or bacteria, we must conclude that orotic acid is not a by-product of pyrimidine biosynthesis in Neurospora, but that the biosynthetic sequence for the formation of uridine-5'-phosphate via orotic acid in Neurospora is entirely analogous to that reported for bacteria and mammals. The interrelations of pyrimidine biosynthesis to arginine and lysine noted by Houlahan and Mitchell (39), and to α-amino-n-butyric acid and threonine(Fairley, 22) were not studied, and at present, are not interpretable.

The thesis of this paper, that uridine-5'-phosphate is synthesized by metabolic reactions analogous to those found in bacterial and mammalian systems, was supported by the study of accumulated pyrimidine intermediates. Mutants pyr-4 and pyr-2 were found to accumulate orotidine, orotic acid, dihydroorotic acid, and ureidosuccinic acid; pyr-1, 28-III, and KS-12 accumulated only dihydroorotic acid and ureidosuccinic acid; and pyr-3, 6-III, and wild-type accumulated none of these compounds. In addition, mutants were obtained whose pyrimidine requirements could be met with ureidosuccinic acid and dihydroorotic acid. That enzymes for the metabolic interconversions of these compounds existed in Neurospora, that pyr-2 and pyr-4 lacked the enzyme orotidine-5'-phosphate decarboxylase, and that pyr-1 accumulated dihydroorotic acid, and ureidosuccinic acid has been noted by Mitchell(77).[1]

Evidences for a hitherto unreported metabolic sequence in Neurospora, involving the reversible sequence, uridine, uracil, dihydrouracil, β-ureidopropionic acid, and β-alanine were observed. The evidences were threefold:

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[1]Personal communication.

1) the positive growth responses of pyrimidine mutants to all of these compounds except β-alanine, 2) demonstration of accumulation of all of these compounds by wild type and all pyrimidine mutants when grown with either uracil or uridine, and 3) demonstration of enzyme activity for dihydrouracil hydrase in acetone powder extracts of wild type, and the mutants, pyr-1 and pyr-3. The view is taken that this pathway is primarily catabolic in wild type Neurospora, but may function biosynthetically in pyrimidine mutants blocked in the orotic acid pathway when the uracil intermediates are supplied in the growth medium.

On the basis of differential growth responses of the pyrimidine mutants to the uracil pathway intermediates, one could postulate metabolic lesions in that pathway; however, all of the mutants were found to reduce uracil to its precursors when cultured on uracil, but not when cultured on orotic acid. The ambiguity of negative growth responses, and the plasticity of growth response that varies with the preculture substrate suggest that the apparent differential growth responses were more apparent than real, and than the observed responses probably were results of modifier genes or secondary effects of a gene mutation whose primary function in the non-mutant state was to control the formation of an enzyme in the orotic acid pathway. At any rate, it seems premature to speculate on the possibility of a single gene controlling two enzymes. To quote St. Lawrence and Bonner(74): ". . .so compelling is the evidence of biochemical genetics, that, if an apparent case of one gene controlling two enzymes were to occur, it would probably be classified as involving secondary effects of a primary mutation or be ascribed to two closely linked genes. Experimental discrimination between these possibilities is clearly exceedingly difficult." Newmeyer(65), in a discussion of the genetic

control of catabolic enzymes, cites reference to point cut that severe (catabolic) enzyme deficiencies can be caused by mutations at numerous widely separated loci, and that many of these loci act by controlling the production of other proteins needed for the induction of the enzyme itself, and suggests that catabolic enzymes are more subject than biosynthetic ones to such multi-gene control. In summary of this aspect, it seems that only after long, carefully systematic study of the genetics and physiology of pyrimidine mutants and the enzymes of the uracil and orotic acid pathways can inferences be made in regard to gene-enzyme relations in the uracil pathway.

A summary of the apparent relationships of pyrimidine mutants of Neurospora to pyrimidine metabolism is shown in PlateXII (see PlateIII for comparison of the metabolic relations with those of bacteria and mammals.) Orotidine probably arises from the phosphorolysis of orotidine-5'-phosphate, as suggested by Kornberg(44). Mutants 28-III, pyr-1, and KS-12 should be found to lack the enzyme, dihydroorotic acid dehydrogenase, whereas the metabolic blocks of pyr-3 and 6-III are most probably before the formation of ureidosuccinic acid. The reaction catalyzed by ureidosuccinic synthetase (carbamyl aspartate transferase) in the formation of ureidocuccinate from carbamyl phosphate and aspartate, has not been studied in Neurospora, to our knowledge; however, Hall, et al.(34) have obtained enzymatic formation of carbamyl phosphate in cell-free homogenates of Neurospora crassa while studying the reaction involving the coupling of carbamyl phosphate with ornithine to form citrulline.

An alternate pathway for the formation of ureidosuccinate has been suggested by the work of Heinrich, et al.(36). They found that when an arginine mutant (46004) (subsequently shown to be blocked in the conversion of arginosuccinate to arginine and fumarate(23)) of Neurospora was grown 4 days at

room temperature in a medium containing arginine and citrulline-ureido-C^{14}, they mycelium yielded a nucleic acid fraction in which most of the activity was in the cytidylic and uridylic fractions. Their conclusion was that argininosuccinate, the immediate product of the ATP catalyzed coupling of aspartate and citrulline, was hydrolytically split to yield ornithine and ureidosuccinate-C^{14} which was then subsequently incorporated into the pyrimidines. However, the reversibility of the formation of citrulline via ornithine and carbamyl phosphate noted by Knivett(42) in extracts of Streptococcus faecalis, could also result in the labeling of pyrimidines via the coupling of labeled carbamyl phosphate with aspartate. These reactions deserve further study in Neurospora to pin-point the lesions of pyr-3 and 6-III.

The author has suggested an experiment to test if argininosuccinate is a precursor of pyrimidines as suggested by the work of Heinrich, et al(36) (described above) which would alleviate the possibility of ureido-C^{14}-citrulline being converted to labeled carbamyl phosphate. That experiment would involve the preparation of the triple mutant derived from arg-3(30300) or arg-2 (33442), pyr-1(263) or 28-III, and any of the mutants blocked in the conversion of argininosuccinate to arginine. Arg-3 and arg-2 are blocked in the conversion of ornithine to citrulline and pyr-1 and 28-III accumulate dihydroorotic acid and ureidosuccinic acid. The procedure would be to grow the triple mutant in medium supplemented with arginine, uridine, and ureido-C^{14}-citrulline. Then the labeled compound would be converted only to argininosuccinate and not split to give ornithine and carbamyl phosphate and the arg-mutation would prevent the conversion of argininosuccinate to arginine and urea. (It would be necessary to use labeled citrulline rather than labeled argininosuccinate since the latter is impermeable(23)). The triple mutant should first be grown

with these compounds without label to determine the time of incubation for maximum accumulation of dihydroorotic acid and ureidosuccinic acid. Isolation of these compounds, after feeding the label, from the culture filtrate and determination of radioactivity should qualitatively and quantitatively indicate the efficiency of argininosuccinate as their precursor.

In connection with the possibility of carbamyl phosphate as a precursor to both pyrimidines and citrulline, it should be possible to isolate single gene mutants in Neurospora which require both citrulline and pyrimidine, and would be blocked in the formation of carbamyl phosphate. This situation was presumably obtained in a mutant of E. coli(85). Ordinarily, the selection techniques for Neurospora preclude the isolation of a mutant requiring two apparently unrelated compounds, unless both compounds are included in the isolation medium. Srb(75) has studied a single gene mutant (30820) which required either citrulline or arginine, but not ornithine, and was markedly stimulated by the addition of any one of the following compounds in the presence of either citrulline or arginine: adenine, guanine, uridine, and cytidylic acid. This mutant may have been blocked in the formation of carbamyl phosphate thus creating a double requirement for citrulline and pyrimidine. Carbamyl phosphate might also possibly be involved in the biosynthesis of adenine and guanine, as well, which would explain the stimulation by those compounds.

In regard to the interrelation of citrulline and pyrimidine biosynthesis, one might speculate on the significance of the functional-spatial relationships of the two mutations, arg-2 (33442) and pyr-3. Both of these mutations are located on the right arm of linkage group IV and are very closely linked (3). Pyr-3 was presumed to be blocked in the conversion of carbamyl phosphate

and aspartate to ureidosuccinate (see previous discussion) and presumably arg-2 is involved in the coupling of carbamyl phosphate and ornithine. It would seem fruitless to pursue this speculation further until further experimental evidence is obtained, but in passing, it should be mentioned that functional-spatial relations have been noted in bacteria and fungi, and, in some cases, the order of the genes on the chromosome corresponds to the order of a biosynthetic sequence controlled by those genes(20 and 21).

The finding of a large number of sites, separable by infrequent crossover, at the pyr-3 locus and of two sites or pseudoalleles at the pyr-1 locus with the apparently indistinguishable growth properties and accumulation products within a group offers the exciting possibility of contributing to the growing number of cases in which the genetic basis of an enzyme is a short chromosome segment, or complex locus, rather than a single gene in the sense of an indivisible unit (see Newmeyer(65) for a short discussion of complex locus-single enzyme relations).

With the detailed information of the spatial relation at the pyr-3 locus, study of the enzyme controlled by the non-mutant locus and examination of the mutant extracts for protein precursors to that enzyme could be revealing in elucidating gene-enzyme relationships.

Yanofsky(84) has studied the enzyme, tryptophan synthetase, in Neurospora and finds a number of mutants controlling that enzyme which are distinguishable in their temperature sensitivity and differential reaction to suppressor genes, but as yet indivisible by cross-over tests. Certain mutants have been found to contain serologically active proteins(CRM) closely related to tryptophan synthetase that are enzymatically inactive. It would seem possible to determine whether different and specific CRM proteins are

formed as the result of damage to particular areas within the tryptophan synthetase locus; however, the inability to obtain detailed spatial relations within that locus would appear to limit the information obtainable on gene-enzyme relations. We propose the pyr-3 locus offers greater possibilities in that regard.

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SUMMARY

Evidence has been presented in this paper to support the hypothesis that the metabolism of pyrimidines in Neurospora crassa is analogous to pyrimidine metabolism in bacteria and mammals. These evidences were obtained from three approaches: 1) growth response of pyrimidine mutants to proposed pyrimidine intermediates, 2) the study of accumulation products of pyrimidine metabolism in mutant culture filtrates and extracts, and 3) enzymatic tests.

A metabolic scheme for uracil metabolism, hitherto unreported in Neurospora, was elucidated. The intermediates in uracil metabolism were identical to those reported for bacteria and mammals. The view was discussed that the uracil pathway was catabolic in the prototroph, but that, in the mutants, could be reversed to function biosynthetically if the appropriate intermediates were fed.

The precursors of orotic acid, dihydroorotic acid, and ureidosuccinic acid, were found to accumulate in certain pyrimidine mutants, and to support growth of other pyrimidine mutants.

On the basis of these experiments, it was possible to relate the mutation effects of the pyrimidine mutants to single metabolic reactions.

The state of knowledge of the relationships of genes to pyrimidine metabolism in Neurospora is now such that detailed enzymatic studies could be undertaken to relate specific mutational events at a given locus to possible variations in the enzyme controlled by that locus.

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LITERATURE CITED

1. Archibald, R. M.
Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. J. Biol. Chem, Nov., 1944, 156:121-142.

2. Amos, H., and B. Magasanik.
Uridine as a Precursor of Bacteriophage Thymidylic Acid. J. Biol. Chem., Dec., 1957, 229:653-657.

3. Barratt, R. W., D. Newmeyer, D. D. Perkins, and L. Gernjobst.
Map construction in Neurospora crassa. Advances in Genetics VI:1-93, 1954. Academic Press Inc., New York, New York.

4. Batt, R. D., J. K. Martin, J. M. Ploeser, and J. Murray.
Chemistry of the dihydropyrimidines. Ultraviolet spectra and alkaline decomposition. J. Am. Chem. Soc., July, 1954, 76:3663-3665.

5. Beadle, G. W., and E. L. Tatum.
Neurospora. II. Methods of producing and detecting mutations concerned with nutritional requirements. Am. J. Bot., Dec., 1945, 32:678-686.

6. Bendich, A.
Chemistry of purines and pyrimidines. Occurrence and distribution of purines and pyrimidines of nucleic acid origin. The Nucleic Acids, Vol. 1. pp. 89-90. 1955. Chargaff, E. and J. N. Davidson, editors. Academic Press Inc., Publishers, New York, New York.

7. Campbell, L. L., Jr.
Reductive Degradation of Pyrimidines. I. The isolation and characterization of a uracil-fermenting bacterium, Clostridium uracilicum Nov. Spec. Jour. Bact., Feb., 1957, 73:220-224.

8. Campbell, L. L., Jr.
Reductive Degradation of Pyrimidines. II. Mechanism of uracil degradation by Clostridium uracilicum. Jour. Bact., Feb., 1957, 73:225-229.

9. Campbell, L. L., Jr.
Reductive Degradation of Pyrimidines. III. Purification and properties of dihydrouracil dehydrogenase. J. Biol. Chem., Aug., 1957, 227:693-700.

10. Canellakis, E. S.
Pyrimidine metabolism. II. Enzymatic pathways of uracil anabolism. J. Biol. Chem., July, 1957, 227:329-338.

11. Canellakis, E. S.
Pyrimidine metabolism. III. The interactions of the catabolic and anabolic pathways of uracil metabolism. J. Biol. Chem., Aug., 1957, 227:701-709.

12. Carter, C. E.
Metabolism of purines and pyrimidines. Ann. Review of Biochem. 1956, 25:123-246. Annual Reviews, Inc., Stanford, California.

13. Cohen, S. S., J. Leichtenstein, H. D. Barner, and M. Green.
Studies of the biosynthesis of bacterial and viral pyrimidines. IV. Utilization of pyrimidine bases and nucleosides by bacterial mutants. J. Biol. Chem., Oct., 1957, 228:611-619.

14. Comar, C. L.
Radioisotopes in Biology and Agriculture. Principles and Practices. McGraw Hill Book Co., New York, New York, p. 364, 1955.

15. Consden, R., A. H. Gordon, and A. J. P. Martin.
Quantitative Analysis of Proteins: a partition chromatographic method using paper. Biochem. J., May, 1944, 38:224-232.

16. Cooper, C., R. Wu, and D. W. Wilson.
Some precursors of pyrimidines. J. Biol. Chem., Sept., 1955, 216:37-49.

17. Crawford, I., A. Kornberg, and E. S. Simms.
Conversion of uracil and orotate to uridine-5'-phosphate by enzymes in Lactobacilli. J. Biol. Chem., June, 1957, 226: 1093-1101.

18. Crick, F. H. C.
The structure of DNA. A Symposium on the Chemical Basis of Heredity. McElroy, W. D., and B. Glass, editors. The John Hopkins Press, Baltimore, pp. 532-539. 1957.

19. Dent, C. E.
A study of the behaviour of some sixty amino acids and other ninhydrin-reacting substances on phenol-'collidine' filter paper chromatograms with notes as to the occurrence of some of them in biological fluids. Biochem. J., May, 1948, 43:169-180.

20. DiCarlo, F. J., A. S. Schultz, and A. M. Kent.
On the mechanism of pyrimidine metabolism by yeasts. J. Biol. Chem., Nov., 1952, 199:333-343.

21. Demerec, M.
Structure of gene loci. Enzymes: Units of Biological Structure and Function. Gaebler, O. H., editor. Academic Press, Inc., New York, New York, pp. 131-134.

22. Fairley, J. L.
The growth promoting effect of certain amino acid for pyrimidineless Neurospora mutants. J. Biol. Chem., Sept., 1954, 210:347-351.

23. Fincham, J. R. S., and J. B. Boylen.
Neurospora crassa mutants lacking argininosuccinase. J. Gen. Microbiol., April, 1957, 16:438-448.

24. Fink, R. M., R. E. Cline, and H. M. G. Koch.
Chromatographic detection of pyrimidine reduction products: microbiological application. Fed. Proc., March, 1954, 13:207-208.

25. Fink, R. M., R. E. Cline, C. McGaughey, and K. Fink.
Chromatography of pyrimidine reduction products. Anal. Chem., Jan., 1956, 28:4-6.

26. Fink, R. M., C. McGaughey, R. E. Cline, and K. Fink.
Metabolism of intermediate pyrimidine reduction products in vitro. J. Biol. Chem., Jan., 1956, 218:1-14.

27. Fink, K.
Excretion of pyrimidine reduction products by the rat. J. Biol. Chem., Jan., 1956, 218:9-14.

28. Friedkin, M., and A. Kornberg.
The enzymatic conversion of deoxyuridylic acid to thymidylic acid and the participation of tetrahydrofolic acid. A Symposium on the Chemical Basis of Heredity, W. D. McElroy and B. Glass, editors. The John Hopkins Press, Baltimore, pp. 609-614, 1957.

29. Fritzon, P.
Distribution of enzymes involved in the breakdown of uracil to B-alanine in rat liver cells. Acta Chem. Scand., Oct., 1956, 10:1674-1675.

30. Fritzon, P.
The catabolism of C^{14}-labeled uracil, dihydrouracil, and B-ureidopropionic acid in rat liver slices. J. Biol. Chem., May, 1957, 226:223-228.

31. Fritzon, P, and A. Phil.
The catabolism of C^{14}-labeled uracil, dihydrouracil, and B-ureidopropionic acid in the intact rat. J. Biol. Chem., May, 1957, 226:229-235.

32. Grisolia, S., and D. P. Wallach.
Enzymic interconversion of hydrouracil and B-ureidopropionic acid. Biochem. Biophys. Acta, Sept., 1955, 18:449.

33. Grisolia, S., and S. S. Cardoso.
The purification and properties of hydropyrimidine dehydrogenase. Biochem. Biophys. Acta, May, 1957, 25:430-431.

34. Hall, L. M., R. L. Metzenberg, and P. P. Cohen.
Isolation and characterization of a naturally occurring cofactor of carbamyl phosphate synthesis. J. Biol. Chem., Feb., 1958, 230:1013-1021.

35. Haskins, F. A., and H. K. Mitchell.
Evidence for a tryptophane cycle in Neurospora. Proc. Natl. Acad. Sci., Sept., 1949, 35:500-506.

36. Heinrich, M. R., V. C. Dewey, and G. W. Kidder.
Citrulline as a precursor of pyrimidines. J. Amer. Chem. Soc., June, 1954, 76:3102-3.

37. Herrmann, R. L., and J. L. Fairley.
The use of aminobutyric acid-3-C^{14} for pyrimidine biogenesis by Neurospora crassa. J. Biol. Chem., Aug., 1957, 227:1109-1116.

38. Houlahan, M. B., and H. K. Mitchell.
A suppressor in Neurospora and its use as evidence for allelism. Proc. Natl. Acad. Sci., Aug., 1947, 33:223-229.

39. Houlahan, M. B., and H. K. Mitchell.
Evidence for an interrelation in metabolism of lysine, arginine, and pyrimidines in Neurospora. Proc. Natl. Acad. Sci., Oct., 1948, 34:465-470.

40. Kalckar, H. M.
Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. J. Biol. Chem., Feb., 1947, 167:461-475.

41. Kihara, H., J. M. Prescott, and Snell.
Enzymatic cleavage of canavanine to homoserine and guanidine. Fed. Proc., March, 1955, 14:235-236.

42. Knivett, V. A.
The anaerobic interconversion of ornithine and citrulline by Streptococcus faecalis. Biochem. J., Nov., 1954, 58:480.

43. Koritz, S. R., and P. P. Cohen.
Colorimetric determination of carbamylamino acids and related compounds. J. Biol. Chem., July, 1954, 209:145-150.

44. Kornberg, A.
Pathways of enzymatic synthesis of nucleotides and polynucleotides. A Symposium on the Chemical Basis of Heredity. McElroy, W. E. and B. Glass, editors. The John Hopkins Press, Baltimore, 1957.

45. Lagerkvist, U., P. Reichard, B. Carlsson, and J. Graborz.
The biogenesis of pentosenucleic acid pyrimidines in the Ehrlich Ascites tumor. Cancer Research, March, 1955, 15:164.

46. Leiberman, I., and A. Kornberg.
Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. I. Dihydroorotic dehydrogenase. Biochem. Biophys. Acta, Feb., 1953, 12:223-234.

47. Leiberman, I., and A. Kornberg.
Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. II. Dihydroorotic acid, ureidosuccinic acid, and 5-carboxymethylhydantoin. J. Biol. Chem., April, 1954, 207:911-924.

48. Leiberman, I., A. Kornberg, and E. S. Simms.
Enzymic synthesis of pyrimidine and purine nucleotides. III. Orotidine-5'-phosphate pyrophosphorylase and decarboxylase. J. Amer. Chem. Soc., May, 1954, 76:2844-5.

49. Leiberman, I., and A. Kornberg.
Enzymatic synthesis and breakdown of a pyrimidine, orotic acid. III. Ureidosuccinase. J. Biol. Chem., Feb., 1955, 212:909-920.

50. Leiberman, I., A. Kornberg, and E. S. Simms.
Enzymatic synthesis of pyrimidine nucleotides. Orotidine-5'-phosphate and uridine-5'-phosphate. J. Biol. Chem., July, 1955, 215:403-415.

51. Loring, H. S.
Estimation of purine and pyrimidine components in PNA. The Nucleic Acids, E. Chargaff and J. N. Davidson. Academic Press Inc., Publishers, New York, New York. Vol. I, 1955, pp. 199-202.

52. Lowenstein, J. M., and P. P. Cohen.
Studies on the mechanism of carbamylaspartic acid synthesis. J. Biol. Chem., April, 1955, 213:689-96.

53. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.
Protein Measurement with the Folin phenol reagent. J. Biol. Chem., Nov., 1951, 193:265-275.

54. Maas, W. K.
Mechanism of the enzymatic synthesis of pantothenate from beta-alanine and pantoate. Fed. Proc., March, 1956, 15: 305-306.

55. McNutt, W. S.
Ribonucleosides as precursors of the carbon skeleton of deoxyribonucleic acid in Neurospora. Plant Physiol., 32 (supp.), xxxvi., 1957.

56. Metzenberg, R. L., L. M. Hall, M. Marshall, and P. P. Cohen.
Studies on the biosynthesis of carbamyl phosphate. J. Biol. Chem., Dec., 1957, 229:1019-1025.

57. Michelson, A. M., W. Drell, and H. K. Mitchell.
A new ribose nucleoside from Neurospora: "Orotidine." Proc. Natl. Acad. Sci., July, 1951, 37:396-399.

58. Mitchell, H. K., and J. F. Nyc.
Intermediates in the synthesis of orotic acid from oxalacetic ester and urea. J. Amer. Chem. Soc., March, 1947, 69:674-677.

59. Mitchell, H. K., and M. B. Houlahan.
Investigations on the biosynthesis of pyrimidine nucleosides in Neurospora. Fed. Proc., Sept., 1947, 6:506-509.

60. Mitchell, H. K., M. B. Houlahan, and J. F. Nyc.
The accumulation of orotic acid by a pyrimidineless mutant of Neurospora. J. Biol. Chem., Feb., 1948, 172:525-529.

61. Mitchell, M. B., and H. K. Mitchell.
Observations on the behavior of suppressors in Neurospora. Proc. Natl. Acad. Sci., March, 1952, 38:205-14.

62. Mitchell, H. K.
Growth factors in relation to studies of genetics of microorganisms. Symposium on Growth Factors, 6th Congr. Int. Microbiol. (1953) pp. 75-96.

63. Mitchell, M. B., and H. K. Mitchell.
Tests for non-allelism at the pyrimidine 3 locus of Neurospora. Genetics, Nov., 1956, 41:319-326.

64. Mokrasch, L. C., and S. Grisolia.
Incorporation of hydropyrimidine derivatives in ribonucleic acid with liver preparations. Biochem. Biophys. Acta, March, 1958, 27:226-227.

65. Neumeyer, D.
Arginine synthesis in Neurospora crassa: genetic studies. J. Gen. Microbiol., April, 1957, 16:449-462.

66. Nyc, J. F., and H. K. Mitchell.
Synthesis of orotic acid from aspartic acid. J. Am. Chem. Soc., June, 1947, 69:1382-1384.

67. Ratner, S., B. Petrack, and O. Rochovansky.
Biosynthesis of Urea V. Isolation and properties of argininosuccinic acid. J. Biol. Chem., Sept., 1953, 204:95-113.

68. Reichard, P.
The enzymatic synthesis of ureidosuccinic acid in rat liver mitochondria. Acta Chem. Scand., 1954, 8:795-805.

69. Reynolds, E. S., I. Leiberman, and A. Kornberg.
The metabolism of orotic acid in aerobic bacteria. J. Bact., March, 1955, 69:250-255.

70. Slotnick, I. J., and H. Weinfeld.
Dihydrouracil as a growth factor for mutant strains of Escherichia coli. J. Bact., Aug., 1957, 74:122-125.

71. Snedecor, G. W.
Statistical Methods, 5th Ed., The Iowa State College Press, Ames, Iowa, 1956, pp. 117-120.

72. Snell, F. D., and C. T. Snell.
Colorimetric Methods of Analysis, 3rd Ed., D. Van Nostrand Co., Inc., New York, New York. 1954, p. 181.

73. Strauss, B. S.
Studies on the vitamin B6-requiring, pH-sensitive mutants of Neurospora crassa. Arch. Biochem., Feb., 1951, 30:292-305.

74. St. Lawrence, P., and D. M. Bonner.
Gene conversion and problems of allelism. A Symposium on the Chemical Basis of Heredity. McElroy, W. E., and B. Glass, editors. The John Hopkins Press, Baltimore. 1957, pp. 114-122.

75. Srb, A. M.
Complex growth requirement of an argininеless mutant of Neurospora. Bot. Gaz., June, 1950, 111:470-476.

76. Suyama, Y.
Unpublished data and observations.

77. Tuttle, A. L., and H. K. Mitchell.
Biosynthesis of pyrimidine nucleotides in Neurospora. "Biology 1957" Calif. Inst. of Technology. A report for the year 1956-57 on the research and other activities of the Div. of Biology, p. 19-20.

78. Wagner, R. P., and H. K. Mitchell.
Genetics and Metabolism, John Wiley and Sons, Inc., New York. 1955, pp. 209-10.

79. Wagner, R. P., A. Berquist, and G. W. Karp.
Some factors influencing valine and isoleucine transaminase activity in Neurospora crassa. Arch. Biochem. and Biophys., March, 1958, 74:182-197.

80. Walker, J. B.
Biosynthesis of canavaninosuccinic acid from canavanine and fumarate in kidney. Arch. Biochem. and Biophys., Nov., 1955, 59:233-245.

81. Wallach, D. P., and S. Grisolia.
The purification and properties of hydropyrimidine hydrase. J. Biol. Chem., May, 1957, 226:277-288.

82. Woodward, V. W., J. R. DeZeeuw, and A. B. Srb.
The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filtration and selective plating. Proc. Natl. Acad. Sci., March, 1954, 40:192-200.

83. Woodward, V. W.
Unpublished data.

84. Yanofsky, C.
Gene interactions in enzyme synthesis. Enzymes: Units of Biological Structure and Function. Gaebler, O. H., editor. Academic Press, Inc., New York, New York, pp. 147-160. 1956.

85. Yates, R. A., and A. B. Pardee.
Pyrimidine biosynthesis in Escherichia coli. J. Biol. Chem., Aug., 1956, 221:743-755.

86. Yates, R. A., and A. B. Pardee.
Control by uracil of formation of enzymes required for orotate synthesis. J. Biol. Chem., Aug., 1957, 227:677-692.

APPENDIX

APPENDIX

1-x Buffered Fries Solution

ammonium tartrate	20 g.
NH_4NO_3	4 g.
$MgSO_4 \cdot 7H_2O$	2 g.
NaCL	0.4 g.
$CaCL_2 \cdot 2H_2O$	0.4 g.
KH_2PO_4	8 g.
Na_2HPO_4	0.165 g.
biotin1/	1.6 ml.
trace elements 2/	10 ml.
sucrose	10 g.
distilled water	1000 ml.

1/ biotin stock solution 25 ug/ml.
2/ trace element stock solution:

Element	mg./ml.	Salt used	mg. salt/l.
B	0.01	$Na_2B_4O_7 \cdot 10H_2O$	0.088
Mo	0.02	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.037
Fe	0.2	$FeSO_4 \cdot 7H_2O$	1.00
Cu	0.1	$CuSO_4.5H_2O$	0.39
Mn	0.02	$MNCl_2.4H_2O$	0.072
Zn	2.0	$ZnSO_4.H_2O$	5.5

Table 1. Growth responses of pyrimidine mutants. Exp. 1.

	mg. dry weight-96 hr.								
Mutant	Supplement : Ud	Cd	U	C	OA	DU	DC	RNA	DNA
KS-1	89	76	61	2	52	19	23	30	2
KS-2	90	95	tr	0	tr	8	9	8	0
KS-3	96	98	2	0	0	21	23	8	0
KS-4	80	50	0	0	0	6	tr	0	0
KS-5	88	91	83	3	0	56	21	0	0
KS-6	94	90	12	0	0	4	3	0	0
KS-7	67	72	tr	0	0	tr	tr	0	0
KS-8	73	75	tr	0	tr	6	9	0	0
KS-9	53	60	tr	0	0	2	4	0	0
KS-10	86	89	tr	0	0	12	4	0	0
KS-11	79	88	tr	0	0	tr	tr	0	0
KS-12	80	66	tr	0	0	4	6	0	0
KS-13	99	78	2	0	tr	4	3	0	0
KS-14	80	86	tr	0	0	tr	tr	0	0
KS-15	55	58	tr	0	0	17	7	0	0
pyr-1	75	73	25	2	25	10	8	13	6
pyr-3	67	66	66	5	57	60	25	23	5

Supplements at 10 uM eq. UD, uridine; Cd, cytidine; U, uracil; C, cytosine; OA, orotic acid; DU, deoxyuridine; DC, deoxycytidine; RNA, sodium ribonucleate; DNA, deoxyribonucleic acid. No mutant responded to the following supplements: oxalacetic acid, 5-methylcytosine, thymine, thymidine, L-dihydroorotic acid, DL-ureidosuccinic acid, and DL-5-carboxymethylhydantoin.

No growth is observed on minimal medium for any of the above mutants with the exception of pyr-1 which yields about 5 mg. in 96 hr. at 27°C.

Table 2. Growth responses of second-backcross generations of KS-mutants to 10 mg. per cent orotic acid. 1/

Strain	Hours Incubation					
	24	48	72	96	120	144
KS 1-23-2a	0	0	0	*	+	77
2- 8-2a	0	3	11	62	-	-
3- 4-4a	0	0	0	+	+	81
4-42-1a	0	0	0	0	0	0
6- 2-4a	0	0	*	+	+	+
7- 3-4a	0	0	*	+	+	+
8- 6-4a	0	0	*	+	+	+
9- 6-7a	0	0	*	+	+	+
11- 1-3a	*	*	+	+	+	+

1/ Precultured on uridine agar slants. None of the strains produce visible growth in 144 hr. on minimal media. + signifies on the order of 5-10 mg. growth. Variable response was obtained between replicate flasks of most strains during the 96 to 144 hr. period, ranging from 0 to +. * signifies the growth was too small to weigh (less than 5 mg.).

Table 3. Direct spectrophotometric analysis of culture filtrates of pyrimidine mutants. Exp. 1.

Mutant	Optical Density 1/	
	280 mu	260 mu
KS 1	0.400	0.810
KS 2	0.250	0.790
KS 3	0.250	0.550
KS 4	0.480	0.620
KS 5	0.650	1.050
KS 6	0.440	0.630
KS 7	0.430	0.500
KS 8	0.640	1.030
KS 9	0.610	0.760
KS 10	0.470	0.630
KS 11	0.550	0.860
KS 12	0.590	0.900
KS 13	0.610	0.940
KS 14	0.600	0.900
KS 15	0.570	0.600
pyr 1	0.450	0.680
pyr 2	3.+	2.75
pyr 2 2/	2.27	1.40
pyr 2 3/	1.18	0.970
pyr 3	0.580	0.580
pyr 4	3.+	3.+

1/ 280 mu. and 260 mu. are the wavelengths of max. and min. absorption for orotic acid and uridine, respectively, at the pH(4.5) of the culture filtrates.

2/ diluted 1:10 with distilled water against minimal media diluted 1:10.

3/ diluted 1:15 with KOH, pH 12.0, and absorption read against minimal media diluted 1:15 with KOH, pH 12.

Table 4. Binary spectrophotometric analysis of the culture filtrates of several pyrimidine mutants. 1/
Exp. 2.

Mutant	Optical Density 2/ 260 mu	277 mu	OA 3/ M x 10^6	ug. OA per ml. culture
KS-4	0.990	0.640	0.01	0.02
KS-5	0.870	0.510	0.14	0.28
KS-6	0.760	0.450	0.04	0.08
KS-9	0.885	0.520	0.32	0.64
KS-10	1.247	0.790	1.36	2.50
KS-11	0.660	0.487	0.25	0.50
KS-13	0.810	0.535	1.20	2.21
KS-14	0.800	0.385	0.13	0.28
pyr-2 4/	2.27	1.40	6.340	116. 5/

1/ grown 96 hr. in 4.3 mg. per cent uridine, standing culture, 27°C.
2/ against minimal media for 100 per cent transmission.
3/ calculated from the equation:

$$COA = \frac{6.29D260 - 10.9D_{277}}{-6.13 \times 10^5}$$

4/ grown 72 hr., diluted 1:10 with distilled water, optical density read against minimal media diluted 1:10 with distilled water.
5/ corrected for dilution.

Table 5. Growth responses of several pyrimidine mutants to orotidine (10 uM eq.) at 27°C.

Mutant 1/	mg. dry wt.	
	48 hr.	96 hr.
KS -1	4	10
KS -4	1	3
KS -5	4	10
KS -6	5	10
KS -7	3	9
pyr-1	++	+++
pyr-3	++	+++
pyr-2 (35°C)	0	0
pyr-4	0	0

1/ response of the pyr mutants according to Mitchell (78).

Table 6. Paper chromatography of concentrated culture filtrates of pyrimidine mutants and wild type.
1/ Exp. 3.

Controls	R_f x 100
ureidosuccinic acid	30*
dihydroorotic acid	30
β-ureidopropionic acid	46*
dihydrouracil	90
urea	68*
β-alanine	45#

Strains	media					
--	minimal	-	-	-	-	
73a	minimal	10*#	-	-	-	
73a	uridine	10*#	24#	42*	94#	94
KS-5	uridine	10*#	24#	42*	44#	94
pyr-3	uridine	10*#	24#	40*	44#	94

1/ phenol-water mixture, lower phase. Average values of two independent growth runs.
* indicates PDAB positive without prior sodium hydroxide spray.
\# indicates ninhydrin positive.

Table 7. Paper chromatography of concentrated culture filtrates of pyrimidine mutants and wild-type. 1/.
Exp. 4.

Controls	R_f x 100					
	U.V.		NaOH + PDAB			
uracil	48					
dihydrouracil					50	
dihydroorotic acid					35	
β-ureidopropionic acid					58	
ureidosuccinic acid					47	
Treatments 2/						
pyr-1-U-96	0	47	3	15	35	51
pyr-1-U-144	0	47	-	15	—	51
pyr-3-U-96	0	47	3	15	—	51
pyr-3-U-120	0	—	3	15	—	—
73a-M-96	0	—	3	15	—	—
73a-M-120	0	—	-	—	—	—
73a-M-144	0	—	-	—	—	—

1/ n-butanol-acetic acid-water solvent (2:1:1 v/v)
2/ U signifies uracil supplement, 2 mg. per cent
M signifies minimal medium
the number designates the growth period.

Table 8. Paper chromatography of concentrated culture filtrates and mycelial extracts 1/. Exp. 5.

	PDAB positive substances R_f x 100									
Strain	28III:	KS-12:	pyr-4:	pyr-3:	pyr-22/:	pyr-1:	73aU:	73aM:	6IIIU:	6IIIM
	16*	16*	16*	16*	16*	16*	16*	16*	16*	16*
	34	37	36	—	37	35	—	—	—	—
	42*	40*	41*	—	41*	43*	—	—	—	—
	57	57	54	(56)	56	57	(56)	—	(56)	—
	61*	60*	64*	(63)*	63*	64*	(64)*	—	(63)*	—

Controls 3/

dihydroorotic acid	35
ureidosuccinic acid	43*
citrulline	42*
dihydrouracil	57
β-ureidopropionic acid	62*
urea	63*

1/ in n-butanol-acetic acid-water (2:1:1 v/v). R_f values of strains are averages from a maximum of 32 observations per strain from media and mycelia, aerated and non-aereated, at the growth intervals, 48, 72, 96, and 120 hr..

2/ grown either at 27°C or 40°C.

3/ averages of at least 6 determinations, corrected for media salt distortion.

* indicates reactive to PDAB without prior treatment with sodium hydroxide.

( ) values in parenthesis were observed only at minimum concentration of about 3 times that of other observations.

— indicates no spot observed at a concentration level 5 times that of other observations.

Table 9. Paper chromatography of concentrated culture filtrates 1/.
Exp. 5.

Ninhydrin Positive Substances R_f x 100									
Strain 28III	KS-12	pyr-4	pyr-3	pry-22/	pyr-1	73aU	73aM	6IIIU	6IIIM
16	16	16	16	16	16	16	16	16	16
51	51	51	51	51	51	51	(51)3/	51	(51)3/

Controls

α-alanine	44	citrulline	42
β-alanine	51	tryptophan	52
asparagine	27	δ-amino-n-butyric acid	58
asparatic acid	31		

1/ in n-butanol-acetic acid-water (2:1:1 v/v).
2/ grown either at 27°C or 40°C.
3/ traces observed only in mycelia.

Table 10 Chromatography of mycelial extracts and culture filtrates of pyrimidine mutants. 1/
Exp. 5.

	R_f x 100 2/ ultraviolet absorbing compounds									
Controls	28III	KS-12	pyr-4	pyr-3	pyr-2	pyr-1	73aU	73aM	6IIIU	6IIIM
orotidine 16	-	-	+	-	+	-	-	-	-	-
orotic acid 26	-	-	+	-	+	-	-	-	-	-
uridine 3/ 46	+	+	+	+	+	+	+	+	-	-
uracil 56	+	+	+	+	+	+	+	+	+	+

1/ in n-butanol-acetic acid-water (2:1:1 v/v).
2/ The variance of the sample R_f values was within the variance of controls (on the order of ± 2); — indicates not observed at concentrations 5 times as great as samples showing +.
3/ observed only in mycelial extracts, 72 hr. cultures.

Table 11. Paper chromatography of concentrated culture filtrates 1/
Exp. 5.

	R_f x 100								
Strain 28III	KS-12	pyr-4	pyr-3	pyr-2	pyr-1	73aU	73aM	6IIIU	6IIIM
21*	21*	21*	—	21*	21*	—	—	—	—
33	33	33	—	33	33	—	—	—	—
73*	73*	73*	(73)*	73*	73*	(73)*	—	(73)*	—
95	95	95	(95)	95	95	(95)	—	(95)	—

Controls	
ureidosuccinic acid	21*
dihydroorotic acid	33
citrulline	56*
β-ureidopropionic acid	73*
urea	71*
dihydrouracil	95

1/ solvent: lower phase of phenol-water mixture.
* indicates reactive with PDAB without prior sodium hydroxide spray.
( ) observed at minimum concentration 3 times that of other strains.
— not observed at concentration 5 times as great as other strains.

Table 12. Paper chromatography of concentrated culture filtrates. 1/
Exp. 5.

	R_f x 100								
Strain	28III	KS-12	pyr-4	pyr-3	pyr-2	pyr-1	73aU	73aM	6IIIU	6IIIM
	0*	0*	0*	-	0*	0*	-	-	-	-
	11	11	10	-	11	11	-	-	-	-
	17*	17*	16*	(17)*	17*	17*	(17)*	-	(17)*	-
	48	48	48	(48)	48	48	(48)	-	(48)	-

Controls

ureidosuccinic acid	0*
citrulline	6*
dihydroorotic acid	11
β-ureidopropionic acid	16*
urea	31*
dihydrouracil	48

1/ solvent: isoamyl alcohol-pyridine-water (35:35:30 v/v, pH 8.0).
* indicates reactive with PDAB without prior sodium hydroxide spray.
( ) observed at minimum concentration 3 times that of other strains.
-- not observed at concentrations 5 times that of other strains.

Table 13. Estimates of concentrations of accumulated dihydroorotic acid and ureidosuccinic acid in the growth media of certain pyrimidineless mutants 1/. Exp. 5.

Strain	non-aereated culture growth period (hours)		
	72	96	120
28III	13/19	10/22	11/7
KS-12	- -	2/-	6/3
pyr-4	- -	4/2	5/4
pyr-2*	5/-	7/5	10/-
pyr-1	-/7	11/15	5/14
	aereated culture		
28III	35/31	21/100+	67/100+
KS-12	- -	7/6	2/1
pyr-4	- -	10/5	1/3
pyr-2#	7/5	15/10	2/4
pyr-1	24/15	58/100+	4/19

* at 40°C.
\# at 27°C.
1/ values expressed in micrograms per milliliter of cultural filtrate. First figure = DHO, Second figure = US.
- indicates not detectable on chromatogram at 10 ul. level of concentrate.

Table 14. Paper chromatography of anion exchange fractions of mycelial extract from pyr-1. Exp. 7.

	R_f x 100	
	Solvent 1 [1]	Solvent 2 [2]
dihydroorotic acid	34	36
ureidosuccinic acid	34	48
β-ureidopropionic acid	53	64
dihydrouracil	90	56
Pool 1 [3], without US	34	36
Pool 1 , with US	34	36,48
Pool 2 [4], without US	34,90	36,56
Pool 2 , with US	34,90	36,48,56

1/ lower phase of phenol-water mixture
2/ n-butanol-acetic acid-water (2:1:1 v/v)
3/ 18-22 resin bed volumes
4/ 1-17 and 23-35 resin bed volumes.

Table 15. Paper chromatography of anion exchange fractions of culture filtrate of pyr-1. (Exp. 7 run no. 1) 1/.

	cm. from origin 24 hr. run*
dihydroorotic acid	11.5
ureidosuccinic acid	18.0
dihydrouracil	21.0
β-ureidopropionic acid	25.0
Pool 1 2/	11.0
Pool 2 3/	11.5

1/ solvent: n-butanol-acetic acid-water (2:1:1 v/v)
2/ 14-22 resin bed volumes
3/ 1-13; 23-45 resin bed volumes
* solvent moves past end of paper in 24 hr. run.

Table 16. Paper chromatography of anion exchange fractions of culture filtrate of pyr-1. (Exp. 7 run no. 2) 1/.

	cm. from origin 24 hr. run*
dihydroorotic acid	11.5
ureidosuccinic acid	18.0
dihydrouracil	21.0
β-ureidopropionic acid	25.0
Pool 1 2/	11.0,21.0
Pool 2 3/	18.0,21.0

1/ solvent: n-butanol-acetic acid-water (2:1:1 v/v)
2/ 5-31 resin bed volumes
3/ 33-41 resin bed volumes
* solvent moves past end of paper in 24 hr. run.

Table 17. Paper chromatography of anion exchange fractions of mycelial extract and culture filtrate of pyr-1. Exp. 8.

Controls		R_f x 100 1/ Samples 2/	
dihydroorotic acid	22	IA	23;44
ureidosuccinic acid	34	IB	21;34;44;59
dihydrouracil	40	IIA	22;44
β-ureidopropionic acid	57	IIB	23;34;43;58
		IIC	34;44;58

1/ solvent: n-butanol-acetic acid-water (2:1:1 v/v)
2/ IA: 12-23 RBV from mycelia
IB: remainder of column (except 1st 4 fractions) to 50 RBV from mycelia
IIA: 1-10 RBV from media
IIB: 11-43 RBV from media
IIC: 45-60 RBV from media.

Table 18. Fractionation of the pooled fractions IA (12-23 RBV) from the mycelial extract of pyr-1 by paper chromatography. 1/ Exp. 8.

Fraction 2/	R_f x 100
IA-1-UV	4
IA-2-UV	17
IA-3-PB	23
IA-4-UV	30
IA-5-N	34
IA-6-PB	47
dihydroorotic acid	23
dihydrouracil	47

1/ solvent: n-butanol-acetic acid-water (2:1:1 v/v)
2/ UV indicates absorbancy under ultraviolet lamp
PB indicates reactive to PDAB after NaOH treatment
N signifies ninhydrin-reactive.

Table 19. Assay for the conversion, DHU ⟶ BUP, in cell-free homogenates of Neurospora. Exp. 10.

Strain	Time (min.)	Optical Density 490 mu. Sample No. 1	Sample No. 2	BUP uM/ml	Total uM BUP formed
74A	0	-	-	-	-
	15	0	1.125	0.712	4.98
	30	0	1.140	0.721	5.05
	60	0.025	1.140	0.721	5.05
	120	0.010	1.140	0.721	5.05
pyr-1	0	-	-	-	-
	15	0.190	0	0.120	0.84
	30	0.200	0	0.127	0.89
	60	0.225	0	0.142	0.99
	120	lost	0	-	-

Table 20. Assay for dihydrouracil hydrase activity in acetone powders of Neurospora. - data and calculations. Exp. 11

Strain	pyr-1		73aU			73aM		
			Optical Density 490 mu.					
Sample	1	2	1	2	ave.	1	2	ave.
Time(min.)								
15	0	0.560	0.540	0.690	0.615	0	0.080	0.04
30	0	0	0.590	0.700	0.645	0	0.300	0.150
60	0.150	0	0.660	0.710	0.685	0.75	0	0.375
120	0	lost	0.720	0.740	0.730	0.76	0.780	0.770
180	0.220	0.590	1.067	1.062	1.064	0.730	0.790	0.760

Controls	Substrate + Buffer	Enzyme + Buffer		
Time(min.)		pyr-1	73aU	73aM
0	1.00	0	-	-
15	0.890	0	0.090	0.125
30	1.590	0	0.080	0.095
60	0.650	0	0.055	0.100
120	1.245	0	0.90	0.110
180	1.470	0	0.070	-

β-ureidopropionic acid	Sample 1	Sample 2	ave.
0.75 uM/ml	0.490	0.460	0.475
1.50 uM/ml	0.870	0.830	0.850

Time(min.)	uM BUP/ml.1/ pyr-1	73aU	73aM
15		0.97	0.01
30		1.02	0.24
60	0.24	1.08	0.48
120		1.15	1.23
180	0.65	1.68	1.23

1/ 1.58×10^{-3} uM/OD unit

Time(min.)	Total uM BUP 2/ pyr-1	73aU	73aM
15		6.79	0.07
30		7.14	1.68
60	1.68	7.56	3.36
120		8.05	8.61
180	4.55	11.76	8.61

2/ per 7 ml. reaction mixture.

Table 21. Paper chromatography of reaction mixtures from the assay for dihydrouracil hydrase activity in acetone powder extracts of Neurospora strains. 1/ (Exp. 11).

	R_f x 100	
Controls		73aM series (15,30,60,90,120,180 min.)
β-ureidopropionic acid	61*	all 47 UV
dihydrouracil	51	
ureidosuccinic acid	47*	all 55 decreasing spot area
dihydroorotic acid	36	
uracil	44 UV	increasing 66*
β-alanine	32	spot area
Controls		73aU series (15,30,60,90,120,180 min.)
β-ureidopropionic acid	66*	all 48 UV
dihydrouracil	58	
ureidosuccinic acid	50*	all 55 decreasing spot area
dihydroorotic acid	39	
uracil	48 UV	increasing 66*
β-alanine	38	spot area
Controls		pyr-1 (30,180 min.)
β-ureidopropionic acid	64*	all 50 UV
dihydrouracil	56	
ureidosuccinic acid	50*	all 56 decreasing spot area
dihydroorotic acid	36	
uracil	50 UV	all 66* increasing spot area
β-alanine	44	
		Zero reaction time, 73aM, 73aU
		50 UV
		58

1/ chromatogramed in n-butanol-acetic acid-water (2:1:1 v/v)
UV indicates spot absorbed under ultraviolet lamp
* indicates reactive to PDAB without prior sodium hydroxide treatment. ninhydrin-absorbing spots were observed in reaction mixtures and zero reaction time mixtures, but were too overlapped and streaked to identify β-alanine.

Table 22. Optical density measurements in the assay for dihydrouracil hydrase activity. Exp. 12.

	Optical Density 490 mu.					
		ave.		ave.		ave.
Time	pyr-3		73aU-1		73aU-2	
0	1.560		1.050		1.280	
	1.335		1.150		0.880	
	1.180		0.750		1.055	
	4.075	1.385	2.950	0.850	3.215	1.072
15	1.430		0.890		1.195	
	1.470		1.230		1.370	
	1.415		---		1.065	
	4.315	1.435	2.120	1.060	3.630	1.210
30	1.455		1.370		1.460	
	1.540		1.370		1.250	
	1.540		1.370		1.760	
	4.535	1.512		1.370	4.470	1.490
60	1.680		1.175		1.520	
	1.490		1.175		1.345	
	---		1.200		1.480	
	3.170	1.585	3.550	1.180	4.345	1.448
120	1.488		1.055		1.165	
	1.335		1.285		1.165	
	1.120		---		1.122	
	3.943	1.314	2.340	1.170	3.452	1.150
180	1.570		0.860		1.093	
	1.370		0.880		1.093	
	1.100		1.05		1.000	
	4.040	1.336	2.790	0.930	3.186	1.061

BUP uM/ml.	Triplicate sample average
0.25	0.143
0.50	0.275
0.75	0.415
1.00	0.430
1.50	0.462

Table 23. Assay for dihydrouracil hydrase activity in acetone powders of Neurospora, calculations. Exp. 12.

Time	Change in Optical Density -490 mu. pyr-3	73aU-1	73aU-2
15	0.050	0.210	0.128
30	0.127	0.520	0.418
60	0.200	0.330	0.376
120	-0.071	0.320	0.078
180	-0.049	0.08	-0.011
		uM BUP/ml	
15	0.08	0.351	0.214
30	0.212	0.868	0.698
60	0.334	0.551	0.628
120	-0.116	0.534	0.130
180	-0.08	0.142	0.021
		Total uM BUP	
15	0.56	2.46	1.47
30	1.48	6.08	4.89
60	2.34	3.86	4.40
120	-0.81	3.74	0.91
180	-0.56	0.99	0.14

Table 24. Assay for protein concentration in acetone powder extracts.- measurements and calculations. Exp. 12.

Protein Assay-Lowry Method

Standards 1/	O.D. 750 mu	
70ug/ml	0.017	
140 ug/ml	0.029	slope 0.025 mg./OD unit
700 ug/ml	0.051	

1/ Human serum standardized by biuret method

Extract	O.D. 750 mu	Ave.	protein mg/ml
pyr-3	0.355		
	0.410	0.387	9.7
73aU-1	0.385		
	0.370	0.378	9.4
73aU-2	0.285		
	0.295	0.290	7.2
Purification steps 73a-U-1			
Homogenate	0.480		
	0.495	0.487	12.2
Acid Treatment	0.270		
	0.275	0.272	6.8
Heat Treatment	0.255		6.4
First Amm. Sulfate	0.210		
	0.155	0.180	4.5
Second Amm. Sulfate	0.050		0.1

Table 25. Purification assay. Exp. 12.

Treatment	Optical Density-490 mu Zero time		90 min.		Change in Optical Density
Crude extract	1.545 1.635	1.590	1.545 1.330	1.437	-0.123
Acid treatment	1.295 1.170	1.232	1.250 1.115	1.182	-0.040
Heat treatment		1.390		1.120	-0.170
First Amm. Sulfate		1.257	1.200 1.190	1.195	-0.262
Second Amm. Sulfate		1.000	1.230 1.265	1.247	0.247

Treatment	Total ml.	mg. protein/ml.	OD units/ mg protein/ 90 min.	Total Activity
Crude	18	12.2	-0.01	-2.10
Acid	23	6.8	-0.006	-0.94
Heat	23	6.4	-0.027	-3.96
First AS	6	4.5	-0.058	-1.33
Second AS	4	0.1	+2.47	0.99

Table 26. Assay for dihydrouracil hydrase activity in acetone powders of Neurospora strain. — Optical density measurements. Exp. 13.

	Optical Density-490 mu							
Strain	pyr-1	ave.	pyr-3	ave.	73a-U	ave.	73a-M	ave.
Time(min.)								
0	0.175		0.118		0.505		0.540	
	0.157		0.103		0.455		0.720	
	0.135		---		---		0.550	
	0.467	0.156	0.221	0.110	0.960	0.480	1.810	0.603
15	0.158		0.122				0.343	
	0.280		0.118				0.105	
	---		0.124				0.290	
	0.438	0.219	0.364	0.121			0.738	0.269
30	0.193		0.145		0.218		0.196	
	0.188		0.175		0.460		0.253	
	---		0.105		0.540		0.125	
	0.381	0.190	0.425	0.142	1.218	0.406	0.574	0.191
60	0.220		0.203		0.200		0.110	
	0.250		0.213		0.182		0.280	
	0.470	0.235	0.272		0.340		0.115	
			0.688	0.239	0.722	0.261	0.505	0.168
90			0.248		0.235		0.280	
			0.340				0.230	
			0.150				0.198	
			0.738	0.266			0.608	0.203
120			0.280		0.158		0.143	
			0.150		0.310		0.360	
			0.316		0.223		0.100	
			0.746	0.273	0.691	0.230	0.603	0.201
Enzyme + Buffer								
0			0.110		0.050		0.010	
120			0.100		0.000		0.045	

Table 27. Assay for dihydrouracil hydrase activity in acetone powders of Neurospora strains. — calculations. Exp. 13.

Change in Optical Density-490 mu				
Strain	pyr-1	pyr-3	73a-U	73a-M
Time(min.)				
15	0.063	0.011	—	-0.334
30	0.034	0.032	-0.074	-0.412
60	0.179	0.129	-0.216	-0.435
90	—	0.156	-0.245	-0.400
120	—	0.163	-0.240	-0.402

Total uM BUP formed in reaction		
Time(min.)	pyr-1	pyr-3
15	1.75	0.175
30	—	1.31
60	2.01	2.89
90	—	3.24
120	—	3.33

Table 28. Growth responses of pyrimidine mutants to pyrimidine intermediates 1/

Supplement 2/	6-III hours incubation					
	24	48	72	96	120	144
Min.	0	*	11	27	45	62
UD	5	50	81	—	—	—
U	*	25	61	86	—	
DHU	*	*	7	50	67	—
BUP	0	*	+	7	49	77
OA	*	*	18	60	70	—
L-DHO	*	*	+	7	38	64
DL-US	*	*	+	6	19	52
			28-III			
Min.	0	0	0	0	0	0
UD	3	48	83	—	—	—
U	*	4	23	42	—	—
DHU	0	*	*	3	8	11
BUP	0	0	0	0	0	0
OA	*	4	18	31	—	—
L-DHO	0	0	0	0	0	0
DL-US	0	0	0	0	0	0
			pyr-1			
Min.	0	0	tr	4	12	20
UD	*	29	70	97	—	—
U	0	*	7	24	52	—
DHU	0	*	*	11	18	42
BUP	0	0	*	5	15	24
OA	*	*	3	14	18	—
L-DHO	0	0	*	4	14	29
DL-US	0	0	*	4	14	26

1/ Values represent mg. of dry weight. Strains were precultured on uridine agar slants.
2/ 10 mg. per cent
* trace of growth (1-5mg.)

Table 29. Growth responses of substrain-3* to orotic acid pathway intermediates. 1/

Supplement	mg. dry weight replication 1	2	3	ave.
minimal medium	13	13	14	13.3
DL-ureidosuccinic acid	18	8	9	11.7
L-dihydroorotic acid	15	35	20	23.3#
orotic acid	38	33	35	35.3

* derived from the cross KS 138-3-1a x KS 12-2-4A

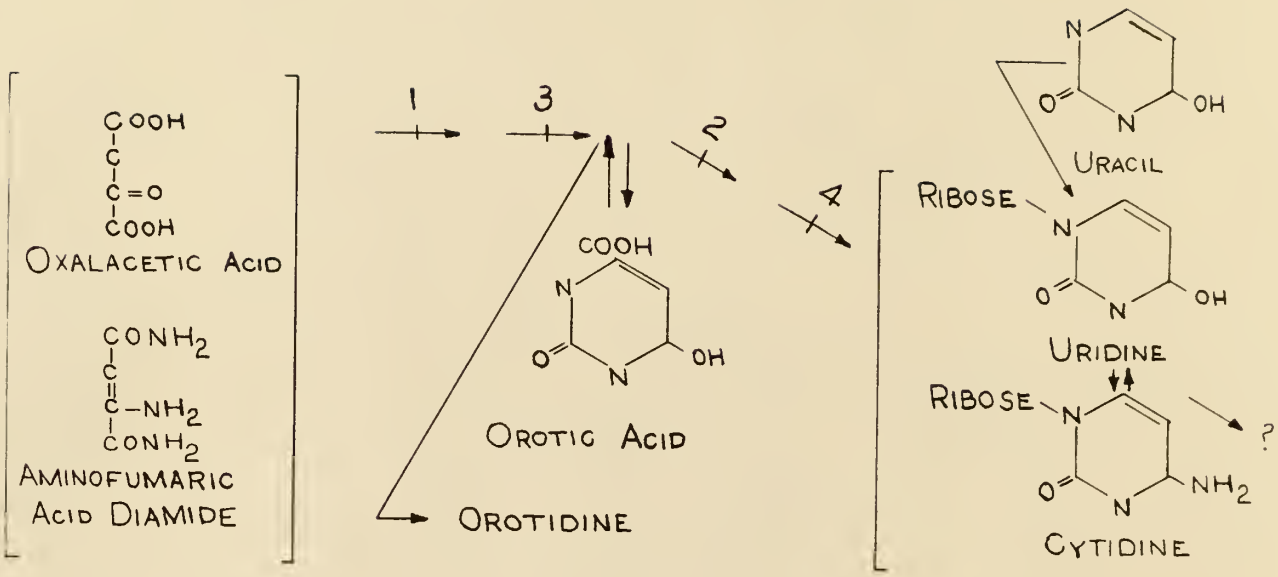
1/ Cultured in 250 ml. flasks containing 40 ml. of medium and 10 mg. per cent supplement for 96 hr. at 27°C. in standing culture.

\# significantly different from minimal medium at the 95 per cent confidence interval by the non-parametric tau-test (71).

EXPLANATION OF PLATE I

Biosynthesis of some of the pyrimidines of nucleic acids. Studies of mutants have not established whether orotic acid and orotidine are true intermediates, but enzyme work has shown that orotic acid goes to orotidine phosphate and thence to the phosphates of uridine and cytidine instead of the free nucleosides as indicated here. (After Wagner and Mitchell (78), 1955).

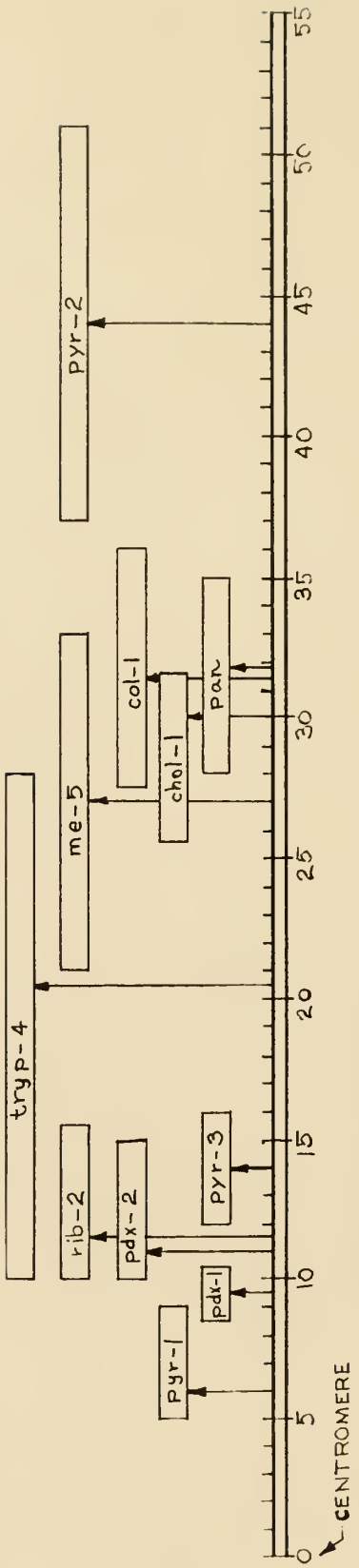
PLATE I


COOH
C
C
C=O
COOH
OXALACETIC ACID
CONH2
C
C=C-NH2
CONH2
AMINOFUMARIC ACID DIAMIDE
1
3
2
4
COOH
N
O
N
OH
OROTIC ACID
OROTIDINE
N
O
N
OH
URACIL
RIBOSE
N
O
N
OH
URIDINE
RIBOSE
N
O
N
NH2
?
CYTIDINE

EXPLANATION OF PLATE II

Linkage map of the right arm of linkage group four in *Neurospora crassa*. Corrected. (After Barratt, et al.(3), 1954). The distances indicated for a given gene represent a statistical confidence interval. Abbreviations: pyrimidine, pyr; pyridoxine, pdx; riboflavin, rib; tryptophan, tryp; methionine, me; choline, chol; colonial, col; pantothenate, pan.

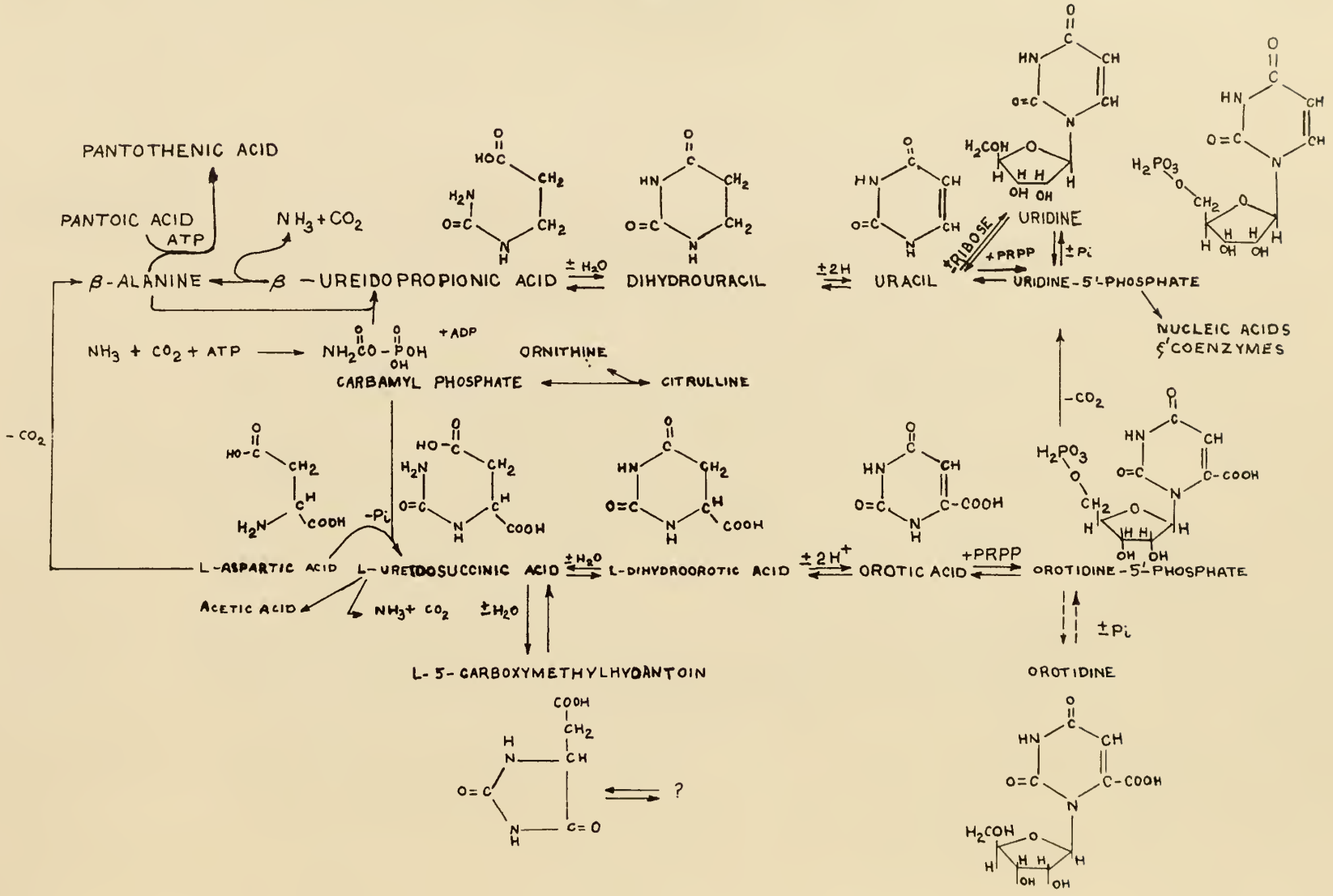
PLATE II


tryp-4
me-5
col-1
chol-1
pan
pyr-2
rib-2
pdx-2
pyr-3
pdx-1
pyr-1
0
5
10
15
20
25
30
35
40
45
50
55
CENTROMERE

EXPLANATION OF PLATE III

Pattern of Pyrimidine Metabolism in Bacterial and Mammalian Systems.

PLATE III


PANTOTHENIC ACID
PANTOIC ACID
ATP
$NH_3 + CO_2$
β-ALANINE
β-UREIDOPROPIONIC ACID
$\pm H_2O$
DIHYDROURACIL
$\pm 2H$
URACIL
URIDINE
RIBOSE
+PRPP
$\pm Pi$
URIDINE-5'-PHOSPHATE
NUCLEIC ACIDS & COENZYMES
$NH_3 + CO_2 + ATP$
+ADP
CARBAMYL PHOSPHATE
ORNITHINE
CITRULLINE
$-CO_2$
L-ASPARTIC ACID
-Pi
L-UREIDOSUCCINIC ACID
$\pm H_2O$
L-DIHYDROOROTIC ACID
$\pm 2H^+$
OROTIC ACID
+PRPP
OROTIDINE-5'-PHOSPHATE
ACETIC ACID
$NH_3 + CO_2$
$\pm H_2O$
$\pm Pi$
L-5-CARBOXYMETHYLHYDANTOIN
OROTIDINE
?

EXPLANATION OF PLATE IV

Experiment 5. Growth response of Neurospora strains in minimal medium (M) and minimal supplemented with 20 mg. per cent of uracil (U). Cultured with aeration at 27°C. Values are average of triplicate 250 ml. Erlenmeyer flasks that contained 40 ml. of medium.

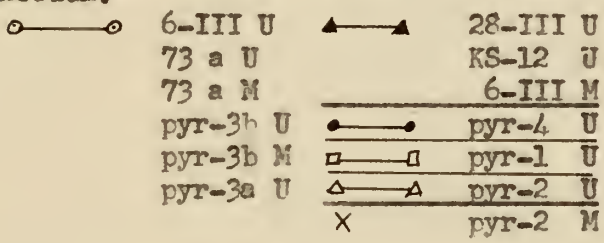
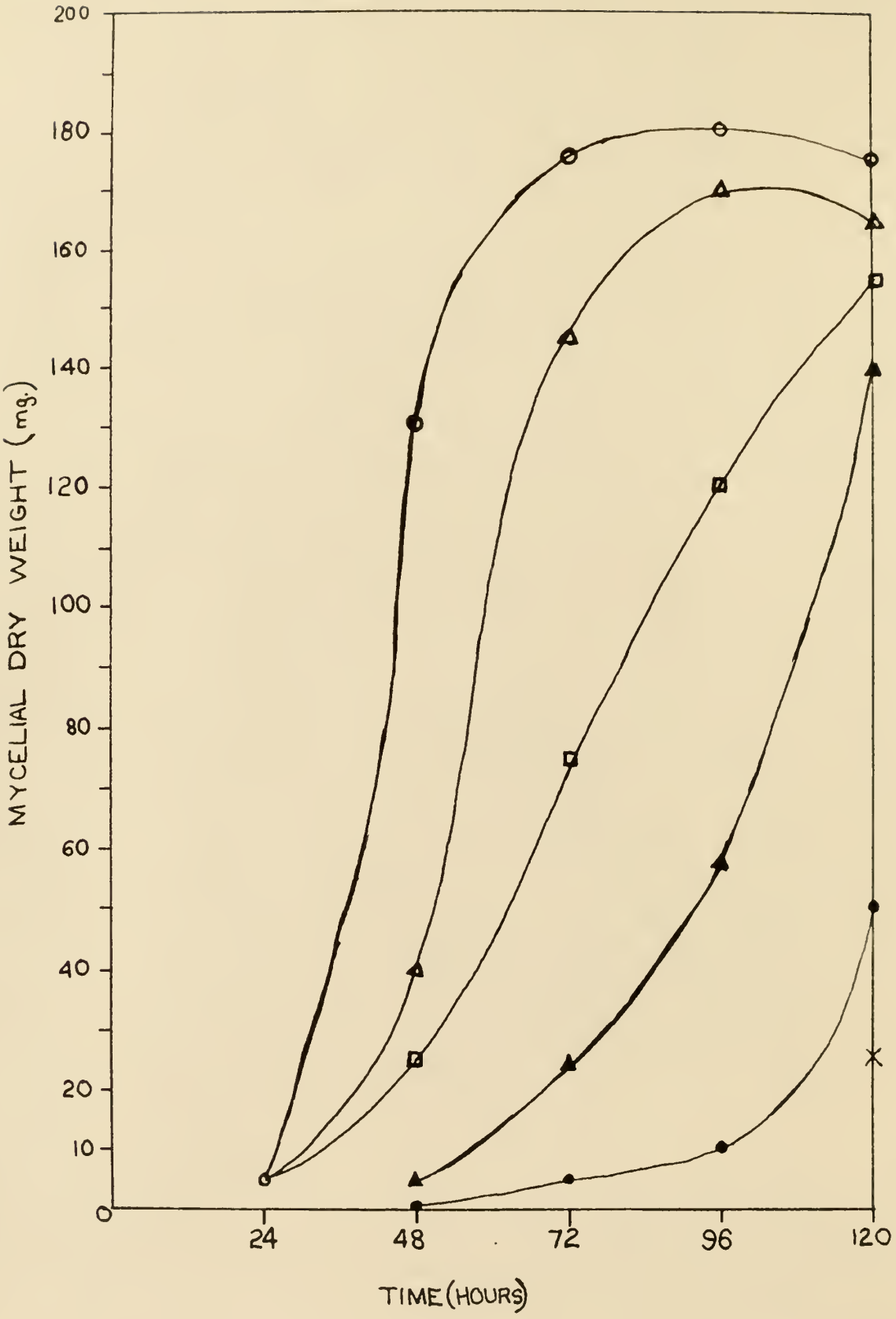


PLATE IV


200
180
160
140
120
100
80
60
40
20
10
0
MYCELIAL DRY WEIGHT (mg.)
24
48
72
96
120
TIME (HOURS)

EXPLANATION OF PLATE V

Experiment 5. Growth responses of Neurospora strains in minimal medium (M) and minimal medium supplemented with 20 mg. per cent uracil (U). Cultured in standing flasks at 27°C, or at 40°C where indicated. Values are averages of triplicate 250 ml. Erlenmeyer flasks that contained 40 ml. of medium.

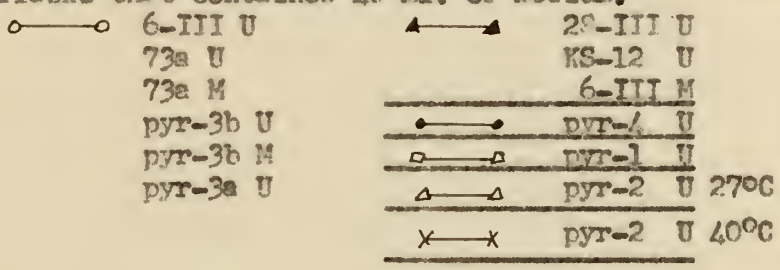
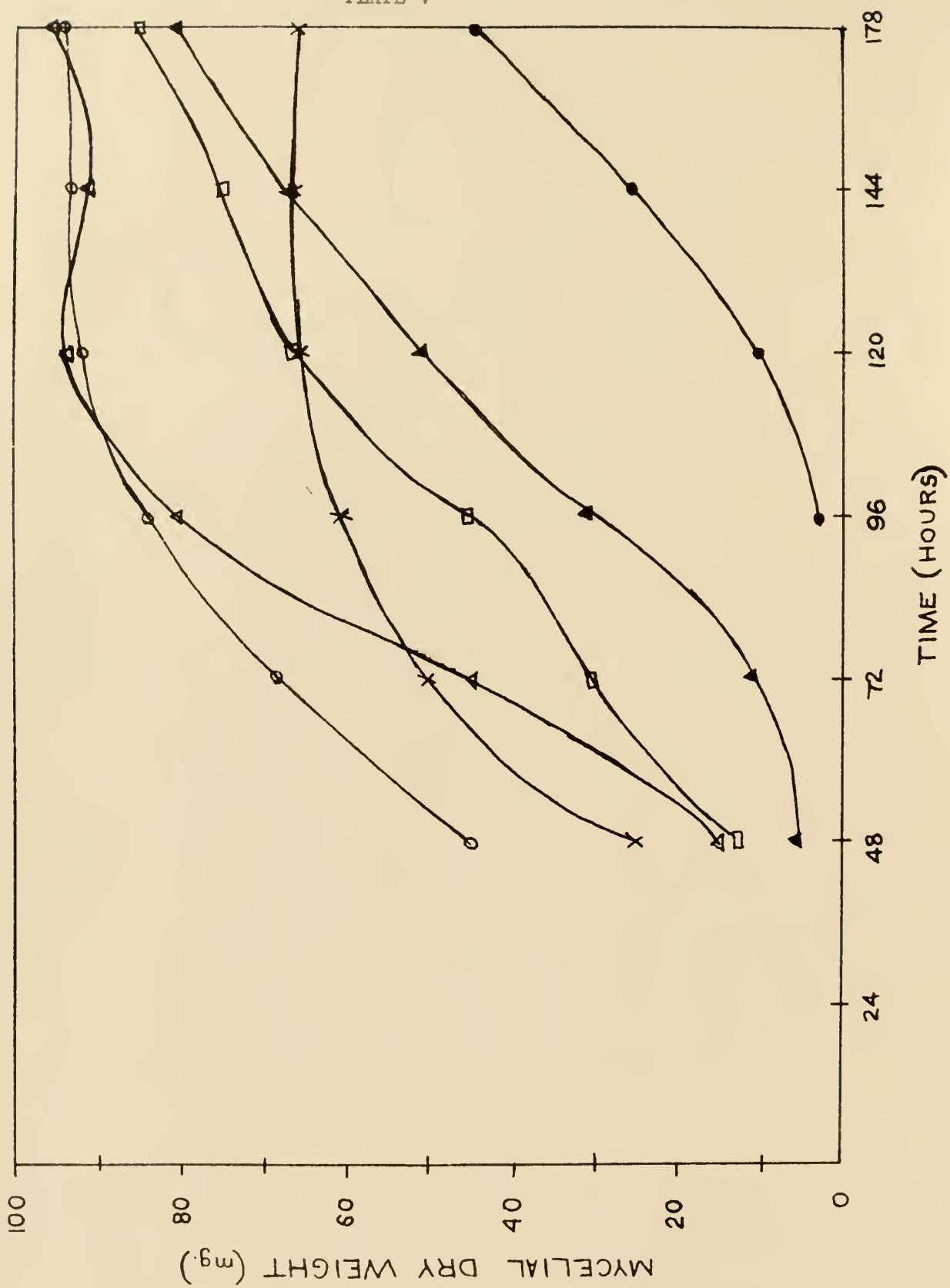


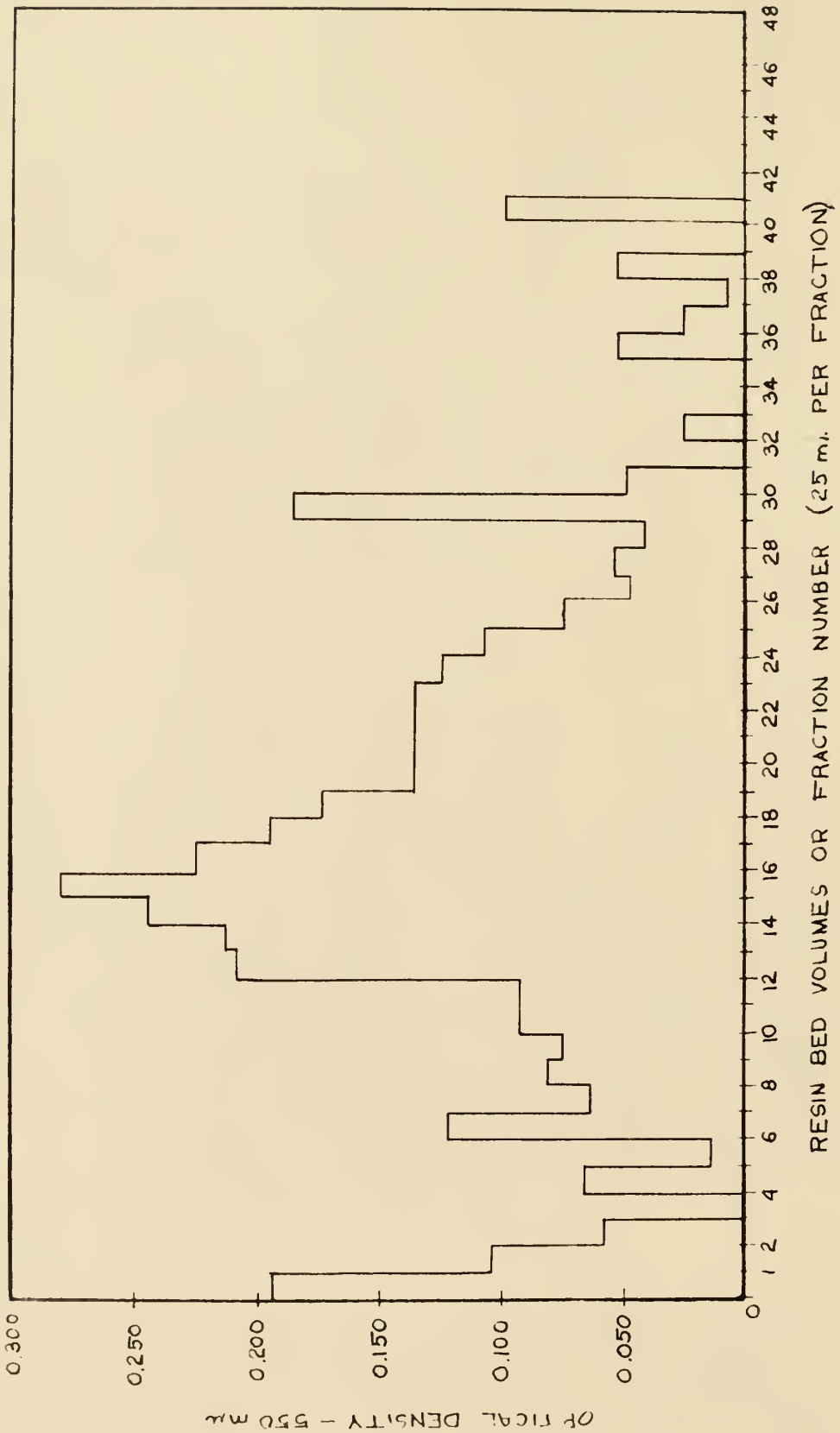
PLATE V


MYCELIAL DRY WEIGHT (mg.)
0
20
40
60
80
100
TIME (HOURS)
24
48
72
96
120
144
178

EXPLANATION OF PLATE VI

Experiment 7-Run No. 2. Effluent pattern from Dowex 1-X10, 200-400 mesh formate-form column (25 cm. x 1 cm.) fractions of pyr-1 culture filtrate. Eluted with sodium formate buffer, 0.055 M, pH 3.2.

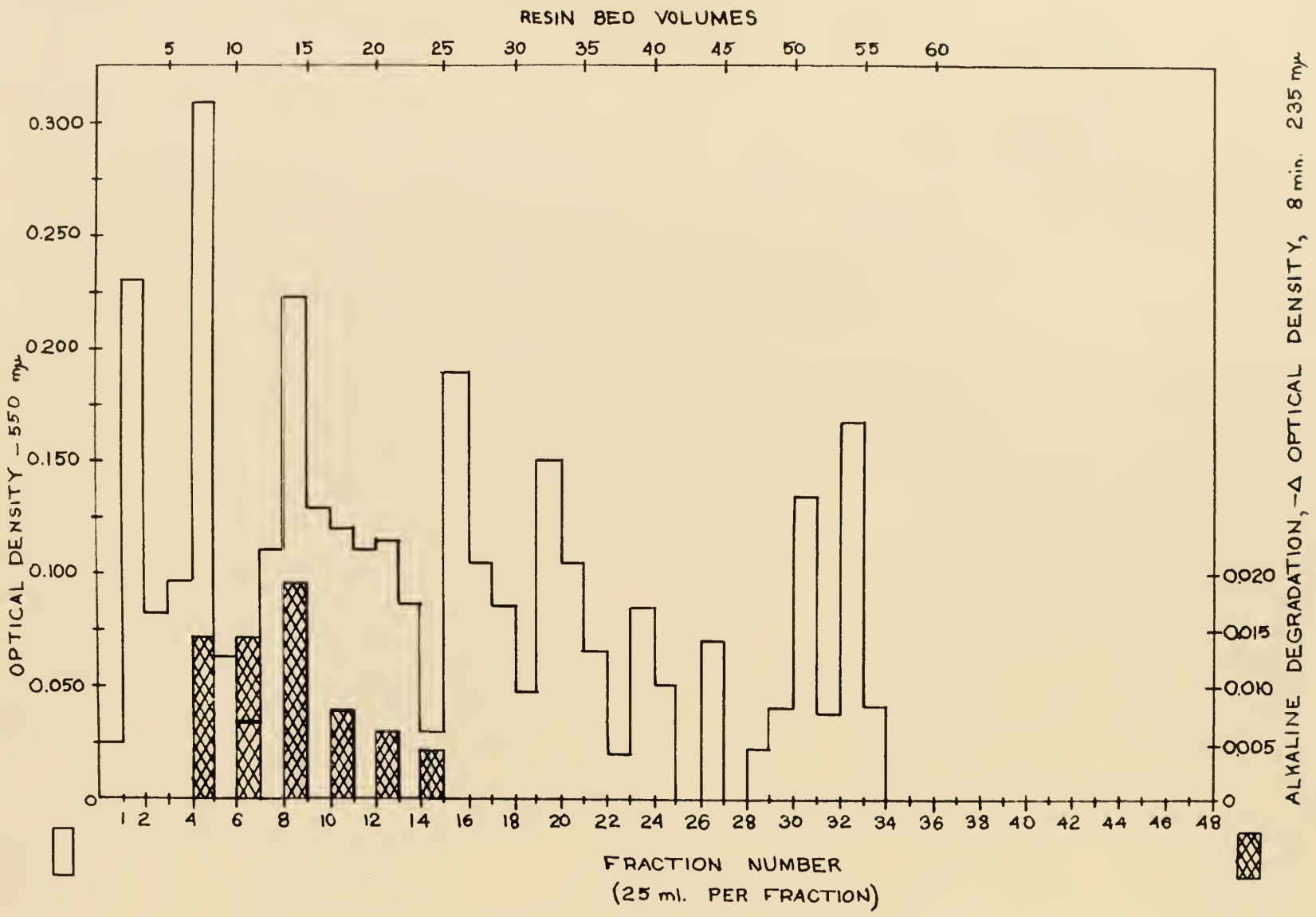
PLATE VI


OPTICAL DENSITY - 550 mμ
0.300
0.250
0.200
0.150
0.100
0.050
0
1 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
RESIN BED VOLUMES OR FRACTION NUMBER (25 ml. PER FRACTION)

EXPLANATION OF PLATE VII

Experiment 8. Effluent pattern from Dowex 1-X10, 200-400 mesh formate-form column (25 cm. x 1 cm.) fractions of pyr-1 culture filtrate. Eluted with sodium formate buffer, 0.055 M, pH 3.2.

PLATE VII

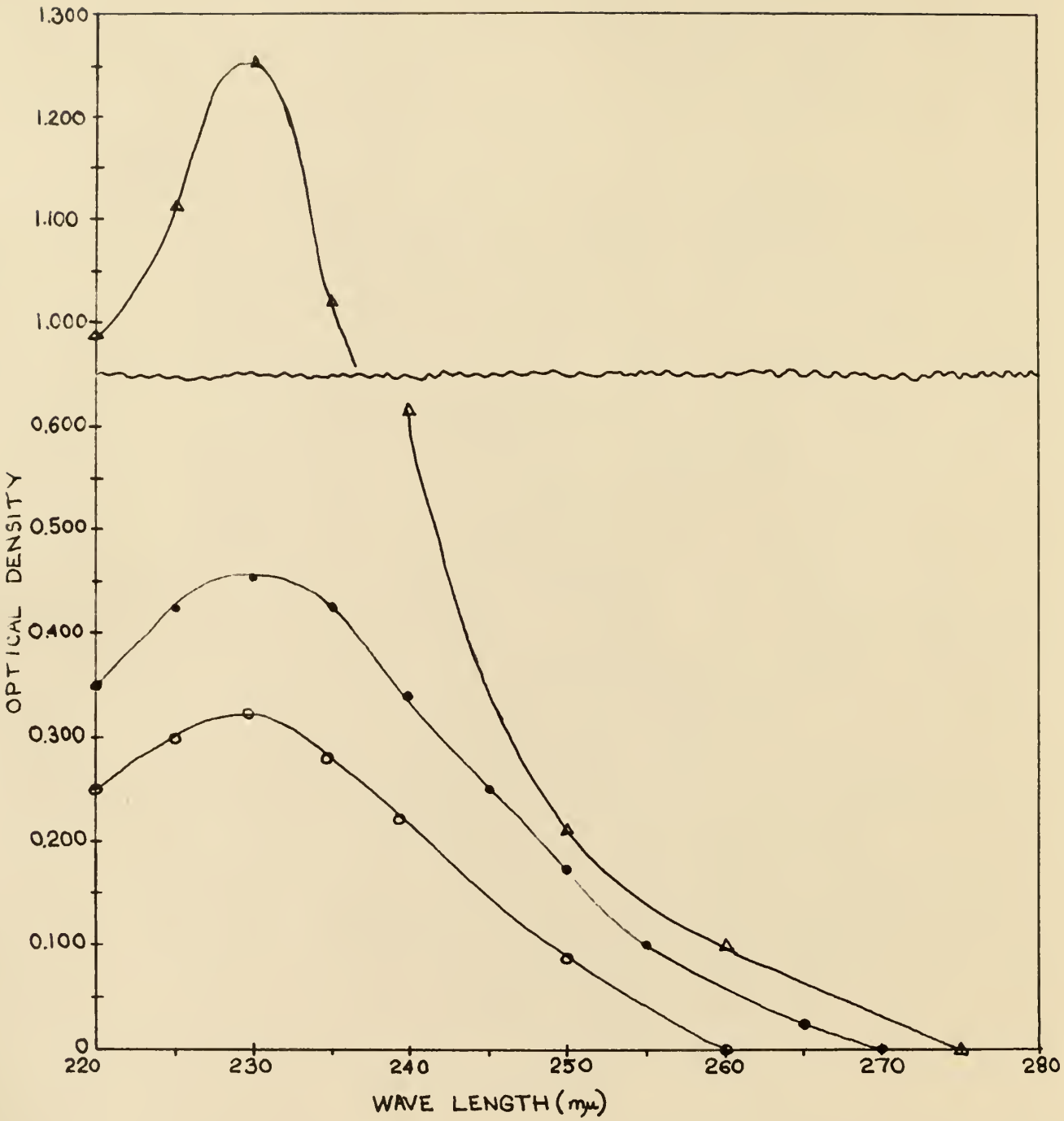

RESIN BED VOLUMES
5 10 15 20 25 30 35 40 45 50 55 60
OPTICAL DENSITY – 550 mμ
0.300
0.250
0.200
0.150
0.100
0.050
0
ALKALINE DEGRADATION, –Δ OPTICAL DENSITY, 8 min. 235 mμ
0.020
0.015
0.010
0.005
0
1 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
FRACTION NUMBER
(25 ml. PER FRACTION)

EXPLANATION OF PLATE VIII

Experiments 8 and 9. Ultraviolet absorption spectra of purified fractions obtained from the mycelia of mutants pyr-1 and 28-III compared with authentic L-dihydroorotic acid, all in KOH, pH 12.0. Purifications procedures described in text.

●——● pyr-1
△——△ 28-III
○——○ L-dihydroorotic acid

PLATE VIII

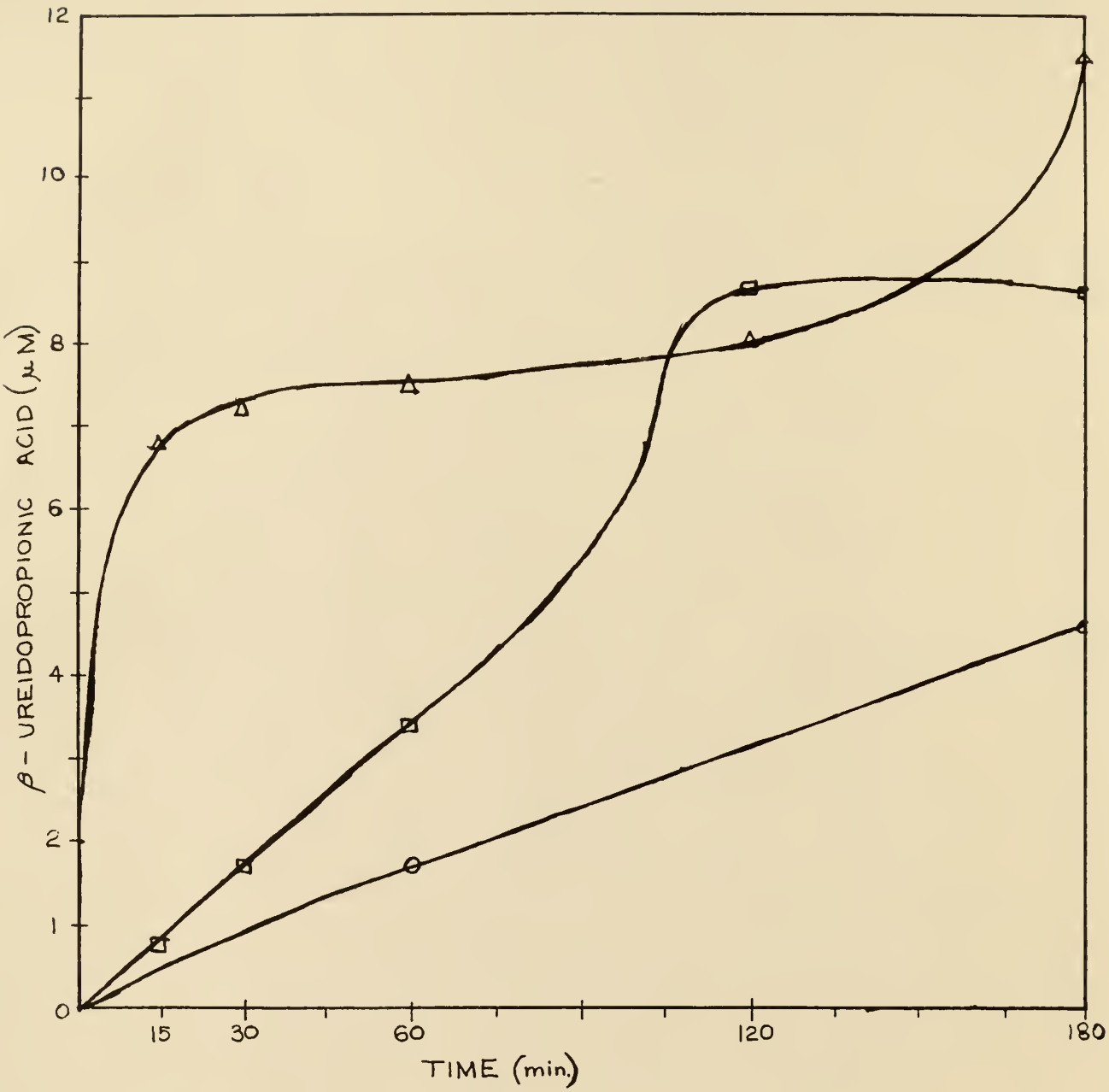

1.300
1.200
1.100
1.000
0.600
0.500
0.400
0.300
0.200
0.100
0
OPTICAL DENSITY
220
230
240
250
260
270
280
WAVE LENGTH (mμ)

EXPLANATION OF PLATE IX

Experiment 11. Assay for dihydrouracil hydrase activity in acetone powder extracts of *Neurospora crassa* strains. The reaction mixture contained: Tris buffer, 0.50 uM, pH 9.0; dihydrouracil, 50 uM; and 1 ml. of acetone powder extract (24 ml. Tris buffer per gm. acetone powder). Final volume 2 ml. Incubation was at 30°C. Reaction mixtures were assayed by the modified method of Archibald (81). Values are corrected for reaction of extract plus substrate at zero time.

o——o pyr-1, Δ——Δ 73a U, □——□ 73a M

PLATE IX

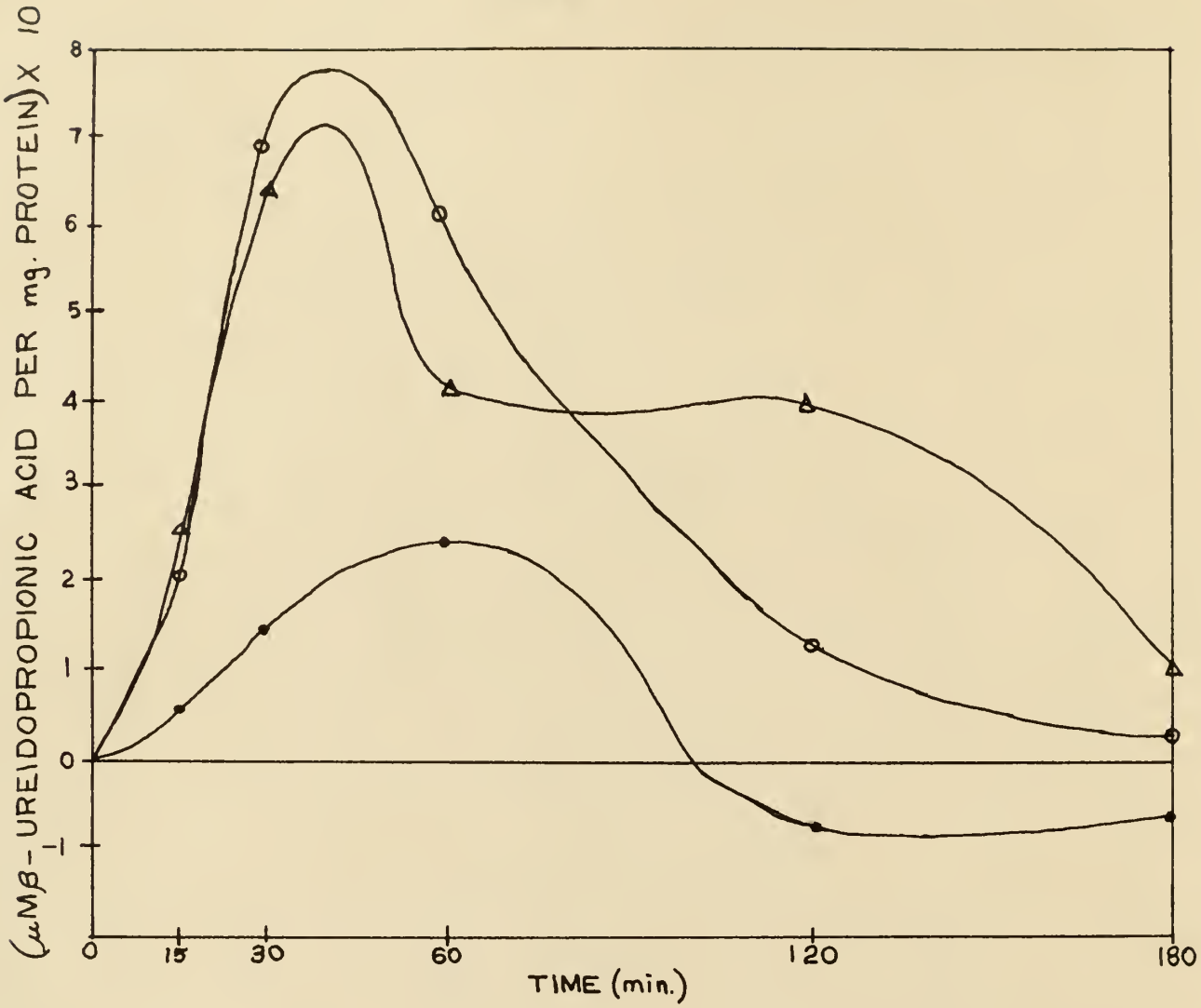

12
10
8
6
4
2
1
0
β - UREIDOPROPIONIC ACID (μM)
15
30
60
120
180
TIME (min.)

EXPLANATION OF PLATE X

Experiment 12. Assay for dihydrouracil hydrase activity in acetone powder extracts of Neurospora crassa strains. The reaction mixtures contained: Tris buffer, 0.25 uM, pH 9.0; dihydrouracil, 50 uM; and protein--pyr-3, 9.7 mg.--73a U-1, 9.4 mg.,--73a-U-2, 7.2 mg., in a final volume of 2 ml.. Incubation at 30°C. Reactions mixtures assayed by modified method of Archibald (81). Values corrected for reaction at zero time and adjusted to an equal protein basis.

•——• pyr-3, o——o 72aU-1, Δ——Δ 73aU-2.

PLATE X

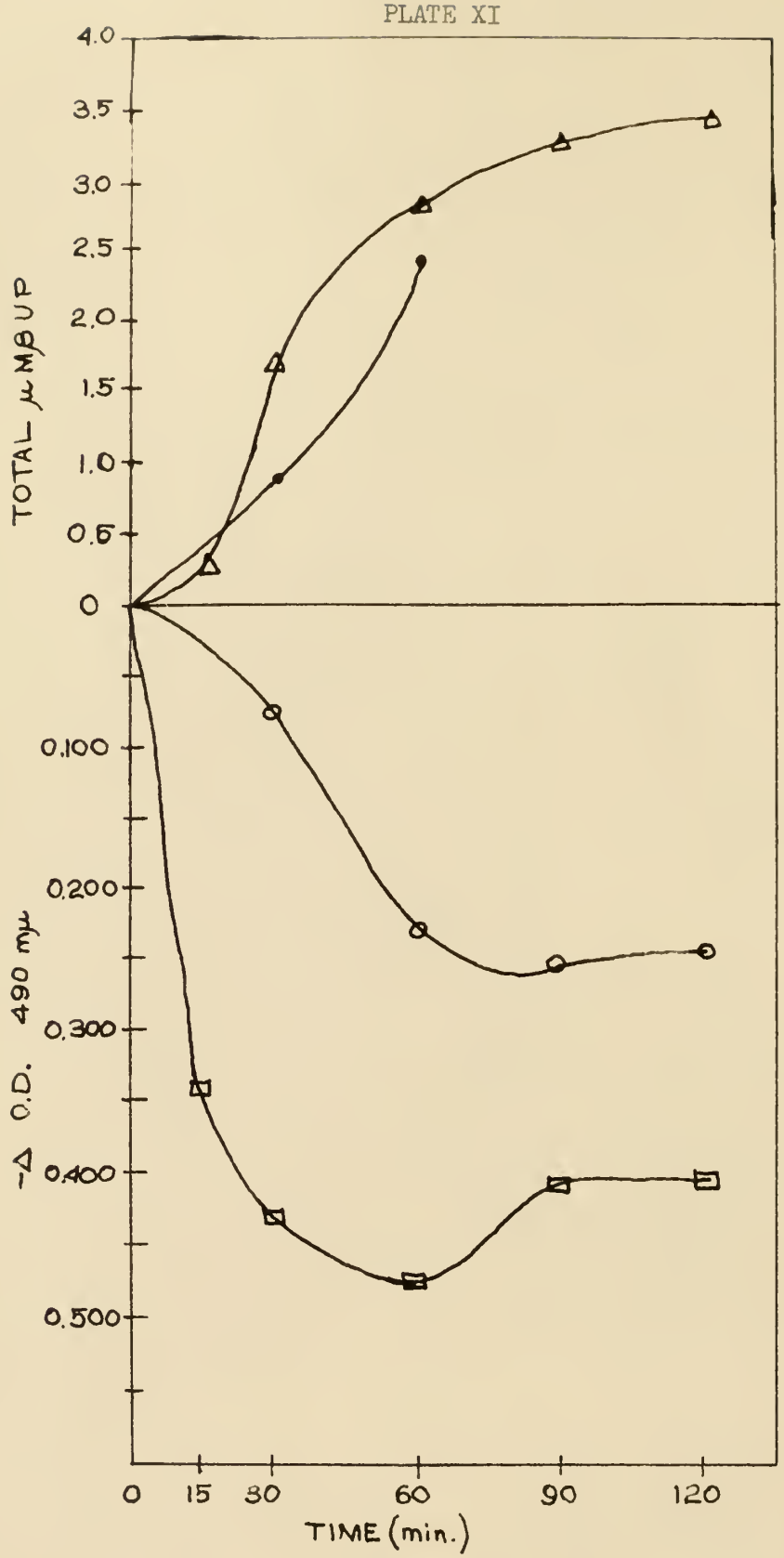

(μMβ-UREIDOPROPIONIC ACID PER mg. PROTEIN) X 10
8
7
6
5
4
3
2
1
0
-1
0
15
30
60
120
180
TIME (min.)

EXPLANATION OF PLATE XI

Experiment 13. Assay of acetone powder extracts of Neurospora for dihydrouracil hydrase activity. The reaction mixture contained: Tris buffer, 0.25 uM, pH 9.0; dihydrouracil, 50 uM; and 1 ml. of acetone powder extract (24 ml. distilled water per gm. of acetone powder) in a final volume of 2 ml. Incubation at 30°C. Reaction mixtures assayed by the modified method of Archibald (81). Values corrected for absorption at zero reaction time.

●——● pyr-1 △——△ pyr-3 ○——○ 72aU □——□ 73aM

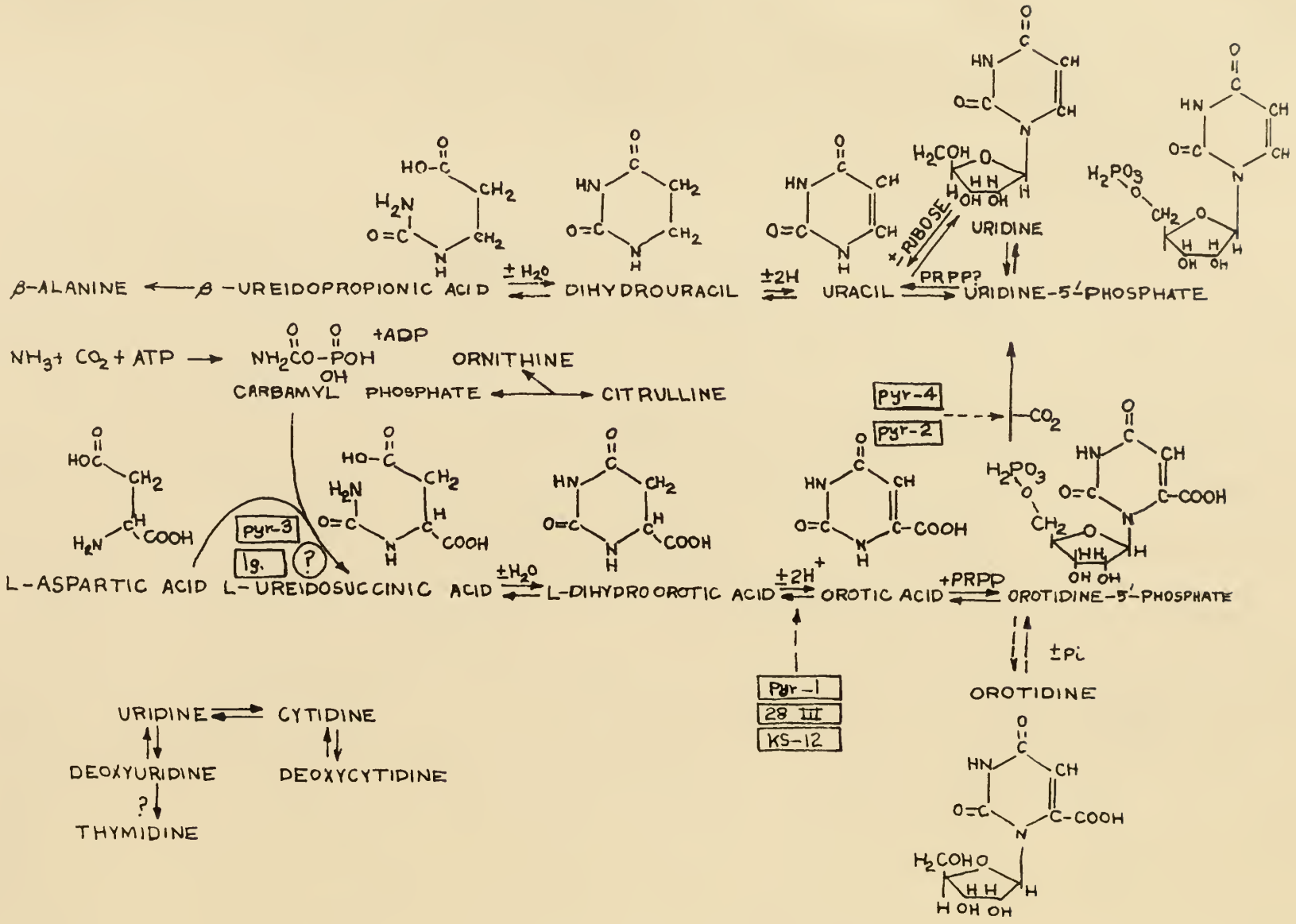
PLATE XI


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
TOTAL μ MβUP
0.100
0.200
0.300
0.400
0.500
-Δ O.D. 490 mμ
0
15
30
60
90
120
TIME (min.)

EXPLANATION OF PLATE XII

Summary of genetic-metabolic relations in Neurospora crassa.

PLATE XII


β-ALANINE
β-UREIDOPROPIONIC ACID
±H2O
DIHYDROURACIL
±2H
URACIL
PRPP?
URIDINE-5'-PHOSPHATE
RIBOSE
URIDINE
NH3+ CO2 + ATP
+ADP
CARBAMYL PHOSPHATE
ORNITHINE
CITRULLINE
L-ASPARTIC ACID
L-UREIDOSUCCINIC ACID
±H2O
L-DIHYDROOROTIC ACID
±2H+
OROTIC ACID
+PRPP
OROTIDINE-5'-PHOSPHATE
±Pi
OROTIDINE
Pyr-4
Pyr-2
Pyr-3
lg.
?
Pyr-1
28 III
KS-12
URIDINE
CYTIDINE
DEOXYURIDINE
DEOXYCYTIDINE
?
THYMIDINE

COMPARATIVE BIOCHEMICAL STUDIES OF PYRIMIDINE MUTANTS OF *NEUROSPORA CRASSA*

by

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B. S., Kansas State College of
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AN ABSTRACT OF A THESIS

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Neurospora crassa, a red bread mold, offers a rather unique tool among organisms for determining genetic-metabolic interrelations in that detailed, classical genetic analyses can be made concomitantly with biochemical analyses.

Among the several areas of metabolism approachable from the biochemical-genetic point of view, pyrimidine metabolism was chosen for three reasons: 1) little was known about pyrimidine metabolism, *per se*, in the fungi, 2) the genetic relations to pyrimidine metabolism were unknown, and 3) several pyrimidines are unique to deoxyribonucleic acid (DNA), the carrier of genetic information. Thus, a study of the biosynthesis of the pyrimidines would, in effect, be a beginning in the study of the autocatalytic properties of genes and DNA. In addition, fundamental principles elucidated by these studies could further studies of eccentric DNA metabolism as found in virus and neoplastic growth.

The thesis of this paper, that pyrimidine metabolism of *Neurospora crassa* is analogous to pyrimidine metabolism in bacteria and mammals, and that the intermediary reactions in pyrimidine biosynthesis are genetically controlled, was supported by evidences obtained from three approaches: 1) growth response of pyrimidine mutants to proposed pyrimidine intermediates, 2) the study of accumulation products of pyrimidine metabolism in mutant culture filtrates and extracts by spectrophotometry, paper chromatography, and ion exchange analysis, and 3) enzymatic studies of cell-free extracts.

A metabolic scheme for uracil metabolism, hitherto unreported in

Neurospora, was elucidated. The intermediates of uracil metabolism were identical to those reported for bacteria and mammals. One of the enzymes in the uracil pathway, dihydrouracil hydrase, was studied in cell-free extracts. The view was discussed that the uracil pathway was catabolic in the prototroph, but that in the mutants, could be reversed to function biosynthetically if the appropriate intermediates were fed.

The precursors of orotic acid--dihydroorotic acid and ureidosuccinic acid--were found to accumulate in certain pyrimidine mutants, and to support growth of other pyrimidine mutants.

On the basis of these experiments, it was possible to relate the mutation effects of given mutants to single-step reactions leading to the biosynthesis of uridine-5'-phosphate.

The state of knowledge of the relationships of genes to pyrimidine metabolism in Neurospora is now such that detailed enzymatic studies could be undertaken to relate specific mutational events at a given locus to possible variations in the enzyme controlled by that locus.